October 2009

LSM 5 MP / LSM 510 / LSM 510 *DuoScan*
LSM 510 META / LSM 510 META *DuoScan*

Upgrade ZEN 2009

Knowledge of this manual is required for the operation of the instrument. Would you therefore please make yourself familiar with the contents of this manual and pay special attention to hints concerning the safe operation of the instrument.

The specifications are subject to change; the manual is not covered by an update service.

How to make best use of the LSM 510 and LSM 510 META operating instructions:

This operating manual also includes the instructions for the LSM 510 META *DuoScan,* the LSM 510 *DuoScan* and the LSM 5 MP systems.

A few symbols in these operating instructions will help you to recognize the nature and purpose of information immediately:

The WARNING symbol warns against hazards for the user that might arise when operating the laser.

This WARNING symbol warns against hazards from dangerously high voltages.

The CAUTION symbol warns against faults and hazards that might arise during operation and which might cause damage to the unit.

The NOTE symbol will help you to optimally solve your work problem. It represents a practical tip which will help you to find out which settings and methods are capable of improving or accelerating a procedure.

The HOT SURFACE symbol warns against hazards for the user that might arise when touching the lamp housing during operation.

The MAINS PLUG symbol remembers service personal to pull the mains plug before opening the device housing.

Depending on the request, these operating instructions will supply you with various possibilities:

• If you want to know where to find certain general areas of information, refer to the following outline of sections to get a general overview.

• You will find a detailed table of contents at the start of every chapter. There you will see at a glance what topics are covered in detail.

Always remember: **The time you invest in getting acquainted with the product will pay for itself many times over in your application task.**

Contents

CHAPTER 1   NOTES ON DEVICE SAFETY

CONTENTS

1   NOTES ON DEVICE SAFETY

1.1   General

The LSM 510 and LSM 510 META laser scanning microscope, including its original accessories and compatible accessories from other manufacturers, may only be used for the purpose of microscopic techniques.

Laser Scanning Microscopes (LSM) are intended for high resolution imaging of biological or material samples, whereby in contrast to wide field microscopy the specimen is illuminated raster-fashion with a focused laser beam and the optical arrangement prevents light from out-of-focus regions of the specimen contributing to image formation.

☞   Installation and commissioning of the LSM 510 and LSM 510 META system must be performed by authorized Carl Zeiss service staff. The system should not be used prior to instruction by a Carl Zeiss representative.

⚠   The manufacturer will not assume liability for any malfunction or damage caused by anything other than the intended use of the LSM 510 or LSM 510 META or individual modules or parts of it, nor by any repair or other service operation performed or attempted by persons other than duly authorized service staff. Any such action will invalidate any claim under warranty, including parts not directly affected by such action. This also includes the modification of the system computer with new cards, etc. by the user. The use of a camera at the base port of AxioObserver.Z1 SP stands with motorized beam path switching is not allowed for reasons of laser safety. Any manipulation will result in the loss of warranty of laser safety.

Please read also the notes on device safety and manuals of the microscope, the HBO, the HAL and additional optional devices, if ordered, as the UV Laser, the piezo focusing device, the heating inserts and the Ti:Sa laser.

⇒   As the system is largely operated via menus on a computer, you should be familiar with the principles of the operating system and its WINDOWS, WINDOWS 2000 or Windows XP graphical user interface. The respective manuals are supplied together with the programs.

☀   The LSM 510 and LSM 510 META are devices that belong to laser hazard class 3B. The systems are equipped with safety interlocks that comply with laser hazard class 3B and 4. If equipped with a Ti:Sa laser (see list in section 1.8), the LSM 510 and LSM 510 META are devices that belong to laser hazard class 4. WHO recommendations concerning health and industrial protection when handling laser devices must be observed. The operator of the unit must also observe all and any relevant statutory accident prevention regulations. The user is referred to the safety data sheet provided together with the manual.

1.2 Regulations

Extensive knowledge of the hardware/the system is indispensable for safe operation of the LSM 510 and LSM 510 META.

☞ Read these operating instructions and all device publications belonging to the system conscientiously **before** operating the LSM 510 or LSM 510 META! You can obtain additional information on the hardware configuration delivered and on optional system extensions from the manufacturer or via the service hotline.

⇒ The LSM 510 and LSM 510 META have been designed, built and tested in conformity with the following regulations and guidelines:

DIN EN 61010-1 (IEC 61010-1) "Safety requirements for electrical equipment for measurement, control and laboratory use"

DIN EN 60825-1 (IEC publication 60825-1) "Safety of laser equipment", taking relevant CSA and UL specifications into account

DIN EN 61326: "Electrical equipment for control technology and laboratory use – EMC-requirements"

Low voltage directive: 2006/95/EG

EMC directive: 89/336/EWG

⇒ The company works according to a certified Environment Management System according to ISO 14001.

The Product was developed, tested and produced in accordance with the valid regulations and guidelines for environmental law of the European Union.

The product and its accessories have been classified as instrument category 9 (laboratory equipment or comparable standard). The product and its accessories agree with the EU-regulations 2002/95/EG (RoHS) and 2002/96/EG (WEEE), if applicable for the product.

Carl Zeiss has installed a process for taking back and recycling the instruments within the member states of the European Union, which takes care of the appropriate utilization according to the said EU guidelines.

For details on the disposal and recycling please refer to your relevant Carl Zeiss sales or service organization.

The product must not be disposed in the household waste or through the municipal disposal organizations. In case of resale the seller is obliged to inform the buyer, that the product has to be disposed according to the said regulations.

1.3   Power Requirements

☞   The LSM 510 and LSM 510 META comes with a mains power supply cord and plug, either CEE red (3/N/PE 400/230V/16A), or NEMA L 14-30P (2/N/Ground 120/240V/30A), and with the matching mains socket outlet.
A ground wire (AWG10 green/yellow) is supplied because it is necessary to ground the system. The connecting part on both ends of the cable is a cable eye with 8 mm inner diameter.
A suitable grounding point must be installed in the room.
For systems (220 ... 240 V AC) equipped with X-Cite 120 the mains socket outlet must be equipped with a fuse having minimum tripping characteristic C according to IEC/EN 60898.

Line voltage	220 ... 240 V AC (±10 %)	100 ... 125 V AC (±10 %)
Line frequency	50...60 Hz	50...60 Hz
LSM incl. VIS laser		
– Max. current	3 phases at 16 A	2 phases at 25 A
– Power	Phase 1 = 1.9 kVA max.	Phase 1 = 3.2 kVA max.
	Phase 2 = 1.5 kVA max.	Phase 2 = 2.8 kVA max.
	Phase 3 = 2.6 kVA max.	
– Power consumption	5000 VA max.	5000 VA max.
Argon UV laser		
– Line Voltage	208...240 V AC	208...240 VAC
	(±10 %) 50 / 60 Hz	(±10 %) 50 / 60 Hz
– Max. current	1 phase at 63 A	1 phase at:
	Note: For Line Voltage 220 V the connector and power plug are rated for 63 Amps, However wiring and fuse should be rated for 32 Amps.	208 V: 34 Amps 230 V: 31 Amps 240 V: 29 Amps
– Power consumption	7000 VA max.	7000 VA max.
Class of protection	I	I
Type of protection	IP 20	IP 20
Overvoltage category	II	II
Pollution degree	2	2

Fig. 1-1   Power connector for LSM 510 / LSM 510 META and Laser Module V. Free/reserve outlets may be used to supply power to additional equipment. No more than 1 A can be provided by each outlet. (Scheme is turned 90° to real system.)

Fuse switches

Argon laser

Fuse 230 V,
Order number: 302-914
or
Fuse 120 V,
Order number: 302-917

Power supply

LVDS

Safety
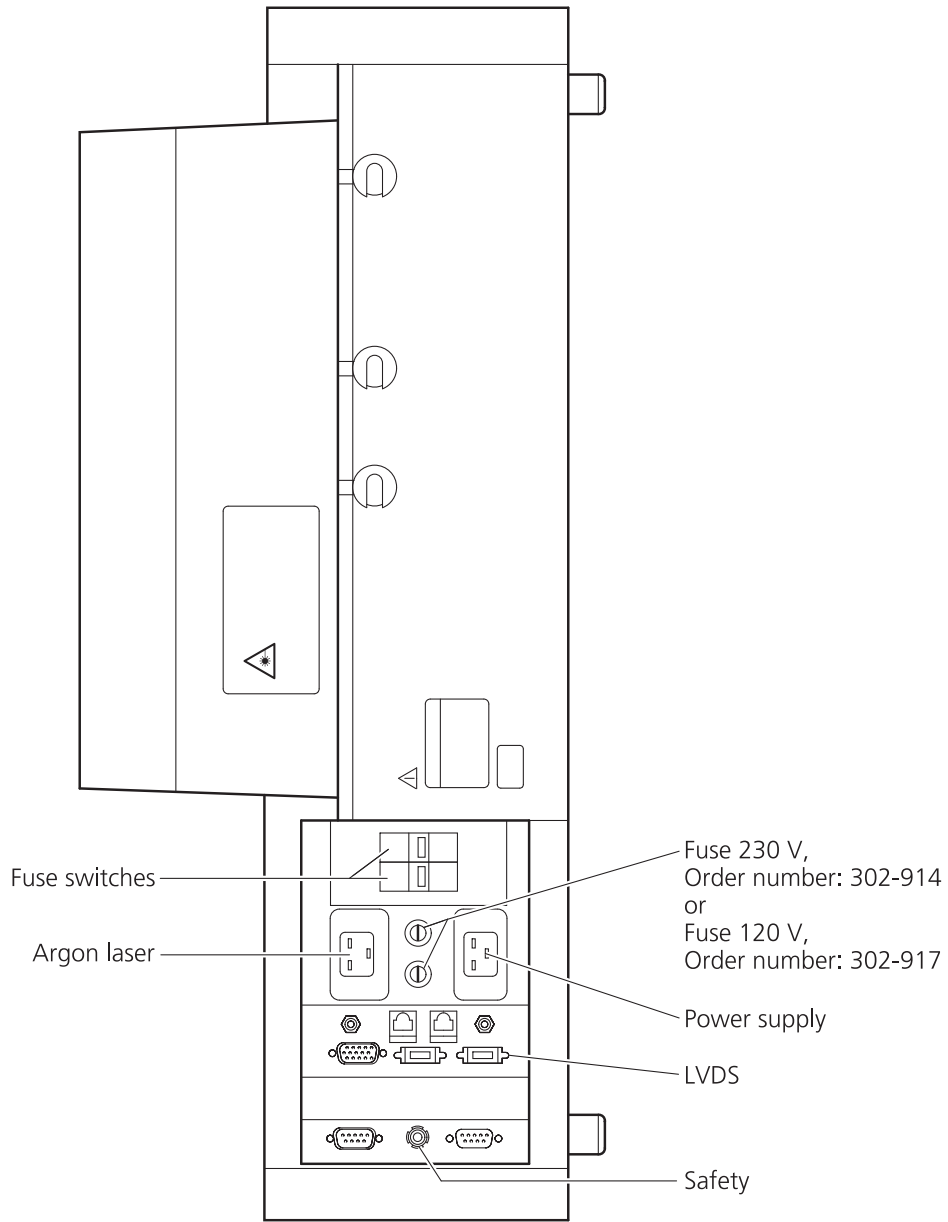
**Fig. 1-2   Power connector for Laser Module RGB
(Scheme is turned 90° to real system.)**
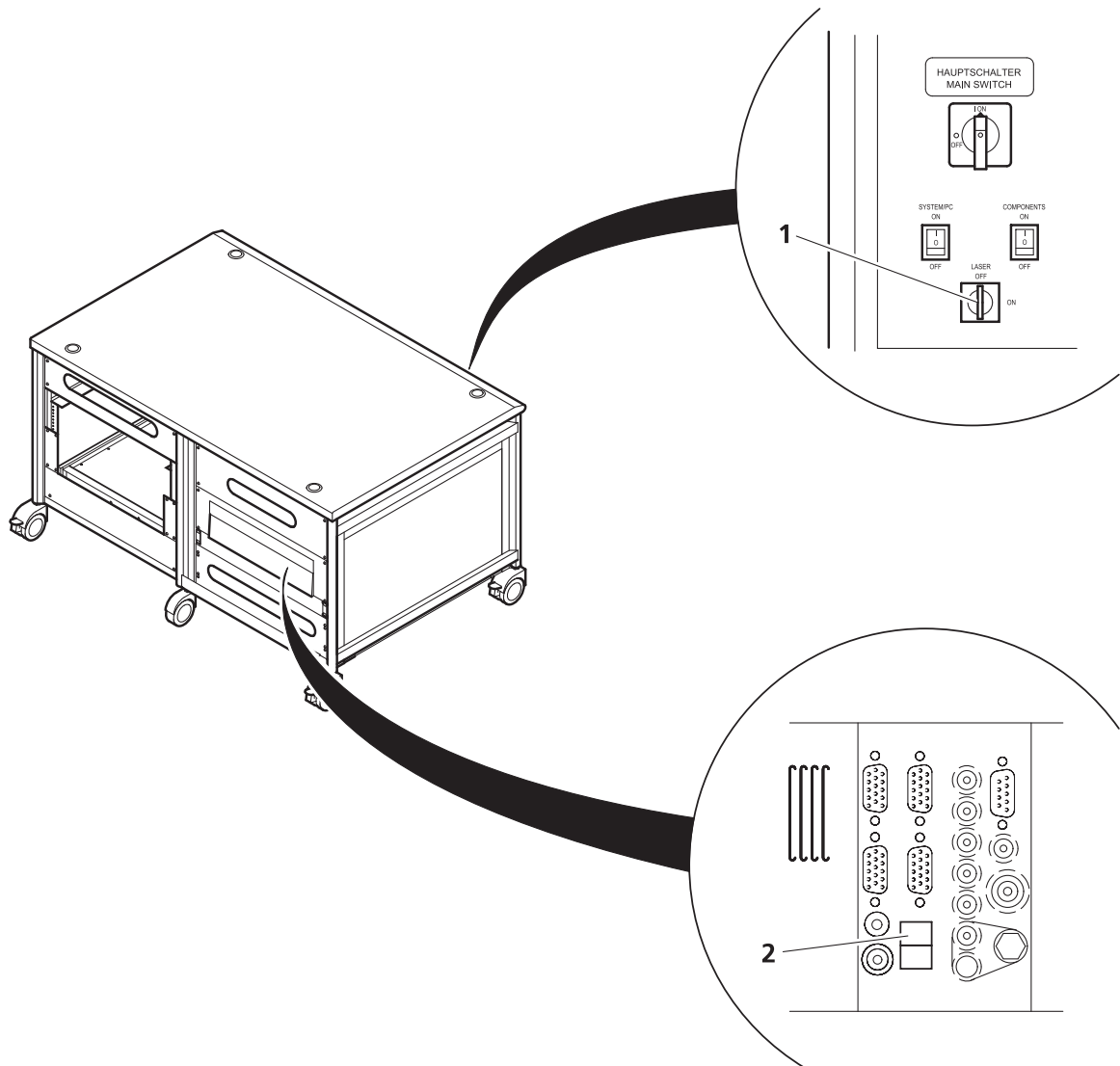
1   Key-interlock Laser ON/OFF
2   Door interlock interface

Fig. 1-3   Key-interlock Laser ON/OFF and interface for connection of door interlock

The door interlock interface is covered with a green plug to bypass a door interlock.

• To use the interface remove the top of the green plug and the bypass wire.

• Then connect the wires of the door interlock at the same position.

Two door interlocks can be connected.

1.4 Physical Dimensions

	Length (cm)	Width (cm)	Height (cm)	Weight (kg)
Large system table	150	80	78	100
Small system table	65	80	78	60
Passively damped anti-vibration table	130	100	75	137
Active anti-vibration table (NLO) for Mai Tai Laser or Chameleon	150	140	75	200
Active anti-vibration table (NLO) for two-microscope configuration	200	140	75	400
Scanning Module LSM 510	25	20	25	15
Scanning Module LSM 510 META	28	27	30.5	13
Scanning Module LSM 5 MP	25	20	25	15
Scanning Module LSM *DuoScan*	40	15	13	8
Microscope	50	35	50	20
Laser Module RGB	110	70	28	95
Laser Module, UV	140	20	20	60
Laser Module V (405 nm)	66	52	22	30
Plug-in unit external laser	66	52	22	9
System Electronic Rack	110	70	58	90
Power supply for Ar (UV)	50	50	30	30
Cooling unit for Ar (UV)	80	45	50	30
Laser Module *LIVE*	66	52	22	58

1.5 Environmental Requirements

1. Operation, specified performance	T = 22 °C ± 3 °C without interruption (24 h a day independently whether system is operated or switched-off)
2. Operation, reduced performance	T = 10 °C to 35 °C, any conditions different from 1. and 5.
3. Storage, less than 16 h	T = -40 °C to 55 °C
4. Storage, less than 6 h	T = -55 °C to 70 °C
5. Temperature gradient	± 0.5 °C/h
6. Warm up time	1 h, for high-precision and/or long-term measurements ≥ 3 h
7. Relative humidity	< 65 % at 30 °C
8. Operation altitude	max. 2000 m
9. Loss of heat	4 kW

☞ These requirements do not include the requirements for high precision measurements. Please refer to the Operator's Manual of the microscope for these requirements.

1.6 Notes on Setting up the Microscope System

☞ Installation and commissioning of the LSM 510 and LSM 510 META system must be performed by authorized Carl Zeiss service staff. The system should not be used prior to instruction by a Carl Zeiss representative.

The LSM 510 or LSM 510 META laser scanning microscope is delivered in several crates.

⚠ The LSM 510 and LSM 510 META must be set up so as to ensure that the minimum clearance between the wall and the rear of the system is no less than 0.5 m. This clearance is needed for adjustment and maintenance operations.

Do not set up the unit in the proximity of heat sources such as radiators or direct sunlight. To avoid heat build-ups, the ventilation slots on the microscope system must not be covered up.

The system must not be set up in areas with potential danger by explosives.

The unit must be connected to a properly installed socket outlet with earthing contact by means of the mains cables supplied. Continuity of PE connection must not be affected by the use of extension leads.

⚡ The system contains components with dangerous voltage. The system must not be opened by anybody else than authorized Carl Zeiss Service staff. Before opening the main plug has to be disconnected.

Before connecting the mains cables, please check whether your mains voltage corresponds to the voltage specified on the rating plate of the laser module.

For reasons of laser safety, all ports must either be equipped with the corresponding device (scan head, camera, HBO lamp etc.) or covered with the counterpart of the laser safety kit provided.

Maintenance, repair, modification, removal or exchange of components, or other interference with the equipment beyond the operations described in this manual may only be carried out by the manufacturer Carl Zeiss or by persons expressly authorized by Carl Zeiss to do so.
This applies especially to the microscope system, the laser scanning module, lasers, the PC system, the power supply units, cable connections and other system components.

Please note that the LSM 510 and LSM 510 META are high-precision opto-electronic instruments. Inexpert handling may easily impair their function or even damage them.

The openings for ventilation must not be covered.

There are hot surfaces on the HBO and HAL lamp.

When sliding the compact Lasermodule V in and out of the System electronic rack take care not to catch your fingers.

After installation or conversion of the LSM system, authorized specialized staff must carefully check that it is in a proper condition and, particularly, that covers protecting against laser radiation are provided.

Tube openings or other unused mounts should always be protected against dust and moisture with the corresponding device components or with termination covers/blind plugs.

By establishing a corresponding workplace environment, please ensure that the formation of electrostatic charges of electronic components is avoided.

To avoid vibrations during operation, the LSM 510 and LSM 510 META should only be operated in conjunction with the system table (vibration damping).

1.7   Warning and Information Labels

⚠ The warning and information labels attached on the LSM 510 and LSM 510 META must be observed. Check whether all of the labels shown below are provided on your instrument, and contact Carl Zeiss Germany or one of the service agencies if you should discover that any of the labels should be missing. You will receive a free replacement.

Description of labels

⚠ Caution: Faults and hazards that might arise during operation which might cause damage to the unit or injury to the user.

☢ Attention: Laser irradiation hazards possible when operating the system.

⚡ Attention: High voltage.

🔌 Pull the mains plug before opening the device housing.

♨ Caution: Hot surface.

☢ Caution: UV radiation.

⚠ Caution: Fingers can be caught.

▭ The arrow points to the opening where laser light comes out during operation of the system.

☞ Other labels on the system include one of the above depicted symbols and a detailed description of the handling instructions. See also the following drawings of the system parts.
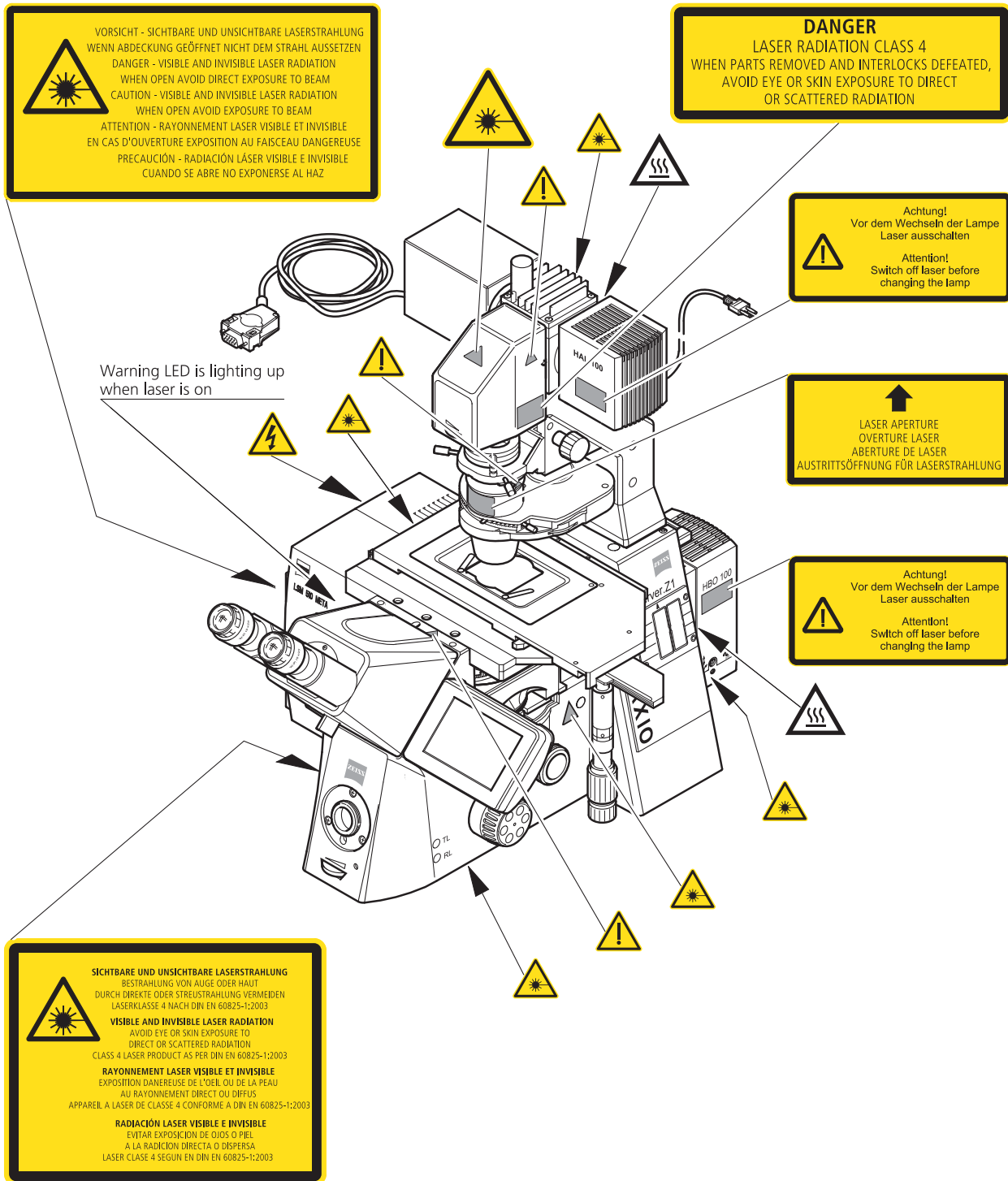
DANGER
LASER RADIATION CLASS 4
WHEN PARTS REMOVED AND INTERLOCKS DEFEATED,
AVOID EYE OR SKIN EXPOSURE TO DIRECT
OR SCATTERED RADIATION

VORSICHT - SICHTBARE UND UNSICHTBARE LASERSTRAHLUNG
WENN ABDECKUNG GEÖFFNET NICHT DEM STRAHL AUSSETZEN
DANGER - VISIBLE AND INVISIBLE LASER RADIATION
WHEN OPEN AVOID DIRECT EXPOSURE TO BEAM
CAUTION - VISIBLE AND INVISIBLE LASER RADIATION
WHEN OPEN AVOID EXPOSURE TO BEAM
ATTENTION - RAYONNEMENT LASER VISIBLE ET INVISIBLE
EN CAS D'OUVERTURE EXPOSITION AU FAISCEAU DANGEREUSE
PRECAUCIÓN - RADIACIÓN LÁSER VISIBLE E INVISIBLE
CUANDO SE ABRE NO EXPONERSE AL HAZ

Achtung!
Vor dem Wechseln der Lampe
Laser ausschalten

Attention!
Switch off laser before
changing the lamp

Warning LED is lighting up
when laser is on

LASER APERTURE
OVERTURE LASER
ABERTURE DE LASER
AUSTRITTSÖFFNUNG FÜR LASERSTRAHLUNG

Achtung!
Vor dem Wechseln der Lampe
Laser ausschalten

Attention!
Switch off laser before
changing the lamp

SICHTBARE UND UNSICHTBARE LASERSTRAHLUNG
BESTRAHLUNG VON AUGE ODER HAUT
DURCH DIREKTE ODER STREUSTRAHLUNG VERMEIDEN
LASERKLASSE 4 NACH DIN EN 60825-1;2003
VISIBLE AND INVISIBLE LASER RADIATION
AVOID EYE OR SKIN EXPOSURE TO
DIRECT OR SCATTERED RADIATION
CLASS 4 LASER PRODUCT AS PER DIN EN 60825-1;2003
RAYONNEMENT LASER VISIBLE ET INVISIBLE
EXPOSITION DANEREUSE DE L'OEIL OU DE LA PEAU
AU RAYONNEMENT DIRECT OU DIFFUS
APPAREIL A LASER DE CLASSE 4 CONFORME A DIN EN 60825-1;2003
RADIACIÓN LASER VISIBLE E INVISIBLE
EVITAR EXPOSICION DE OJOS O PIEL
A LA RADICION DIRECTA O DISPERSA
LASER CLASE 4 SEGUN EN DIN EN 60825-1;2003

Fig. 1-4   Warning and information labels on the Axio Observer.Z1 microscope with the LSM 510 META scanning module
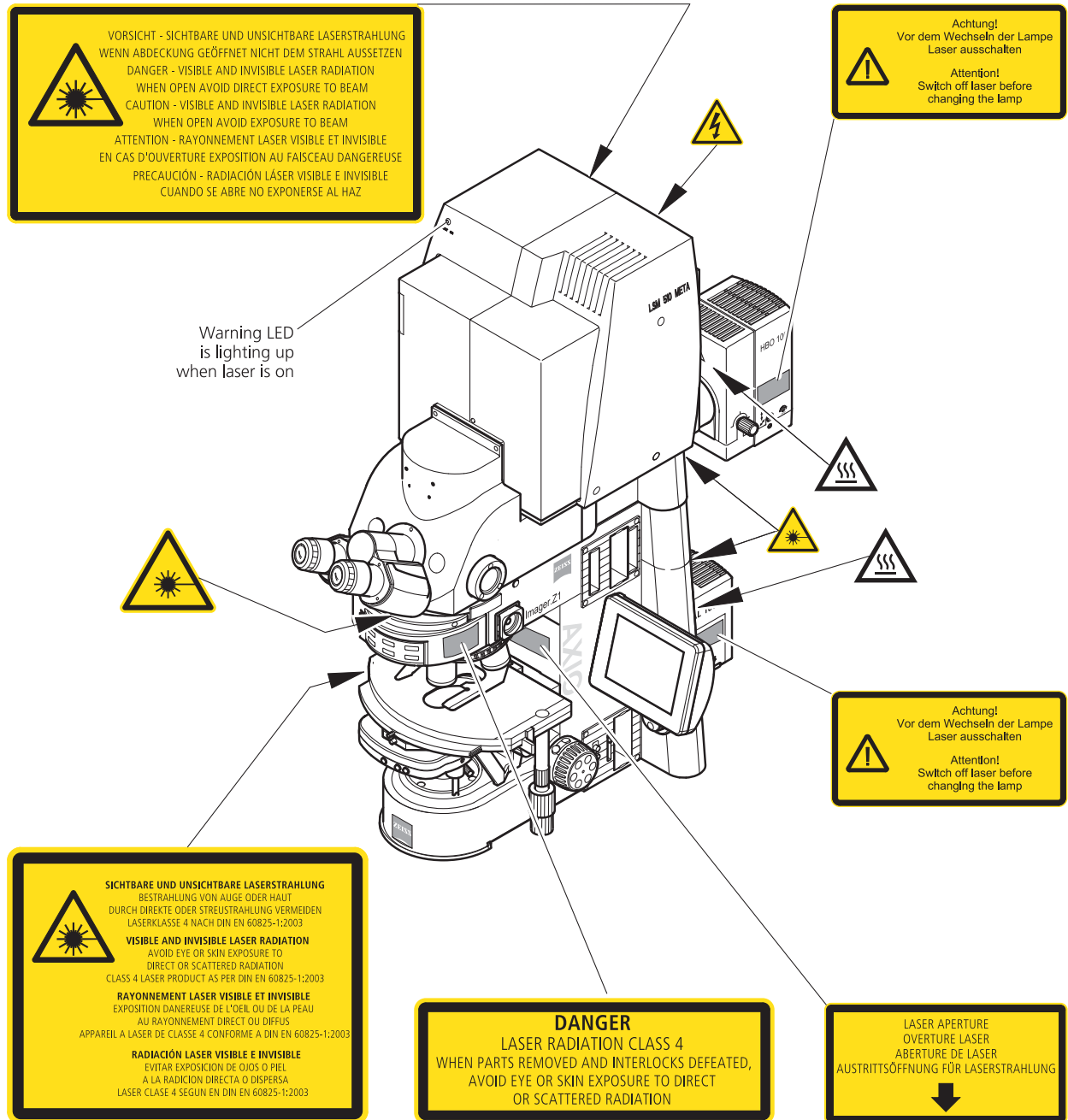
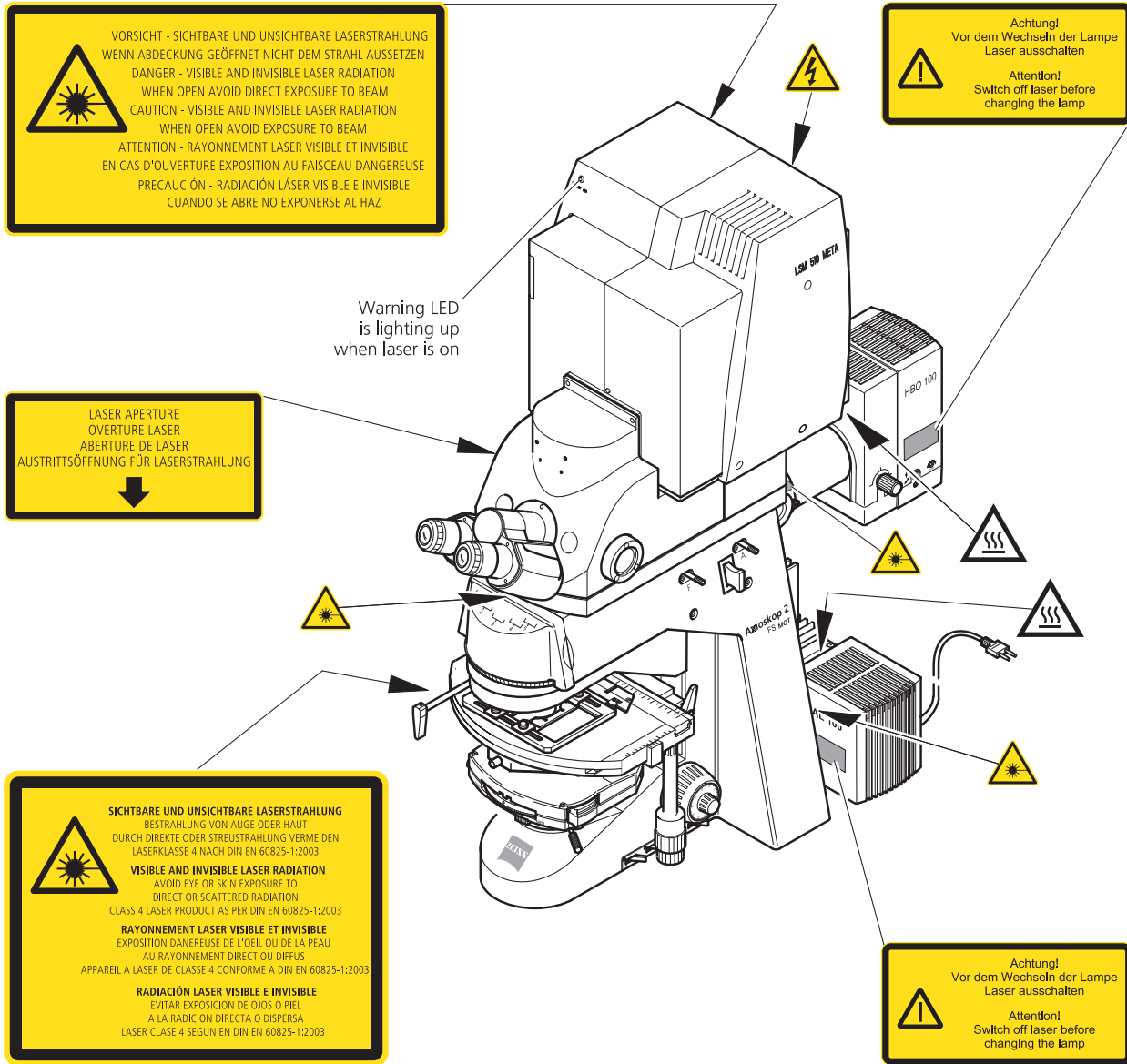**Fig. 1-5   Warning and information labels on the Axio Imager.Z1 microscope with LSM 510 META scanning module**

Fig. 1-6 **Warning and information labels on the Axioskop 2 FS мот microscope with the LSM 510 META scanning module**
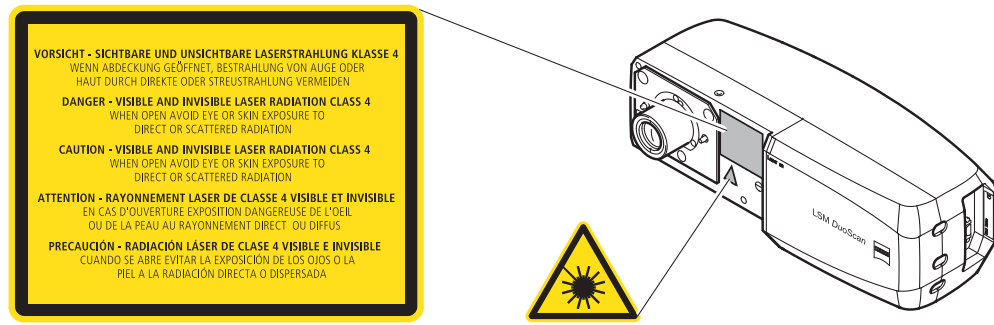
VORSICHT - **SICHTBARE UND UNSICHTBARE LASERSTRAHLUNG KLASSE 4**
WENN ABDECKUNG GEÖFFNET, BESTRAHLUNG VON AUGE ODER
HAUT DURCH DIREKTE ODER STREUSTRAHLUNG VERMEIDEN

DANGER - VISIBLE AND INVISIBLE LASER RADIATION CLASS 4
WHEN OPEN AVOID EYE OR SKIN EXPOSURE TO
DIRECT OR SCATTERED RADIATION

CAUTION - VISIBLE AND INVISIBLE LASER RADIATION CLASS 4
WHEN OPEN AVOID EYE OR SKIN EXPOSURE TO
DIRECT OR SCATTERED RADIATION

ATTENTION - RAYONNEMENT LASER DE CLASSE 4 VISIBLE ET INVISIBLE
EN CAS D'OUVERTURE EXPOSITION DANGEREUSE DE L'OEIL
OU DE LA PEAU AU RAYONNEMENT DIRECT OU DIFFUS

PRECAUCIÓN - RADIACIÓN LÁSER DE CLASE 4 VISIBLE E INVISIBLE
CUANDO SE ABRE EVITAR LA EXPOSICIÓN DE LOS OJOS O LA
PIEL A LA RADIACIÓN DIRECTA O DISPERSADA

Fig. 1-7   **Warning and information labels on LSM *DuoScan* (systems LSM 510 *DuoScan* or LSM 510 META *DuoScan* only)**

Vor Öffnen Netzstecker ziehen

Caution, disconnect
power supply
before opening

Débrancher la fiche
d'alimentation secteur
avant d'ouvrir

Achtung!
Hauptschalter erst ausschalten, wenn
Lasermodulkühlung abgeschaltet.

Attention!
Don't turn off main power
until laser cooling has finished.

DANGER

VISIBLE AND INVISIBLE LASER RADIATION
AVOID DIRECT EXPOSURE TO BEAM

Laser (400-700nm) 0.5W max. output

CLASS IIIB LASER PRODUCT

SICHTBARE UND UNSICHTBARE LASERSTRAHLUNG
NICHT DEM STRAHL AUSSETZEN LASER KLASSE 3B NACH DIN EN 60825-1;2003

VISIBLE AND INVISIBLE LASER RADIATION
AVOID EXPOSURE TO BEAM
CLASS 3B LASER PRODUCT AS PER DIN EN 60825-1:2003

RAYONNEMENT LASER VISIBLE ET INVISIBLE
EXPOSITION AU FAISCEAU DANGEREUSE APPAREIL À
LASER DE CLASSE 3B CONFORME À DIN EN 60825-1:2003

RADIACIÓN LÁSER VISIBLE E INVISIBLE
NO EXPONERSE AL HAZ LÁSER CLASE 3B SEGÚN EN DIN EN 60825-1:2003

LASER (400-700)nm 0.5W max. output

Fig. 1-8   **Warning and information labels on the system electronic rack**

Laser module V (405 nm)

Laser module RGB
(458, 477, 488, 514,
543/561, 594, 633 nm)

Fig. 1-9   Warning and information labels on laser components
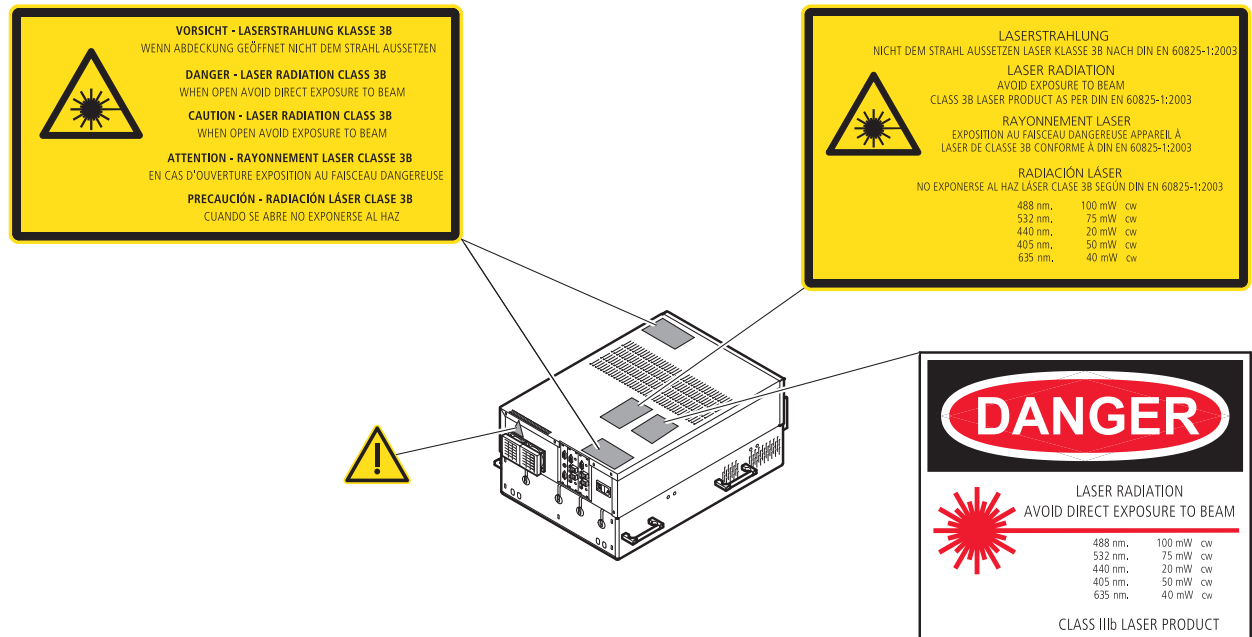
Fig. 1-10   Warning and information labels on laser module LSM 5 *LIVE*

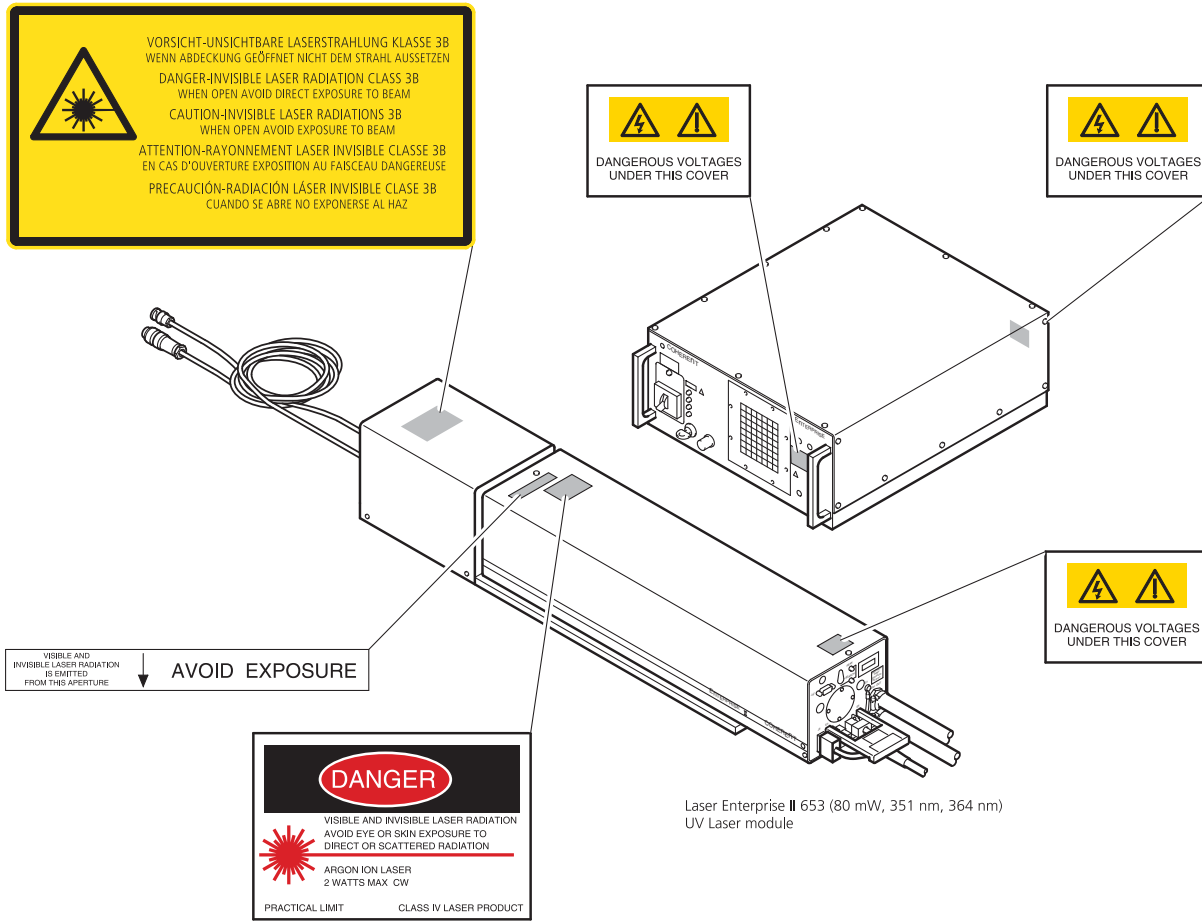VORSICHT-UNSICHTBARE LASERSTRAHLUNG KLASSE 3B
WENN ABDECKUNG GEÖFFNET NICHT DEM STRAHL AUSSETZEN

DANGER-INVISIBLE LASER RADIATION CLASS 3B
WHEN OPEN AVOID DIRECT EXPOSURE TO BEAM

CAUTION-INVISIBLE LASER RADIATIONS 3B
WHEN OPEN AVOID EXPOSURE TO BEAM

ATTENTION-RAYONNEMENT LASER INVISIBLE CLASSE 3B
EN CAS D'OUVERTURE EXPOSITION AU FAISCEAU DANGEREUSE

PRECAUCIÓN-RADIACIÓN LÁSER INVISIBLE CLASE 3B
CUANDO SE ABRE NO EXPONERSE AL HAZ

DANGEROUS VOLTAGES
UNDER THIS COVER

DANGEROUS VOLTAGES
UNDER THIS COVER

DANGEROUS VOLTAGES
UNDER THIS COVER

VISIBLE AND
INVISIBLE LASER RADIATION
IS EMITTED
FROM THIS APERTURE

AVOID EXPOSURE

DANGER

VISIBLE AND INVISIBLE LASER RADIATION
AVOID EYE OR SKIN EXPOSURE TO
DIRECT OR SCATTERED RADIATION

ARGON ION LASER
2 WATTS MAX CW

PRACTICAL LIMIT   CLASS IV LASER PRODUCT

Laser Enterprise II 653 (80 mW, 351 nm, 364 nm)
UV Laser module

Fig. 1-11   Warning and information labels on laser components

1.8 Notes on Handling the Laser Components and Illumination Systems

The LSM 510 and LSM 510 META are laser hazard class 3B instruments. If equipped with a Ti:Sa Laser, the LSM 510 and LSM 510 META are devices that belong to laser hazard class 4.
These moderate and high-risk classes embrace medium-power and high power lasers. You must take care not to expose yourself to the radiation of such lasers. In particular, never look into the laser beam! Only personnel which has been instructed on laser safety is allowed to operate the system.

The following laser types are currently intended for use in the LSM 510 and LSM 510 META. The use of any other lasers as the ones listed below is not authorized.

Laser	Class	Power
1 Ar 351/364 nm (UV)	4*	80 mW
2 Diode laser 405 nm	3B	30 mW
3 Ar/ML 458/477/488/514 nm	3B	30 mW
4 HeNe 543 nm	3B	1 mW
5 DPSS 561 nm	3B	10 mW
6 HeNe 594 nm	3B	2 mW
7 HeNe 633 nm	3B	5 mW
8 Titanium:Sapphire Laser Mai Tai (Spectra Physics) 690-1040 nm (depending on the model)	4	minimum 3 W
9 Titanium:Sapphire Laser Chameleon (Coherent) 690-1064 (depending on the model)	4	minimum 3 W
10 Diode laser 405 nm	3B	50 mW
11 OPSS laser 488 nm	3B	100 mW
12 DPSS laser 532 nm	3B	75 mW
13 DPSS laser 561 nm	3B	40 mW

* Laser type class 4, if mounted on laser module with fiber output class 3B.

Please note that for the maintenance of the UV Laser it is recommended to run the laser at maximum power once a day if the laser is not used frequently or only at low power levels. This enables the Autofill process which keeps up the correct tube gas pressure. This operation prolongs the life time of the tube and prevents complete tube failure if the laser is not used for a prolonged period of time. For details please refer to the Operator's Manual of the UV laser.

☞   Please contact Carl Zeiss if you intend to use a different laser other than the ones above.

If used properly, the LSM 510 and LSM 510 META will not pose any laser radiation risks for operating staff. Nevertheless, you should observe the following warnings:

- If necessary - insofar as specified by law - inform the laser protection officer before commissioning the laser.

- The laser modules are equipped with a key-interlock.

- Always store keys for laser key switches and, if applicable, keys for further laser power supply units, where they are inaccessible to persons not authorized to operate the laser.

- A red LED on the front of the scan head lights up when one or all of the lasers are switched on.

- Do not place any reflecting objects into the beam path.

- Never open any covers or panels.

- Never look into the laser beam, not even to simply view the specimen, whether with the aid of optical instruments or without. Otherwise you risk going blind!

- Do not leave any empty objective positions of the nosepiece uncovered.

- If a class 4 laser is attached to the system, already stray light can impose danger to the operator.

- With class 4 lasers take special care of fire protection requirements. Do not use or store flammable or explosive solids, fluids or gases in the vicinity of the system.

- Class 4 lasers can inflame also flammable materials like cloth or paper. Do not put such materials into the beam path.

- Do not reach into the process beam inside the sample area whenever the Class 4 laser is active!

☞   Suitable protective measures must be taken if gases, dust or vapors hazardous to health, secondary radiation or explosive objects should arise on the specimen as a result of laser radiation.

When using a high power broadband illumination (HBO, X-Cite, HXP) in the incident light path in combination with a neutral density reflector module in the microscope stand, there are operating conditions which lead to strong reflections of excitation light into the eyepiece. According to regulations for radiation load the maximal values are below the permissible threshold and do not lead in all probability to any permanent irritation of the eyes also because the natural averting reaction limits the exposure to the bright light. Nevertheless we recommend not to look through the eyepiece when rotating the reflector turret. Especially, we do not recommend the use of the reflector turret controls located on the microscope stand, since this leads to an observation position close to the eyepiece. Instead, use the TFT-Touchscreen or the LSM software to switch the reflector turret. In case the reflector turret has to be rotated by hand avoid looking into the eyepiece while doing so.

For NLO systems equipped with a specific push and click filter for NDD imaging be aware that the NDD reflector cube in the reflector turret leads to a strong back reflection of HBO light into the specimen plane and the eyepiece. When observing the specimen through the ocular lens the use of the NDD reflector cube should be avoided. The light flash is not harmful but unpleasant. The reflex of closing the eyelid is sufficiently protective. To completely avoid this situation an additional filter (#1261-345) can be mounted into the NDD reflector cube which prevents the back reflection of the HBO light in the ocular plane.

1.9   Notes on Handling the Computer and Data Media

The computer used as standard in your LSM system is an IBM-compatible high-end Pentium computer with WINDOWS XP operating system.

Do make sure, though, that you receive your LSM system with the operating system installed, with initialization and start files set up and with the LSM program also installed.

When working with the hard disk, it is important to know that the more data it contains, the slower its operation will become. Therefore, data that you do not need permanently should be stored on other external devices.

When handling diskettes and USB sticks, avoid data losses by protecting them against extreme temperatures, moisture and magnetic fields. The data on a diskette is stored in the form of magnetic signals. To some extent, monitors, telephones or even lamps generate magnetic fields that might destroy this data. Also, never open the metal cover on diskette cases. A diskette's surface can also be destroyed by touching it.

When handling CDs, CD ROMs or DVDs, do not touch the data side of the disc (the side of the disc with no label or printing).

Do not apply paper labels or write on any part of the disc, data side or label side. If dust or fingerprints get on the disc, wipe it with a soft cloth from the center to the edge, but do not use benzine, paint thinner, record cleaner, or static repellent. This can damage the disc.

Do not place the disc in any place where it is exposed to direct sunlight or high temperatures.

Backup your data on a regular basis.

Do not install any other software without talking to your Carl Zeiss representative.

Never turn your computer off before you have terminated the LSM program and run down the WINDOWS XP operating system. Otherwise, the program and/or data files may get lost.

The LSM computer is a system computer controlling a high end microscope system. The computer must not to be used as a general workstation with frequent updates of windows patches or security patches. The LSM software is released with a specific windows version and patch and this must not be changed unless otherwise released by Carl Zeiss MicroImaging.

1.10 Notes on Care, Maintenance and Service

The manufacturer of the unit cannot be held liable for damage resulting from operating errors, negligence or unauthorized tampering with the device system, particularly as the result of removal or replacement of parts of the unit or as the result of the use of unsuitable accessories from other manufacturers. Any such action will render all warranty claims null and void and also laser safety is no longer warranted.

You are well advised to arrange a service agreement with your nearest Carl Zeiss representative to guarantee perfect functioning of the microscope system in the long term.

Use only original spare parts.

The customer has the possibility to change defective fuses. The positions of the fuses are shown in Fig. 1-1 and Fig. 1-2. The fuses are inserted into fuse holders (normally used for power supply units), bayonet type fuses carriers or screw type fuse carriers. The fuse type is given on the hardware itself and also in Fig. 1-1 and Fig. 1-2.

Modifications and conversion work on the components of the system must only be carried out by the manufacturer, by the service agency or by persons authorized and trained for this purpose by the manufacturer.

Damaged units or parts may only be repaired or maintained by the responsible service agency.

During maintenance or repair carried out by the service personnel the customer is requested to stand aside and wear a pair of laser safety goggles if needed.

⚠ Before opening the housing of the halogen lamp switch off all laser units.

Care operations that may be carried out by operating staff are limited to cleaning painted and glass surfaces.

- Before cleaning the instrument make sure the main power supply is disconnected.

- Cleaning painted surfaces
  To do this, use a clean cloth that has been moistened in a mixture of water and some detergent; do not use any solvent, however. Dry with a lint-free cloth.

- Cleaning glass surfaces
  Glass surfaces that have become soiled or which are marked with fingerprints may be rubbed with a clean optical cleaning cloth.
  If soiling is persistent, dip the optical cleaning cloth into a mixture of distilled water and a small quantity of detergent.
  To complete cleaning, lightly breathe on the glass surface and rub it dry with a clean cloth. Lint or dust is best removed with a clean brush.

- Make sure that no cleaning liquid penetrates into the system.

- Dust filters in the ventilation entries of the system electronic rack have to be replaced every 6 month. For replacement please contact your local service representative.

1.11 User Interface

![laser warning symbol] All user interface ports are equipped with a safety interlock system which warrants laser safety. These interlock devices must not be manipulated. Other interfaces which are not described here are service interfaces and are only to be operated by authorized Carl Zeiss service personnel. The following devices can be mounted and dismounted by the user:

- – Halogen and HBO lamp
- – Transmission PMT
- – Switching Mirror
- – Scan head

1.11.1 Mounting and Dismounting Lamps, TPMT and Switching Mirror

The ports of the lamps, the switching mirror and the transmission PMT are equipped with hardware interlock devices. At most ports (all ports of the Axioskop 2 FS MOT and the Axio Imager.Z1 as well as the HBO port of the Axio Observer.Z1) the following interlock devices are present and have to be operated in the following way:

Interlock with sensor ring and contact ring:
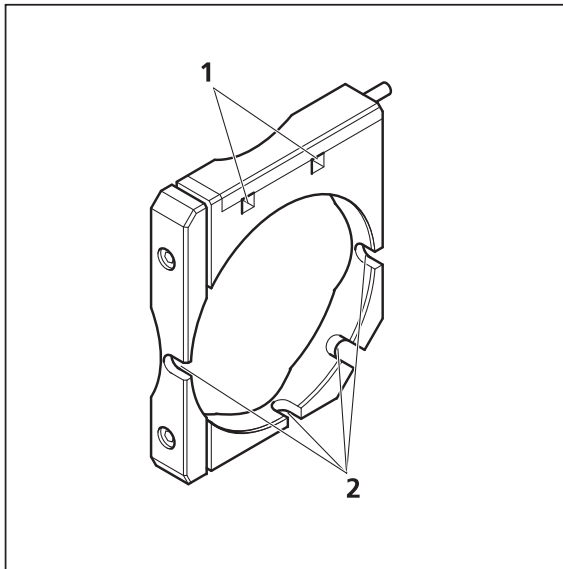
Fig. 1-12   **Sensor ring mounted to the interface ports on the microscope side**
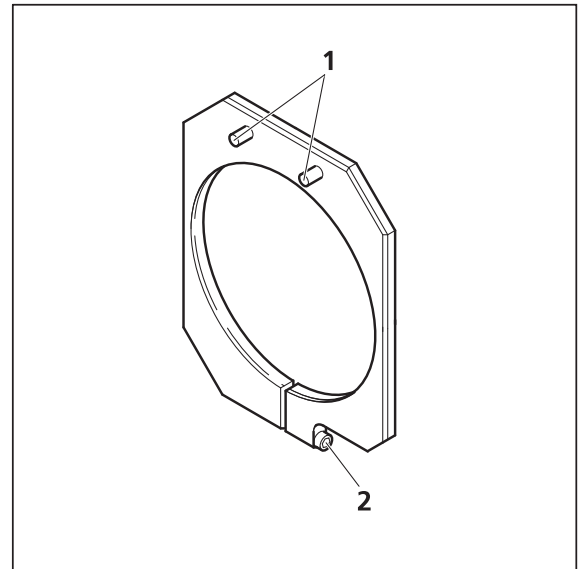
Fig. 1-13   **Contact ring mounted to the lamp, TPMT or switching mirror**

The interlock is working when the sensors of the sensor ring (Fig. 1-12/**1**) are depressed by the pins on the contact ring (Fig. 1-13/**1**). Whenever this is not the case, for example if the distance between the two devices is too large, the laser will be blocked and the system cannot be used.

☞ In case the system is not operating following the removal or attachment of any device on a port with safety interlock check again the connection of the Contact ring to the Sensor ring.
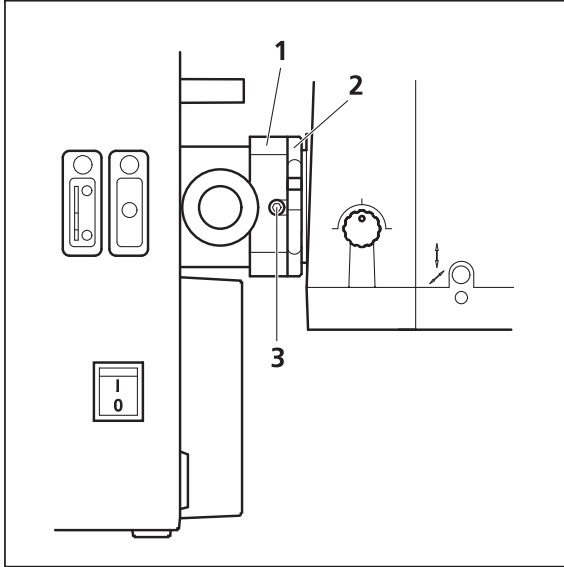
Fig. 1-14   HBO lamp mounted to Axio Observer.Z1 with sensor ring and contact ring for laser safety

For dismounting the lamps, TPMT or switching mirror slightly unscrew the contact ring first (Fig. 1-13/**2** or Fig. 1-14/**2**) so it can be moved away from the sensor ring (Fig. 1-14/**1**). Then unscrew the lamp, TPMT or switching mirror (main screw

Fig. 1-14/**3**) which is in one of the recesses of the sensor ring (Fig. 1-12/**2**). Hold the device to be dismounted with one hand while unscrewing to keep it from dropping. The now empty port has to be closed with the blind cap to restore the functionality of the system. Use the main screw of the port to fix the cap. Make sure the pins of the cap depress the sensors of the sensor ring.

☞   Do not remove the sensor ring from the microscope. This might result in failure of laser safety and a non operating system.
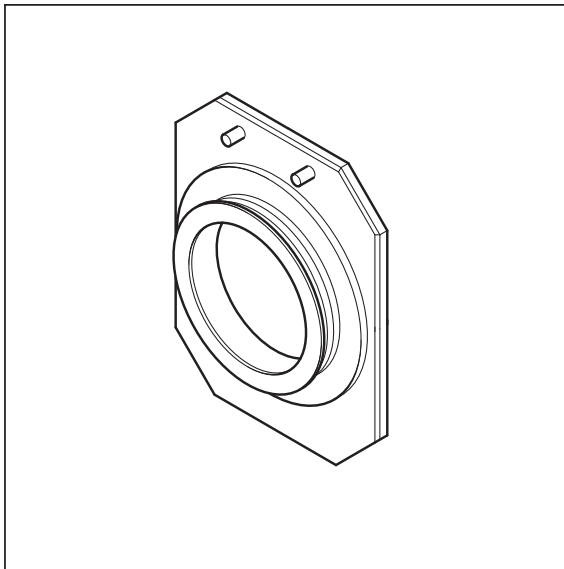
Fig. 1-15   Blind cap for closing any port equipped with an interlock device

For mounting any lamp, TPMT or the switching mirror back onto the microscope reverse the steps for dismounting the device. Be careful not to bend the pins on the contact ring when screwing the device onto the microscope port.

For the Axio Observer.Z1 transmission port and the two ports available on the motorized switching mirror no sensor ring is present. Instead the sensors are directly installed at the Axio Observer.Z1 transmission port or the two ports of the motorized switching mirror.

1.11.2   Mounting and Dismounting the Scan Heads

The scan heads are connected to the microscope via an integrated safety interlock. They can be moved between two microscopes. Make sure the system is shut off completely before starting the following procedure:

☞   Be aware that the scan head weights up to 15 kg.

Moving the scan heads between Axio Observer.Z1, Axioskop 2FS мот and Axio Imager.Z1:

- LSM 510 META scan head: Remove the blue cover from the back of the scan head (Fig. 1-16 and Fig. 1-17).
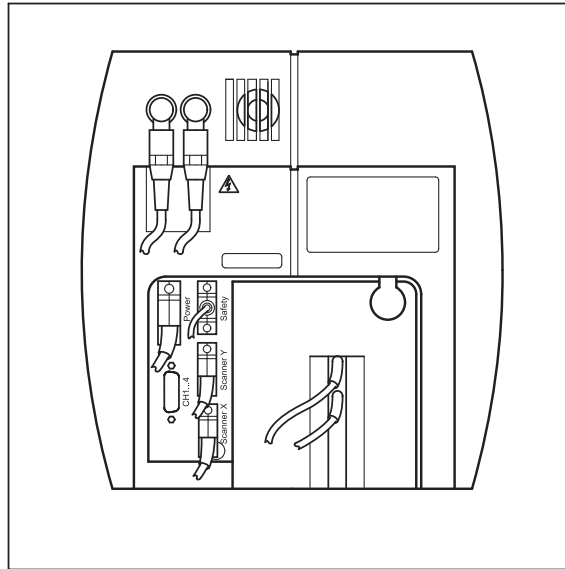
Fig. 1-16   Rear view of META scan head
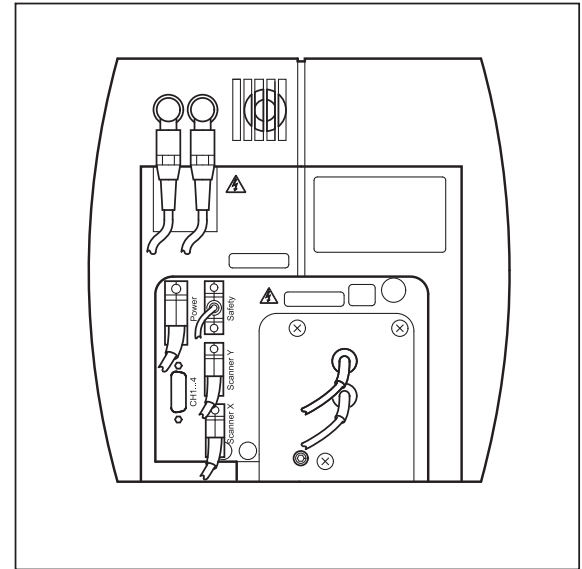
Fig. 1-17   Rear view of META scan head with blue cover removed

- Loosen the screw on the lower end of the scan head (Fig. 1-18/**1** and Fig. 1-19/**1**)
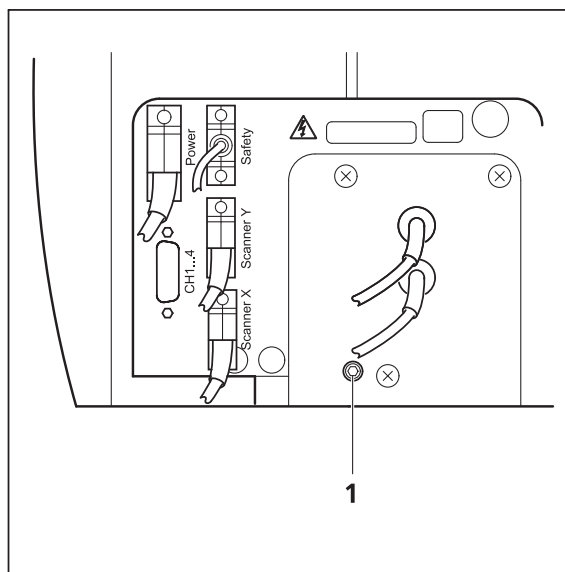
Fig. 1-18   Position of the screw on the LSM 510 META scan head
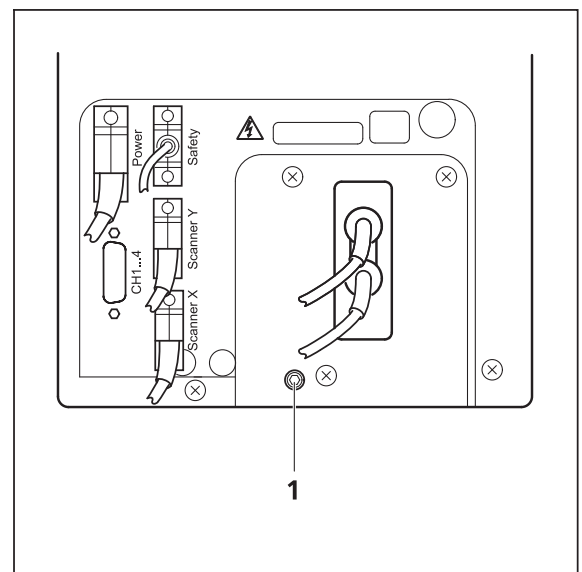
Fig. 1-19   Position of the screw on the LSM 510 scan head

- Loosen the screws on the port to the microscope for Axio Observer.Z1 (Fig. 1-20/**1**) or to the tube on Axio Imager.Z1 and Axioskop 2 FS MOT (Fig. 1-21/**1**).
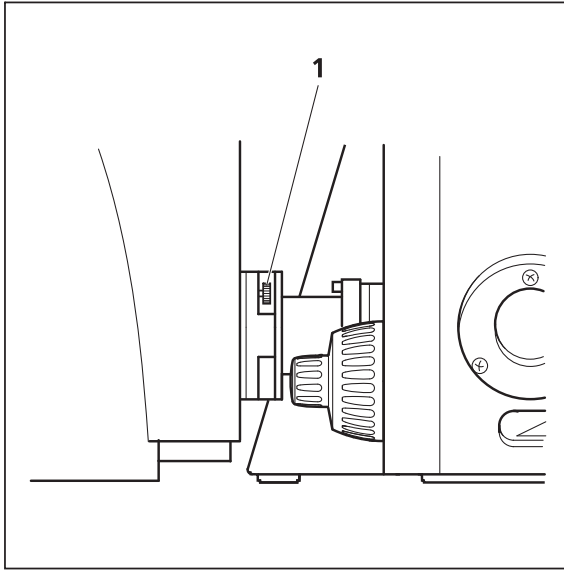
Fig. 1-20   Port connection between LSM and Axio Observer.Z1
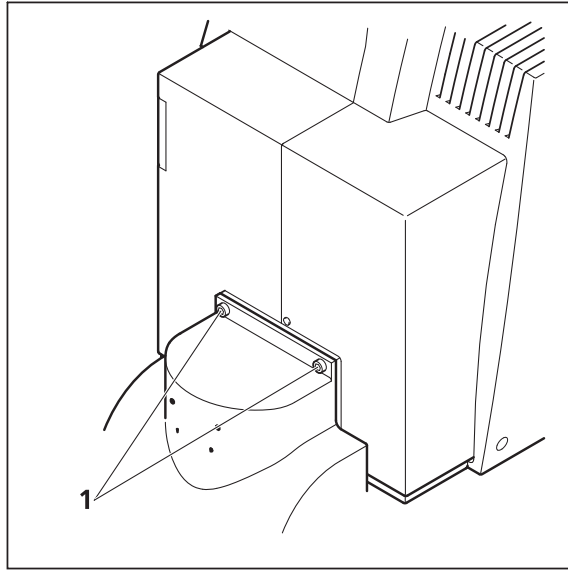
Fig. 1-21   Fastening screws of the scan head at the front of the tube on Axioskop 2 FS MOT and Axio Imager.Z1

- Slowly pull the scan head away from the microscope port or the tube. For mounting the scan head onto a microscope, make sure the pins and the electronic connections of the safety interface match closely. Fasten the screws on the front of the tube (Fig. 1-21/**1**) or at the microscope port (Fig. 1-20/**1**) followed by fastening the screw on the back of the scan head (Fig. 1-18/**1** and Fig. 1-19/**1**).

- Use the three fastening screws (Fig. 1-22/**1**) to attach or detach the LSM *DuoScan* to or from the microscope stand.
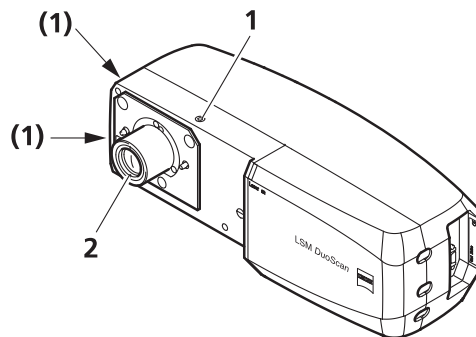
Fig. 1-22   Fastening screws and connections on LSM DuoScan

For NLO systems which are set up together with a Multiphoton laser, the beam tube connecting the periscope and the scan head, has to be removed before the scan head can be dismounted. Perform the following steps for the removal of the tube:

- First pull out the tube from the periscope cover (Fig. 1-24/**5**) by sliding the inner tube into the outer tube (Fig. 1-24/**4**).

- Turn the tube assembly counter clockwise to unscrew the tube (Fig. 1-24/**4**) from the small black tube (Fig. 1-24/**2**) which is fixed to the scan head (Fig. 1-24/**3**).

- Now the tube can be removed and you can proceed as described above.

- For rearranging the beam housing, first screw the tube onto the small black tube (Fig. 1-24/**2**) at the back of the scan head (Fig. 1-24/**3**). It should not be fastened completely to allow the inner tube to fit the side pins into the recess of the ring on the periscope (Fig. 1-24/**5**).

- Open the shutter in the periscope using an Allen key (Fig. 1-23/**1**) and slide in the tube completely, this will hold the shutter open.
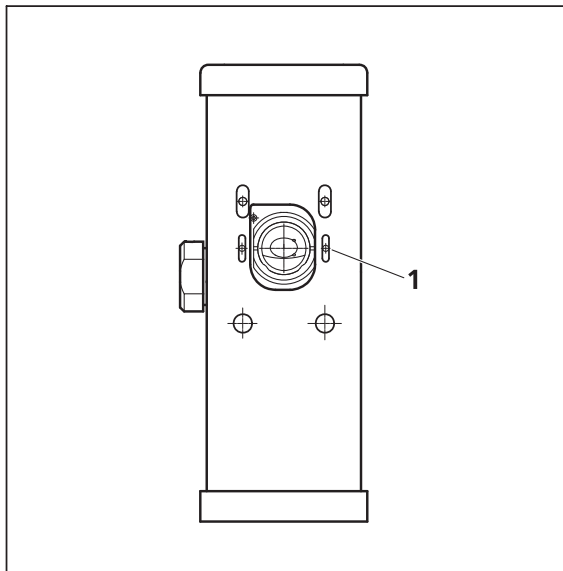
Fig. 1-23   Shutter opening on the periscope. Also applies for the large periscope for upright microscopes.
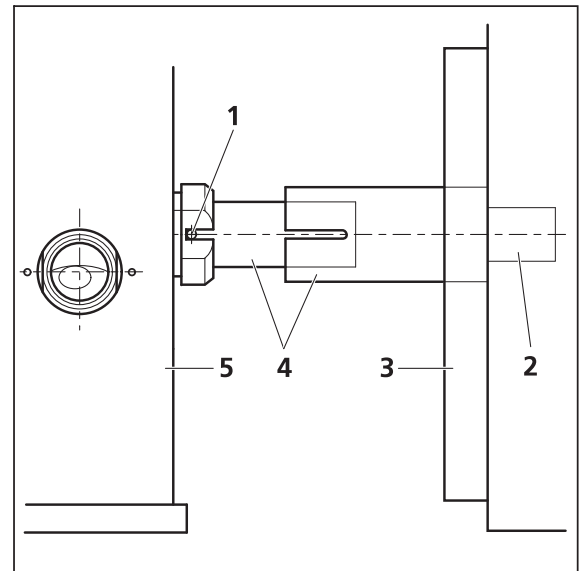
Fig. 1-24   Tube connection from the periscope housing (5) to the scan head (3). Applies for upright and inverted microscopes.

To ensure functioning of the system and laser safety the following connections have to be changed:

1. The connection of the microscope to the safety interface of the system is located either on the additional Safety-Box (Axioskop 2 FS MOT, Fig. 1-25/**1**) or on the rear side of the microscope (Axio Imager.Z1 or Axio Observer.Z1; Fig. 1-26/**1** and Fig. 1-27/**1**). This connection has to be unplugged from the microscope which is not in use and plugged into the microscope to be used following the exchange of the scan head.
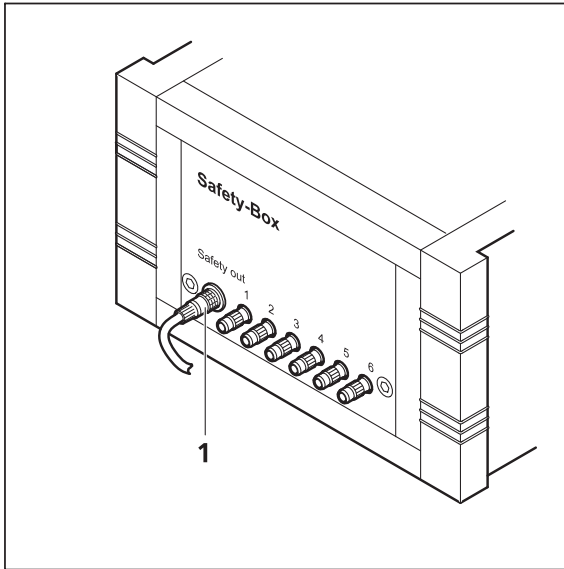
Fig. 1-25   Safety-Box of Axioskop 2 FS MOT with main connection to safety interface (1)
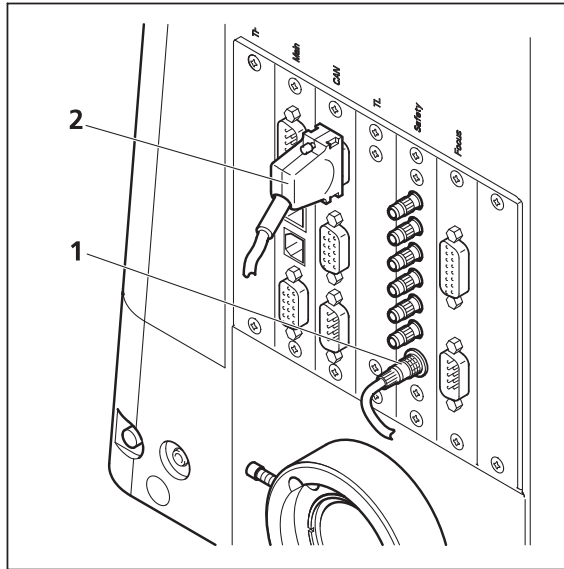
Fig. 1-26   Connection of Axio Imager.Z1 to safety interface (1) and electronics (2)
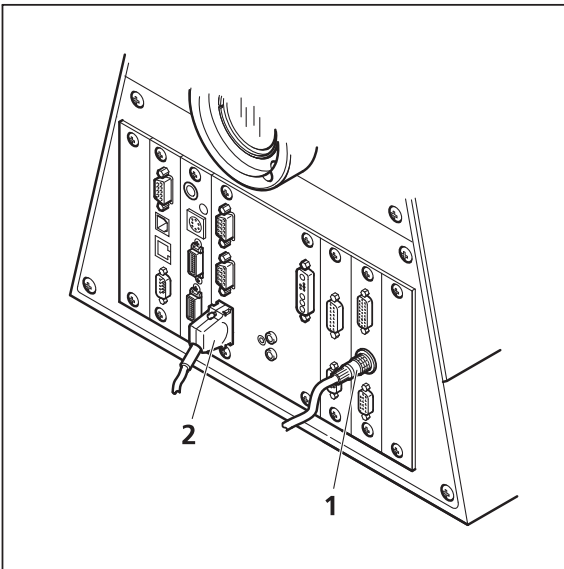
Fig. 1-27   Connection of Axio Observer.Z1 to safety interface (1) and electronics (2)
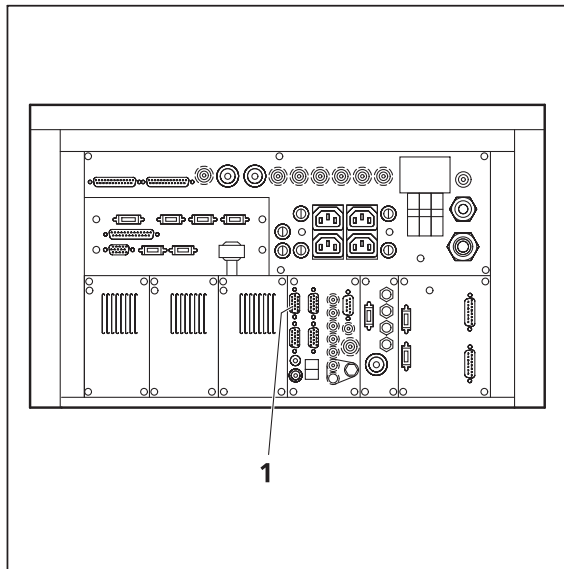
Fig. 1-28   Four CAN connections (1) are available on the rear side of the electronics module. The microscope in use has to be connected to one of them

2.　The plug for the main connection of the microscope to the electronics is situated on the rear side of the electronics rack. It is either one of the four CAN connections shown in Fig. 1-28. Only ONE microscope can be connected to the electronics at a time. The connection has to be plugged in and unplugged at the electronics rack. The corresponding connections on the microscopes are shown in Fig. 1-26/**2**, Fig. 1-27/**2** and Fig. 1-29/**1**. Remove the connection of the microscope no longer in use at the electronics rack and plug in the connection of the microscope which should be used instead.

3.　After the connections of the non used micro-scope are disconnected and the ones of the used microscope are connected, the system can be switched on again. Before initializing the system with the LSM Software make sure to use the right database according to the microscope in use. It can be chosen via the icon Stand Select.
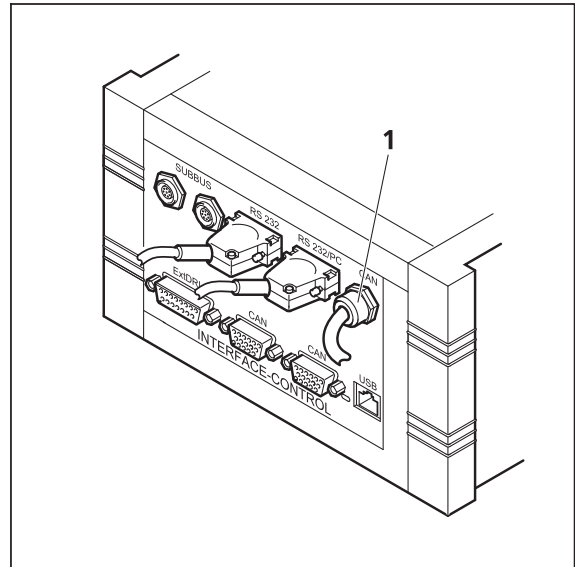
Fig. 1-29　　**The Axioskop 2 FS** ᴍᴏᴛ **is connected to the electronics via the Interface Control. The CAN connection is fixed (1) to the control box**

CHAPTER 2   SETUP REQUIREMENTS

CONTENTS

Page

2   SETUP REQUIREMENTS

2.1   Space Requirements

2.1.1   LSM on large system table (one microscope, upright or inverted): 2 m × 3.50 m
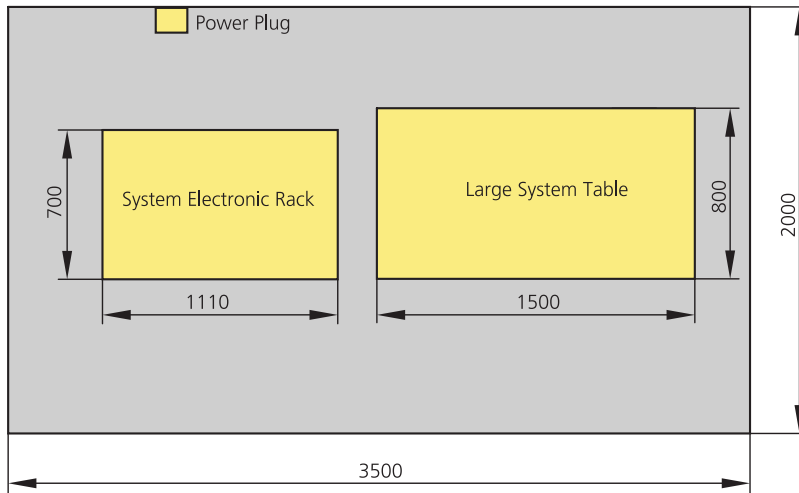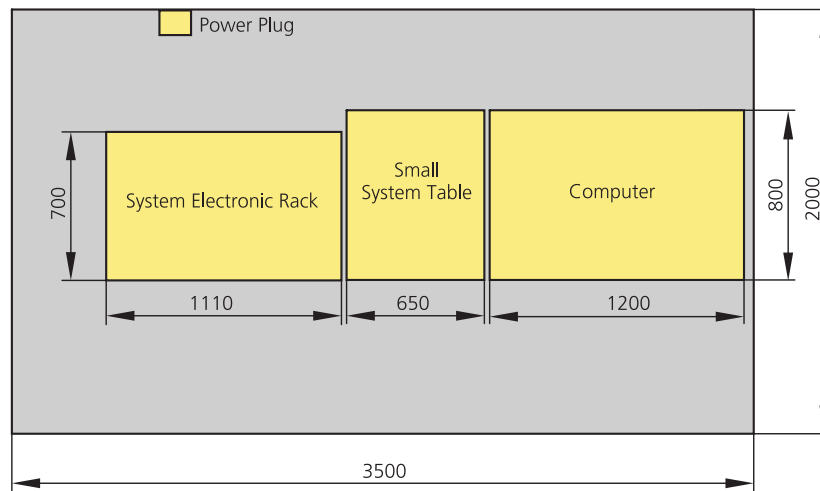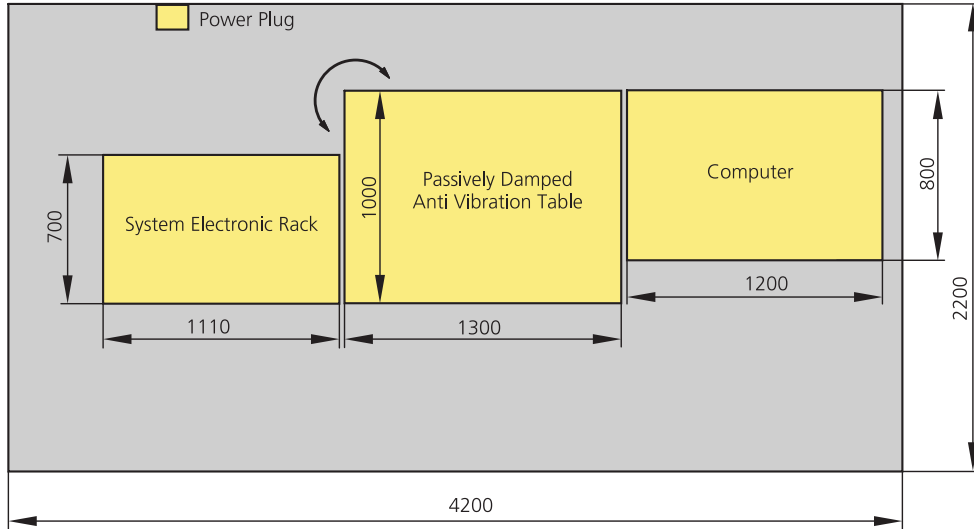

**Fig. 2-1   Space requirements for LSM on large system table
                    (measurements in mm)**

The System Electronic Rack contains all electronics for control of the hardware components of the LSM system, the power supply for the microscope, the scanning unit, the Laser Module V and the Laser Module RGB equipped with the choice of lasers. The Laser Module RGB is set on top of the System Electronic Rack.

☞   On this table the extension of the system with LSM *DuoScan* is not possible!

2.1.2   LSM on small system table (one microscope, upright or inverted): 2 m x 3.5 m

Power Plug

| System Electronic Rack | Small System Table | Computer |
700
1110   650   1200
800 2000
3500

**Fig. 2-2   Space requirements for LSM on small system table
(measurements in mm)**

The System Electronic Rack contains all electronics for control of the hardware components of the LSM system, the power supply for the microscope, the scanning unit, the Laser Module V and the Laser Module RGB equipped with the choice of lasers. The Laser Module RGB is set on top of the System Electronic Rack.

☞   On this table the extension of the system with LSM *DuoScan* is not possible!

2.1.3   LSM on passive anti vibration table (one microscope, upright or inverted): 2.2 m x 4.2 m

Fig. 2-3   Space requirements for LSM on Passively Damped Anti Vibration Table (system table with breadboard).
Depending on the ordered table it might be turned by 90°.
(measurements in mm)

The System Electronic Rack contains all electronics for control of the hardware components of the LSM system, the power supply for the microscope, the scanning unit, the Laser Module V and the Laser Module RGB equipped with the choice of lasers. The Laser Module RGB is set on top of the System Electronic Rack. With the extension of LSM *DuoScan* (VIS and/or 405 nm) the Laser Module *LIVE* is situated in the System Electronic Rack. It substitutes the Laser module V. With the extension of LSM *DuoScan* UV the system has to be set up according to chapter 2.1.5

2.1.4   LSM with Ar UV Laser

☞   We recommend placing the cooling unit of the Ar laser (UV) in a separate room to prevent heat accumulation and vibration. Length of the water hose: 400 cm

One microscope: 2.5 m x 3 m
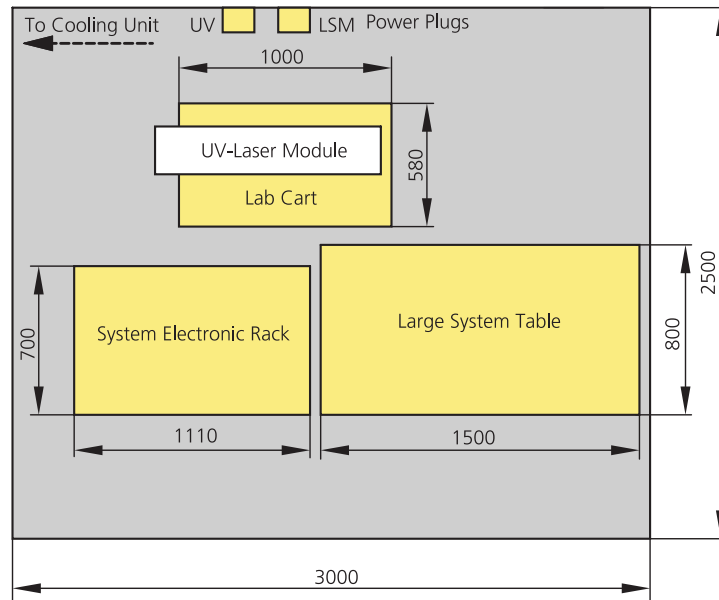
Fig. 2-4   Space requirements for LSM using one microscope and AR UV Laser (measurements in mm)
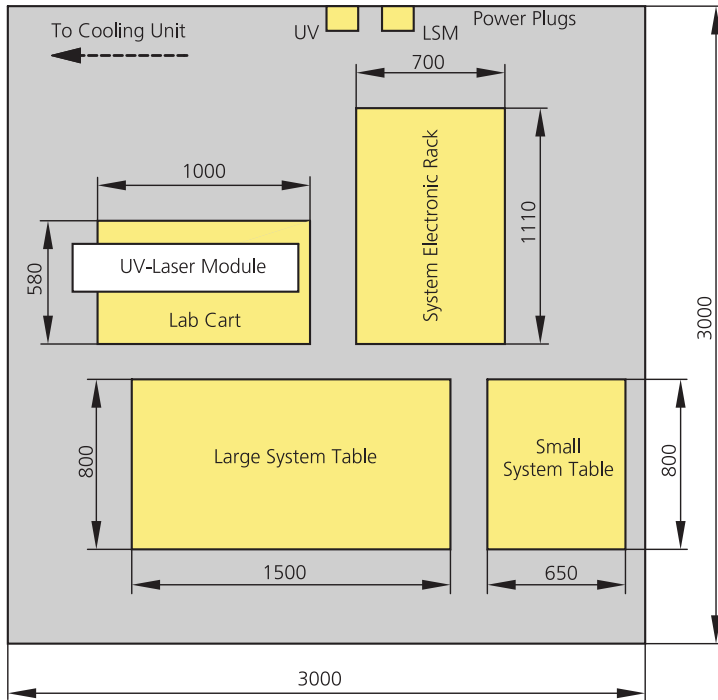
Two microscopes: 3 m x 3 m

To Cooling Unit
UV · LSM · Power Plugs
700
1000
System Electronic Rack
1110
580
UV-Laser Module
Lab Cart
3000
800
Large System Table
Small System Table
800
1500
650
3000

Fig. 2-5 **Space requirements for LSM with two microscopes and AR UV Laser (measurements in mm)**

The System Electronic Rack contains all electronics for control of the hardware components of the system, the power supply for the microscope, the scanning unit and the Laser Module RGB equipped with the choice of lasers. The Laser Module RGB is set on top of the System Electronic Rack. The lab cart holds the power supply for the UV laser and the UV Laser module.

☞ On this table the extension of the system with LSM *DuoScan* is not possible (see chapter 2.1.5)!

2.1.5 LSM 510 or LSM 510 META equipped with LSM *DuoScan* UV
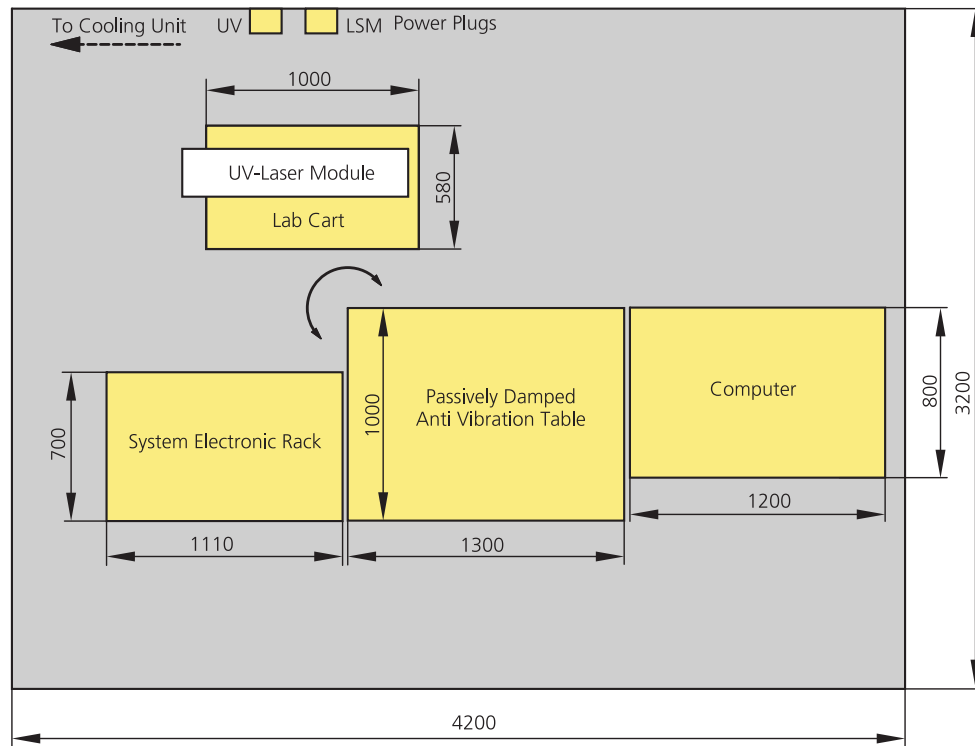
Fig. 2-6 **Space requirements for LSM equipped with LSM *DuoScan* and AR UV Laser (measurements in mm)**

The System Electronic Rack contains all electronics for control of the hardware components of the LSM system, the power supply for the microscope, the scanning unit and the Laser Module RGB equipped with the choice of lasers. The Laser Module RGB is set on top of the System Electronic Rack.

An additional lab cart is recommended which then contains the plug in unit for the UV laser and on top of it the UV laser itself.

2.1.6   LSM 5 MP or LSM equipped with Two Photon Laser (NLO)

2.1.6.1   Coherent "Chameleon" or Spectra Physics "Mai Tai". Directly-coupled to upright or inverted Microscope
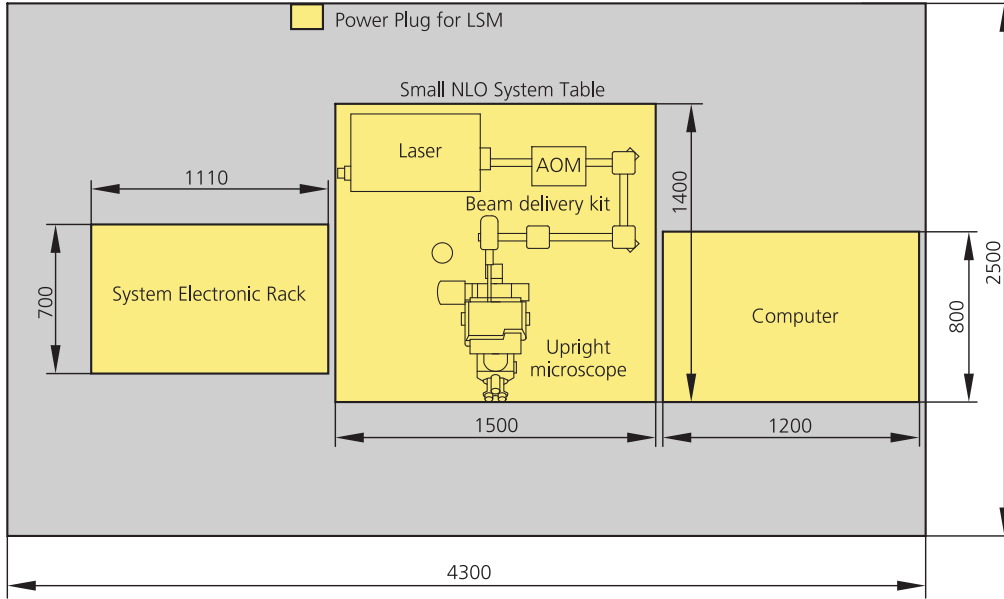
Fig. 2-7   Space requirements for Two Photon Laser and Small NLO System Table with upright microscope (measurements in mm)
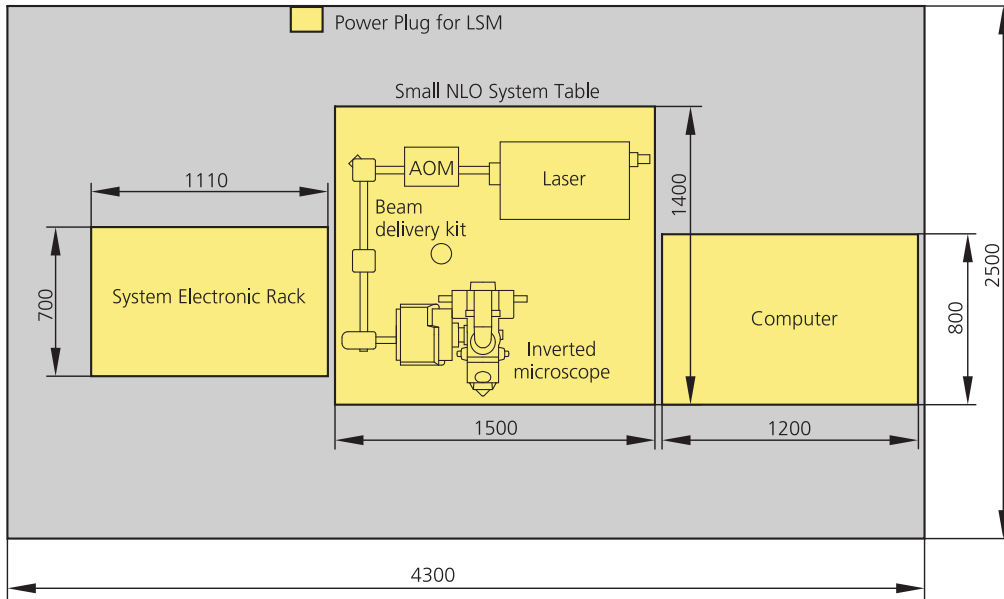
Fig. 2-8   Space requirements for Two Photon Laser and Small NLO System Table with inverted microscope (measurements in mm)

The System Electronic Rack contains all electronics for control of the hardware components of the system, the power supply for the microscope, the scanning unit and the Laser Module RGB equipped with the choice of lasers. The Laser Module RGB is set on top of the System Electronic Rack. Power supply and cooling unit of the NLO Laser can be stored under the system table. With the extension of LSM *DuoScan* (VIS and/or 405 nm) the Laser Module LIVE is situated in the System Electronic Rack. The electronics for the NLO laser have to be set aside the System Electronic Rack. An additional lab cart (000000-0465-515) is recommended. With the extension of the LSM *DuoScan* UV only additional space is required to set up the UV laser (see chapter 2.1.4).

2.1.6.2   Coherent "Chameleon" or Spectra Physics "Mai Tai". Directly-coupled to upright and inverted Microscope
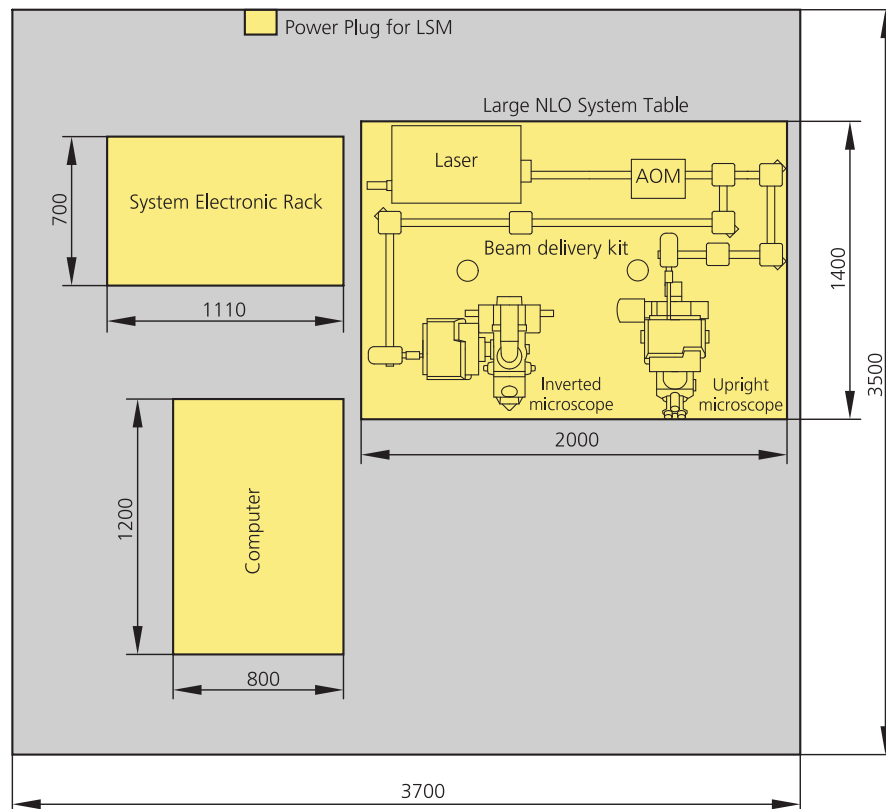
**Fig. 2-9   Space requirements for Two Photon Laser and
                Large NLO System Table. Set up of one LSM system with
                two microscopes (measurements in mm)**
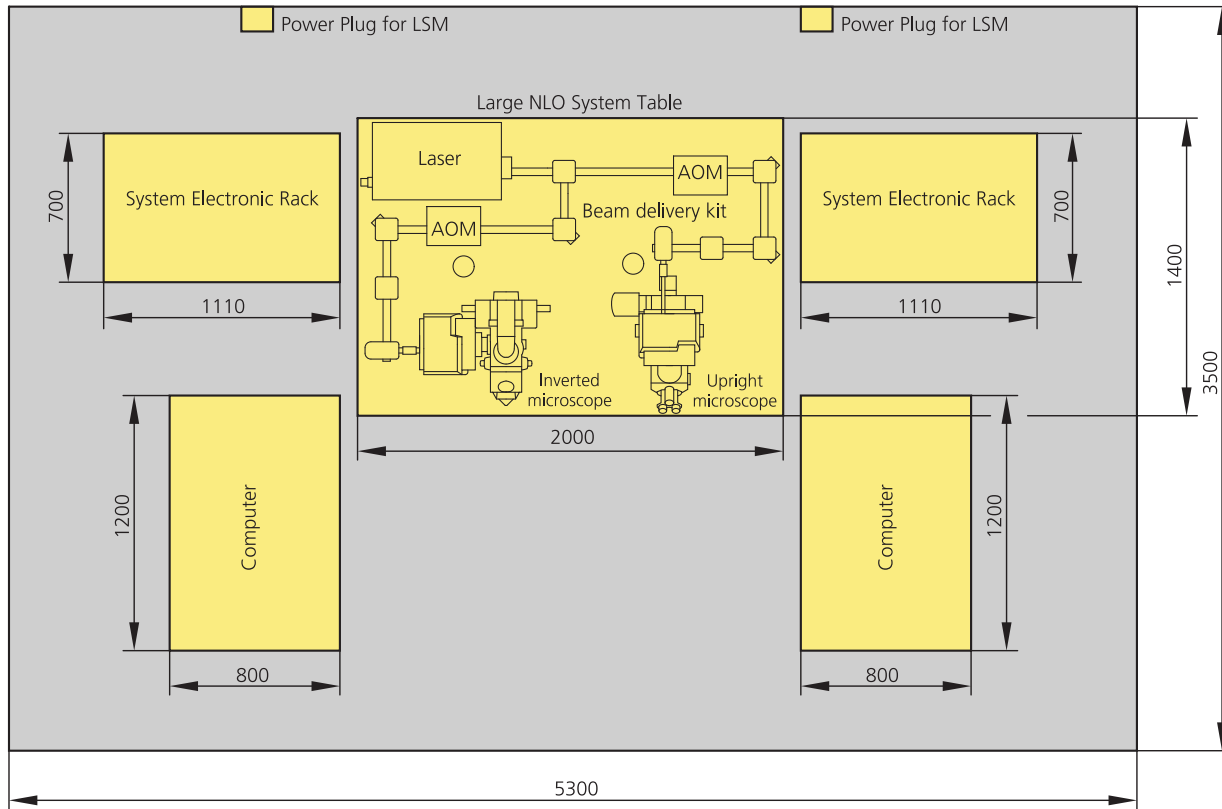
Fig. 2-10   Space requirements for Two Photon Laser and Large NLO System Table. Set up of two LSM system with two microscopes (measurements in mm)

The System Electronic Rack contains all electronics for control of the hardware components of the system, the power supply for the microscope, the scanning unit and the Laser Module RGB equipped with the choice of lasers. The Laser Module RGB is set on top of the System Electronic Rack. Power supply and cooling unit of the NLO Laser can be stored under the system table. This set up can hold two microscopes. Coherent "Chameleon" or Spectra Physics "Mai Tai" lasers can also be used. With the extension of LSM DuoScan (VIS and/or 405 nm) the Laser Module LIVE is situated in the System Electronic Rack. The electronics for the NLO laser have to be set aside the System Electronic Rack. An additional lab cart (000000-0465-515) is recommended. With the extension of the LSM DuoScan UV only additional space is required to set up the UV laser (see chapter 2.1.4).

2.2   Power Requirements

☞   The LSM 510 and LSM 510 META comes with a mains power supply cord and plug, either CEE red (3/N/PE 400/230V/16A), or NEMA L 14-30P (2/N/Ground 120/240V/30A), and with the matching mains socket outlet.

A ground wire (AWG10 green/yellow) is supplied because it is necessary to ground the system. The connecting part on both ends of the cable is a cable eye with 8 mm inner diameter.

A suitable grounding point must be installed in the room. For systems (220 ... 240 V AC) equipped with X-Cite 120 the mains socket outlet must be equipped with a fuse having minimum tripping characteristic C according to IEC/EN 60898.

Line voltage	220 … 240 V AC (±10 %)	100 … 125 V AC (±10 %)
Line frequency	50...60 Hz	50...60 Hz
LSM incl. VIS laser		
– Max. current	3 phases at 16 A	2 phases at 25 A
– Power	Phase 1 = 1.9 kVA max.	Phase 1 = 3.2 kVA max.
	Phase 2 = 1.5 kVA max.	Phase 2 = 2.8 kVA max.
	Phase 3 = 2.6 kVA max.	
– Power consumption	5000 VA max.	5000 VA max.
Argon UV laser		
– Line Voltage	208...240 V AC	208...240 VAC
	(±10 %) 50 / 60 Hz	(±10 %) 50 / 60 Hz
– Max. current	1 phase at 63 A	1 phase at:
	Note: For Line Voltage 220 V the connector and power plug are rated for 63 Amps, However wiring and fuse should be rated for 32 Amps.	208 V: 34 Amps 230 V: 31 Amps 240 V: 29 Amps
– Power consumption	7000 VA max.	7000 VA max.
Class of protection	I	I
Type of protection	IP 20	IP 20
Overvoltage category	II	II
Pollution degree	2	2

2.3   Physical Dimensions

	Length (cm)	Width (cm)	Height (cm)	Weight (kg)
Large system table	150	80	78	100
Small system table	65	80	78	60
Passively damped anti-vibration table	130	100	75	137
Active anti-vibration table (NLO) for Mai Tai Laser or Chameleon	150	140	75	200
Active anti-vibration table (NLO) for two-microscope configuration	200	140	75	400
Scanning Module LSM 510	25	20	25	15
Scanning Module LSM 510 META	28	27	30.5	13
Scanning Module LSM 5 MP	25	20	25	15
Scanning Module LSM *DuoScan*	40	15	13	8
Microscope	50	35	50	20
Laser Module RGB	110	70	28	95
Laser Module, UV	140	20	20	60
Laser Module V (405 nm)	66	52	22	30
Laser Module *LIVE*	66	52	22	58
Plug-in unit external laser	66	52	22	9
System Electronic Rack	110	70	58	90
Power supply for Ar (UV)	50	50	30	30
Cooling unit for Ar (UV)	80	45	50	30
Water hose for Ar (UV)	700			
Fiber optic cable, VIS(ible)	300			
Fiber optic cable, UV	300			
Fiber optic cables at NLO systems	400			
Cables	350			
SCSI cable	350			

2.4 Dimension of Shipment Crates

Crate containing	Length (cm)	Width (cm)	Height (cm)	Weight (kg)
Large system table	160	85	95	120
Small system table	90	75	80	80
Passively damped anti-vibration table	145	115	115	150
Active anti-vibration table (NLO) for Mai Tai Laser or Chameleon	160	160	110	350
Active anti-vibration table (NLO) for two-microscope configuration	220	160	110	480
System Electronic Rack and Laser module	135	90	100	300
LSM, Microscope, Computer	135	90	100	150
Additional Hardware Components	135	90	61	100
UV laser unit	125	55	50	100
UV cooling unit	120	60	90	50

2.5 Environmental Requirements

1. Operation, specified performance	T = 22 °C ± 3 °C without interruption (24 h a day independently whether system is operated or switched-off)
2. Operation, reduced performance	T = 10 °C to 35 °C, any conditions different from 1. and 5.
3. Storage, less than 16 h	T = -40 °C to 55 °C
4. Storage, less than 6 h	T = -55 °C to 70 °C
5. Temperature gradient	± 0.5 °C/h
6. Warm up time	1 h, for high-precision and/or long-term measurements ≥ 3 h
7. Relative humidity	<65 % at 30 °C
8. Operation altitude	max. 2000 m
9. Loss of heat	4 kW

2.6   **Vibrations**

Vibrations under operation conditions (with system table)	**Shipping shock (LSM 510 box)**
5 µm pp at 5 Hz 10 µm pp at 10 Hz 10 µm pp at 20 Hz	3 g

2.7   **Microscopes**

Inverted Axio Observer.Z1 BP or SP

Upright Axioskop 2 FS MOT

Upright Axio Imager.Z1

Upright Axio Imager.M1

All ICS objectives from Carl Zeiss and their accessories can be accommodated.

Z motor   DC servomotor, opto-electronically coded

Least Z interval: 25 nm (Axio Observer.Z1 BP or SP)
                            100 nm (Axioskop 2 FS MOT)
                             10 nm (Axio Imager.Z1)
                             25 nm (Axio Imager.M1)
measured according to VDI 3441

Piezo Objective focus   Piezo-driven single objective drive

Max. travel 250 µm; resolution 15 nm

Z-Piezo stage   Piezo driven stage insert for scanning stages

Max. travel 250 µm; resolution 5 nm

In the unlikely case of extreme fluctuations of the external power net or electromagnetic radiation, the piezo crystal will vary and disturbance in the image is visible. Note that this is not a defect and the piezo drive will not be damaged.

2.8   **Scanning Module LSM 510 and LSM 510 META**

Scanners	2 individually driven galvanometric scanners
Scanning speed	Up to ~5 frames/sec (512 × 512 pixels)
Field resolution	Max. 2048 × 2048 pixels (individually adjustable for each axis)
Field of view	10 × 10 mm² with a 1.25× objective
Zoom	1× ... 40×, continuous control
Channels	a) Up to 4 confocal reflection/fluorescence channels (PMT) simultaneously **or**
	b) 2 confocal reflection/fluorescence channels (PMT) and 1 META detector
	1 transmitted light channel (PMT) and 2 NDD **or**
	3 - 4 NDD (Non descanned detectors, PMT)
	1 reference monitor diode
	Fiber-optic adaptation of external detectors
Dynamic range	12-bit DAC for each detection channel
Pinholes	4 individual variable pinholes (one per confocal channel or META detector)
	Computer controlled automatic alignment

2.9   **Scanning Module LSM *DuoScan***

Scanners	2 individually driven galvanometric scanners
Scanning speed	Up to ~5 frames/sec (512 × 512 pixels)
Field of view	10 × 10 mm² with a 1.25× objective

2.10   Laser Module RGB (458, 477, 488, 514, 543, 561,594, 633 nm)

Single-mode polarization preserving fiber

Laser beam attenuation for all lasers by VIS-AOTF

HeNe laser (543 nm, 1 mW)

HeNe laser (594 nm, 2 mW)

HeNe laser (633 nm, 5 mW)

DPSS laser (561 nm, 10 mW)

Ar laser (458, 477, 488, 514 nm, 30 mW)

2.11   Laser Module V (405 nm)

Single-mode polarization preserving fiber

Laser beam attenuation by UV-AOTF

Diode laser (405 nm, 30 mW)

2.12   Laser Module UV (351, 364 nm)

Single-mode polarization preserving fiber

Laser beam attenuation for all lasers by UV-AOTF

Ar laser (351, 364 nm, 80 mW)

2.13   Laser Module *LIVE* for LSM *DuoScan*

Single-mode polarization preserving fiber

Laser beam attenuation (AOTF) for all lasers

Diode laser (405 nm, 50 mW)

OPSS laser (488, 100 mW)

DPSS laser (532 nm, 75 mW)

DPSS laser (561 nm, 40 mW)

2.14 System Overview LSM 510 META

Laser module RGB
(458, 477, 488, 514, 543/561, 594, 633 nm)

Laser Enterprise II 653 (80 mW, 351 nm, 364 nm)
UV Laser module

VIS, VIS/UV, VIS/NLO,
VIS/405 scan module LSM 510 or
Scan module LSM 5 MP

Upgrade kits LSM 510 to
LSM 510 META

NLO kit for direct coupling

Laser module V
(with output coupling)

VIS, VIS/UV, VIS/NLO or
VIS/405 scan module LSM 510 META
Option:
Fiber decoupling channel 4
Fluorescence Correlation
Spectrosccopy (FCS)

System electronic rack

Plug-in unit for
external laser

System table NLO with active absorption
width 2000 mm, height 750 mm, depth 1400 mm

System table NLO with active absorption
width 1500 mm, height 750 mm, depth 1400 mm

LCD TFT flat screen monitor 19"

Large system table (1500x800)
with stone plate for vibration attenuation

Small system table (650x800)
with stone plate for vibration attenuation

System table with breadboard
Wide: 1000x750mm (1200x950 overall)
Narrow: 750x1000mm (950x1200 overall)

Control computer

PIEZO objective focus

XY-stage controller PIEZO
XY-Joystick
for stage controller PIEZO

Scanning stage 130x85 PIEZO
for upright stand

PIEZO objective focus

Scanning stage DC 120x100
with mounting frame
for inverted stand

VIS, VIS/UV, VIS/NLO
or VIS/405 scan module
LSM 510 META

AxioCam HRm
AxioCam HRc
AxioCam HSm
AxioCam HSc

Motor control MCU 28

2-axes control panel

Piezo objective focus

AxioCam HRm
AxioCam HRc
AxioCam HSm
AxioCam HSc

VIS, VIS/UV, VIS/NLO
or VIS/405 scan module
LSM 510 META

Microscope stand
Axio Imager
with TFT monitor
and light control mot.

AxioCam HRm
AxioCam HRc
AxioCam HSm
AxioCam HSc

Axio Observer.Z1
Several solutions for incubation
will be offered.

VIS, VIS/UV, VIS/NLO
or VIS/405 scan module
LSM 510 META

Axioskop 2 FS mot

HBO 100 illuminator
with lamp mount and collector
HBO 100 illuminator self-adjusting,
with lamp mount and collector
Power supply unit for HBO 100
FluoArc variable intensity lamp control for HBO 100
X-Cite 120 fiber coupled illuminator

Detection module
external PMT for Non
Descanned Detection

Non Descanned
Detection kit
motorized NDD module
with shutter

HAL 100 illuminator
with collector
Halogen lamp 12 V 100 W

Switching mirror mot

Transmitted-light
channel for LSM 5

HAL 100 illuminator
with collector
Halogen lamp 12 V 100 W

Detection module
external PMT for Non
Descanned Detection

Non Descanned
Detection kit
motorized NDD module
with shutter
(not for Axio Imager)

Detection module
external PMT for Non
Descanned Detection

HAL 100 illuminator
with collector
Halogen lamp 12 V 100 W
Power supply 12V DC 100 W,
stabilized

Switching mirror mot

Transmitted-light
channel for LSM 5

2.15   System Overview LSM 510 META *DuoScan*

Laser module RGB
(458, 477, 488, 514, 543/561, 594, 633 nm)

Laser Enterprise II 653 (80 mW, 351 nm, 364 nm)
UV Laser module

LSM *DuoScan*

VIS, VIS/UV, VIS/NLO,
VIS/405 scan module LSM 510 or
Scan module LSM 5 MP

NLO kit for direct coupling

Upgrade kits LSM 510 to
LSM 510 META

Laser module LSM 5 *LIVE*
(with output coupling)

VIS, VIS/UV, VIS/NLO or
VIS/405 scan module
LSM 510 META
Option:
Fiber decoupling channel 4
Fluorescence Correlation
Spectrosccopy (FCS)

System electronic rack

Laboratory cart

Plug-in unit for
external laser

LCD TFT flat screen
monitor 19"

System table NLO with active absorption
width 2000 mm, height 750 mm, depth 1400 mm

System table NLO with active absorption
width 1500 mm, height 750 mm, depth 1400 mm

System table with breadboard
Wide: 1000x750mm (1200x950 overall)
Narrow: 750x1000mm (950x1200 overall)

Control computer

PIEZO objective focus

XY-stage controller PIEZO

XY-Joystick
for stage controller PIEZO

Scanning stage 130x85 PIEZO
for upright stand

PIEZO objective focus

Scanning stage DC 120x100
with mounting frame
for inverted stand

VIS, VIS/UV, VIS/NLO
or VIS/405 scan module
LSM 510 META

AxioCam HRm
AxioCam HRc
AxioCam HSm
AxioCam HSc

Motor control MCU 28

2-axes control panel

Piezo objective focus

AxioCam HRm
AxioCam HRc
AxioCam HSm
AxioCam HSc

VIS, VIS/UV, VIS/NLO
or VIS/405 scan module
LSM 510 META

Microscope stand
Axio Imager *DUO*
with TFT monitor
and light control mot.

AxioCam HRm
AxioCam HRc
AxioCam HSm
AxioCam HSc

Axio Observer.Z1 *DUO*
Several solutions for incubation
will be offered.

VIS, VIS/UV, VIS/NLO
or VIS/405 scan module
LSM 510 META

Axioskop 2 FS mot *DUO*

LSM *DuoScan*

X-Cite 120 fiber coupled illuminator

Lamp housing HAL 100
with collector

Halogen lamp 12 V 100 W

Switching mirror mot

Transmitted-light
channel for LSM 5

Lamp housing HAL 100
with collector

Halogen lamp 12 V 100 W

Lamp housing HAL 100
with collector

Halogen lamp 12 V 100 W

Power supply 12V DC 100 W,
stabilized

Switching mirror mot

Transmitted-light
channel for LSM 5

Quick Guide

LSM 5 MP, LSM 510 and LSM 510 META

Laser Scanning Microscopes

LSM Software ZEN 2009

October 2009

ZEISS

We make it visible.

Contents

Introduction

This LSM 510 / LSM 510 META / LSM 510 NLO Quick Guide describes the basic operation of the LSM 510 / LSM 510 META / LSM 510 NLO Laser Scanning microscope with the ZEN 2009 software. The purpose of this document is to guide the user to get started with the system as quick as possible in order to obtain some first images from his samples. This Quick Guide does NOT replace the detailed information available in the full user manual or in the manual of the respective microscopes (Axio Imager, Axio Observer, Axioskop 2 FS MOT). Also, this Quick Guide is written for a user who is familiar with the basics of Laser Scanning Microscopy.

For your safety!
Observe the following instructions:

– The LSM 510 / LSM 510 META / LSM 510 NLO laser scanning microscope, including its original accessories and compatible accessories from other manufacturers, may only be used for the purposes and microscopy techniques described in this manual (intended use).

– In the Operating Manual, read the chapter *Safety Instructions* carefully before starting operation.

– Follow the safety instructions described in the operating manual of the microscope and X-Cite 120 lamp / HBO 100 mercury lamp.

Starting the System
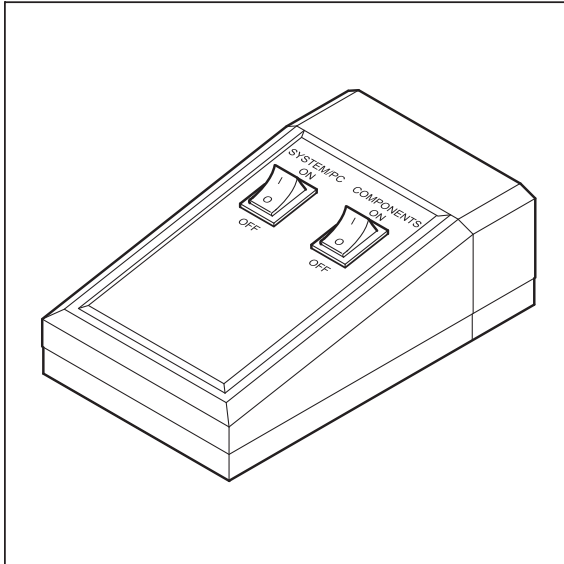
Fig. 1   **REMOTE CONTROL switch**

Switching on the LSM system

- When set to **ON** the REMOTE CONTROL switch labeled **System/PC** provides power to the computer. This allows use of the computer and ZEN software offline (Fig. 1).

- To completely switch on the system, now press the **Components** switch to **ON**. This starts the other components and the complete system is ready to be initialized by the ZEN software.

Switching on the X-Cite 120 or the HBO 100 mercury lamp

- Switch on the main switch of the X-Cite 120 / HBO 100 lamp for reflected light illumination via the power supply as described in the respective operating manual.
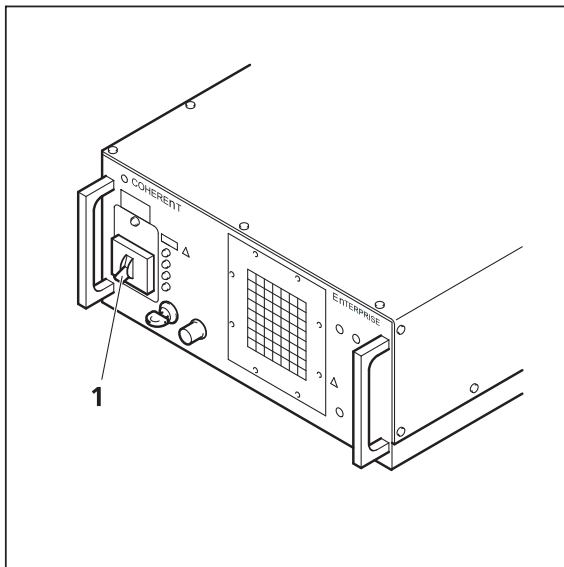
Fig. 2   **Power supply of UV-Ar laser**

Switching on the Enterprise UV-Ar Laser

- If the UV laser is required, switch it on via the toggle switch (Fig. 2/**1**) on the power supply.
    - It will be ready for operation after a few seconds.

Starting the ZEN software

- Double click the ZEN 2009 icon on the WINDOWS desktop to start the Carl Zeiss LSM software.

The **ZEN Main Application** window and the **LSM 510 Startup** window appear on the screen (Fig. 3)

a) ZEN 2009 Main Application window

b)

c) LSM 510 Startup window
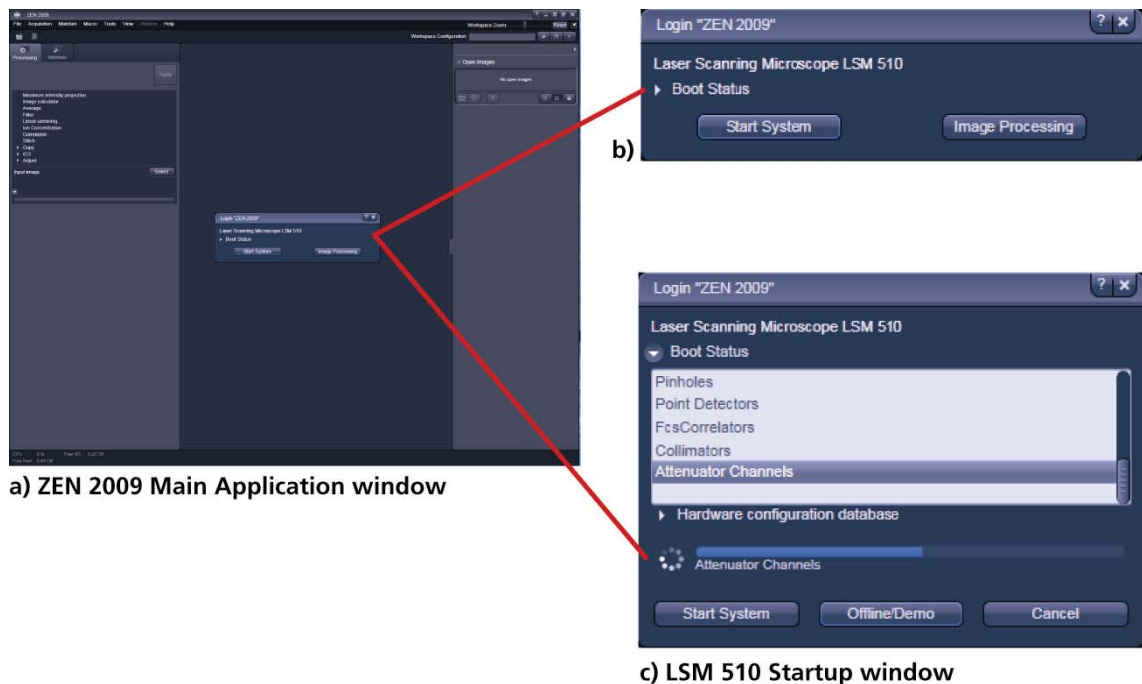
Fig. 3   ZEN Main Application Window at Startup (a) and the LSM 510 Startup Window (b and c)

In the small startup window, choose either to start the system (**Start System** hardware for acquiring new images) or in **Image Processing** mode to edit already existing images. Toggle the little 🔘 symbol to view the Boot Status display and get the additional **Offline / Demo** button option:

– Choosing **Start System** initializes the whole microscope system and activates the entire software package for new image acquisition and analysis.

– The **Image Processing** mode ignores all hardware and activates only data handling and image processing functionality for already acquired images.

– The **Offline / Demo** mode reads the current hardware database but does not activate the system hardware for use. Instead, it simulates the system hardware for training purposes.

– Upon clicking the **Start System** button, the **Image Processing** button changes to a **Cancel** button. Click **Cancel** to interrupt/stop the **Startup** of the system.

After Startup, the ZEN Main Application window (Fig. 4 and Fig. 5) opens. To benefit from all of Zen's features, run the window in its full screen mode.

A Application Bar
B Menu Bar
C Main Toolbar
D Left Tool Area
E Center Screen Area
   (hosts up to 3
   3 Containers)
F Right Tool Area
G Status Area
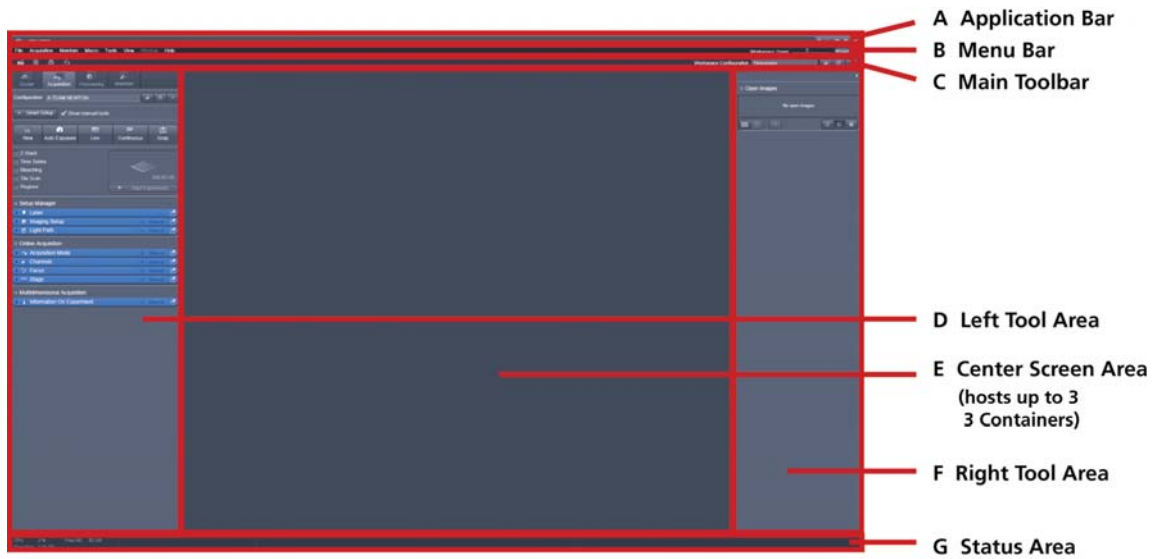
Fig. 4　　ZEN Main Application window after Startup with empty image container
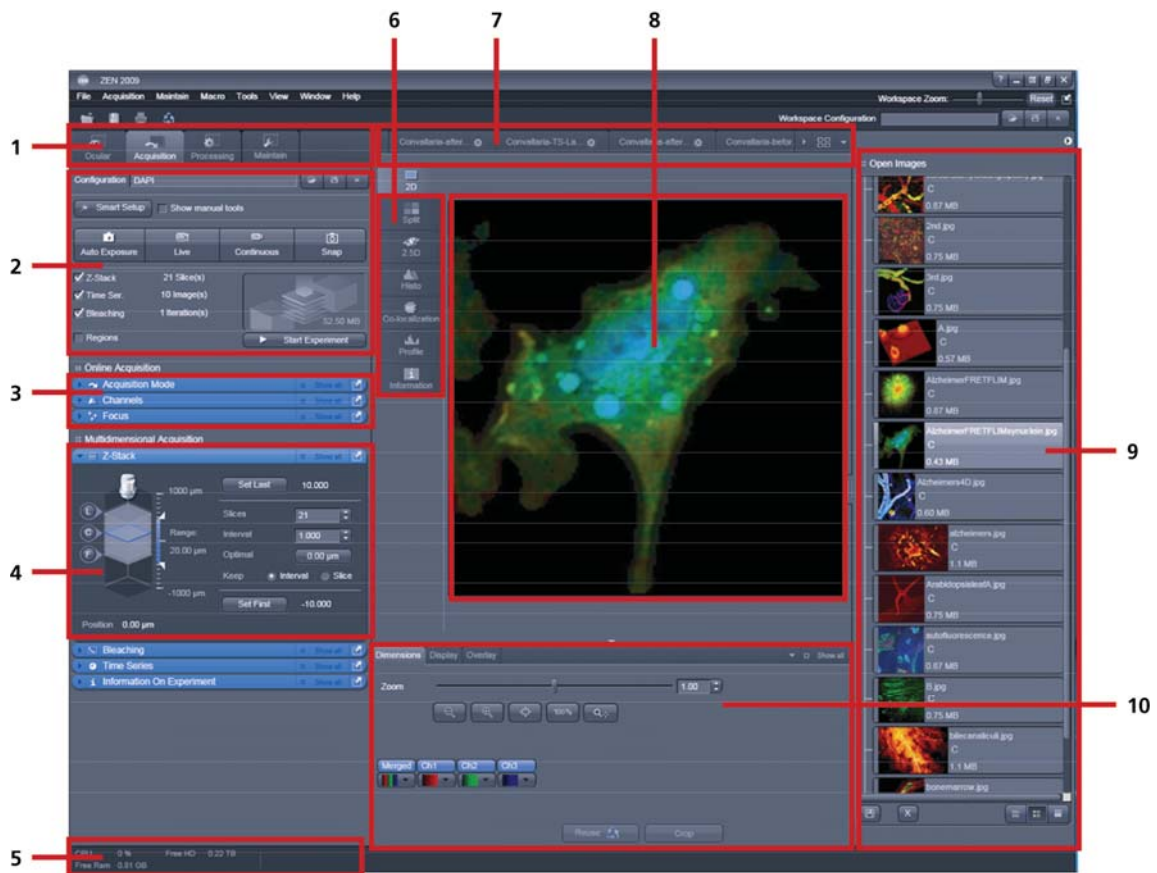
1 Tabs to switch between the tool types
2 Start buttons
3 Tool group
4 Tool
5 Status bar

6 View tabs
7 Image tabs
8 Displayed image
9 File handling area
10 View controls

Fig. 5　　ZEN Main Application window after Startup with several images loaded

Introduction to ZEN – Efficient Navigation

The **ZEN 2009** interface is clearly structured and follows the typical workflow of the experiments performed with confocal microscopy systems:

On the **Left Tool Area** (Fig. 4/**D**) the user finds the tools for sample observation, image acquisition, image processing and system maintenance, easily accessible via four **Main Tabs** (Fig. 5/**1**). All functions needed to control the microscope can be found on the **Ocular Tab**, to acquire images use the **Acquisition Tools** (Fig. 5/**3** and **4**). Arranged from top to bottom they follow the logic of the experimental workflow. The area for viewing and interacting with images is centered in the middle of the **Main Application Window**: the **Center Screen Area**. Each displayed image can be displayed and/or analyzed with many view options available through view tabs which can be found on the left side of the image. According to the chosen view tab, the required view controls appear in View Control Tabs below each image. File management and data handling tools are found in the **Right Tool Area** (see Fig. 4 and Fig. 5).

Color and brightness of the interface have been carefully adjusted to the typical light conditions of the imaging laboratory, guaranteeing optimal display contrast and minimal stray light for high-sensitivity detection experiments. The **ZEN** software is optimized for a 30" TFT monitor but can also be used with dual-20" TFT setups.
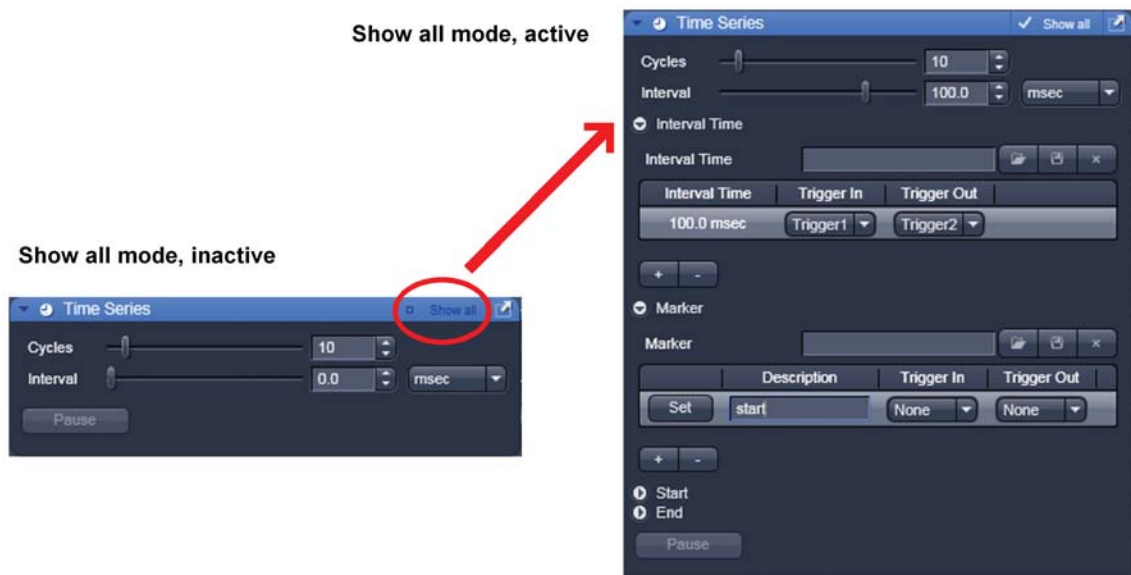
Fig. 6   **Basic and Pro Mode**

A focus in the development of **ZEN 2009** was to fulfill the needs of both basic users and microscopy specialists. Both types of users will appreciate the set of intuitive tools designed to make the use of a confocal microscope from Carl Zeiss easy and fast:

The **Show all** concept ensures that tool panels are never more complex than needed. With **Show all** de-activated, the most commonly used tools are displayed. For each tool, the user can activate **Show all mode** to display and use additional functionality (Fig. 6).
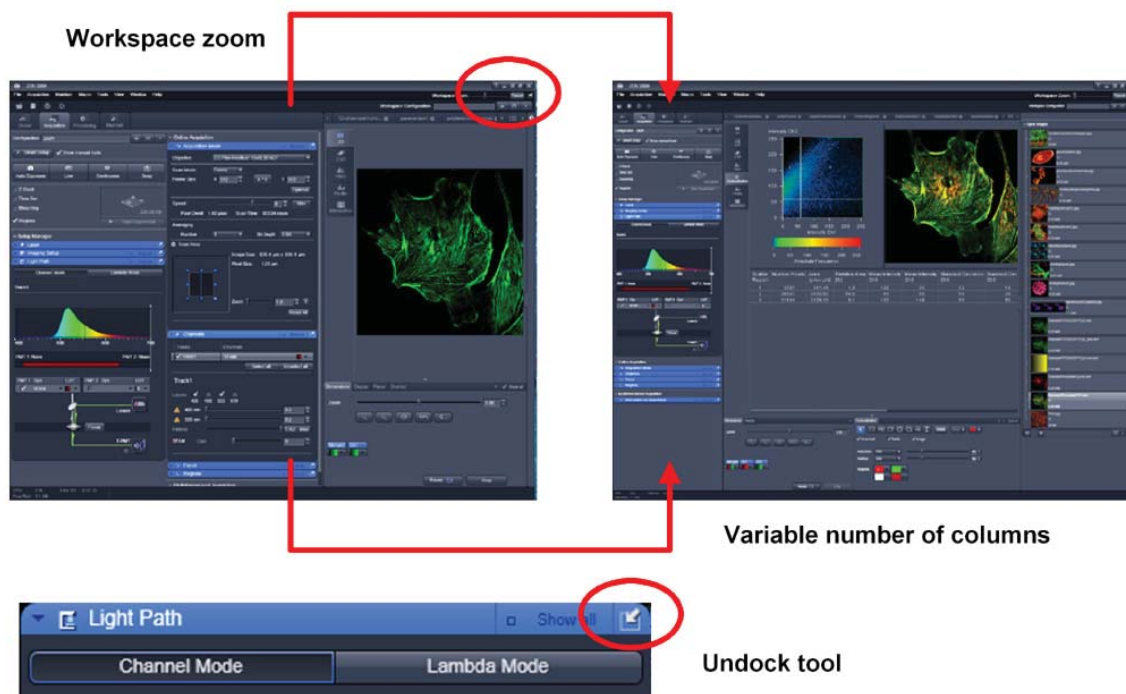
Fig. 7   **ZEN Window Layout configuration**

More features of **ZEN 2009** include:

- The user can add more columns for tools to the **Left Tool Area** or detach individual tools to position them anywhere on the monitor. To add a column, drag a tool group by the title bar (e.g., "Online Acquisition") to the right and a new tool column automatically opens. Alternatively use the context menu "move toolgroup to next column". To detach a tool, click on the little icon on the very right end of the blue tool header bar (Fig. 7).

- Another unique feature in Imaging Software is the scalable ZEN interface. This **Workspace Zoom** allows adjustment of the **ZEN 2009** window size and fonts to the situational needs or your personal preferences (Fig. 7).

- Setting up conventional confocal software for a specific experiment can take a long time and is often tedious to repeat. With ZEN these adjustments have to be done only once – and may be restored with just two clicks of the mouse. For each type of experiment one can now set-up and save the suitable **Workspace Layout**. These configurations can also be shared between users.

- For most controls, buttons and sliders, a **tool tip** is available. When the mouse pointer is kept over the button, a small pop-up window will display which function is covered by this tool/button.

These are just some of the most important features of the ZEN interface. For a more detailed description of the functionality for the **ZEN 2009** software, please refer to the User Manual that is provided with your system.

Setting up a new image document and saving your data

To create a new image document in an empty image container, click the **Snap** [Snap] or the **Auto Exposure** [Auto Exposure] button. For an empty image document press the **New** [New] button.

The new document is immediately presented in the **Open Images Area.** Remember, an unsaved 2D image in the active image tab will be over-written by a new scan. Multi-dimensional scans or saved images will never be over-written and a new scan will then automatically create a new image document.

Acquired data is **not** automatically saved to disc. Make sure you save your data appropriately and back it up regularly. The ZEN software will ask you if you want to save your unsaved images when you try to close the application with unsaved images still open.

☞ There is no "image database" any more like in the earlier Zeiss LSM software versions.
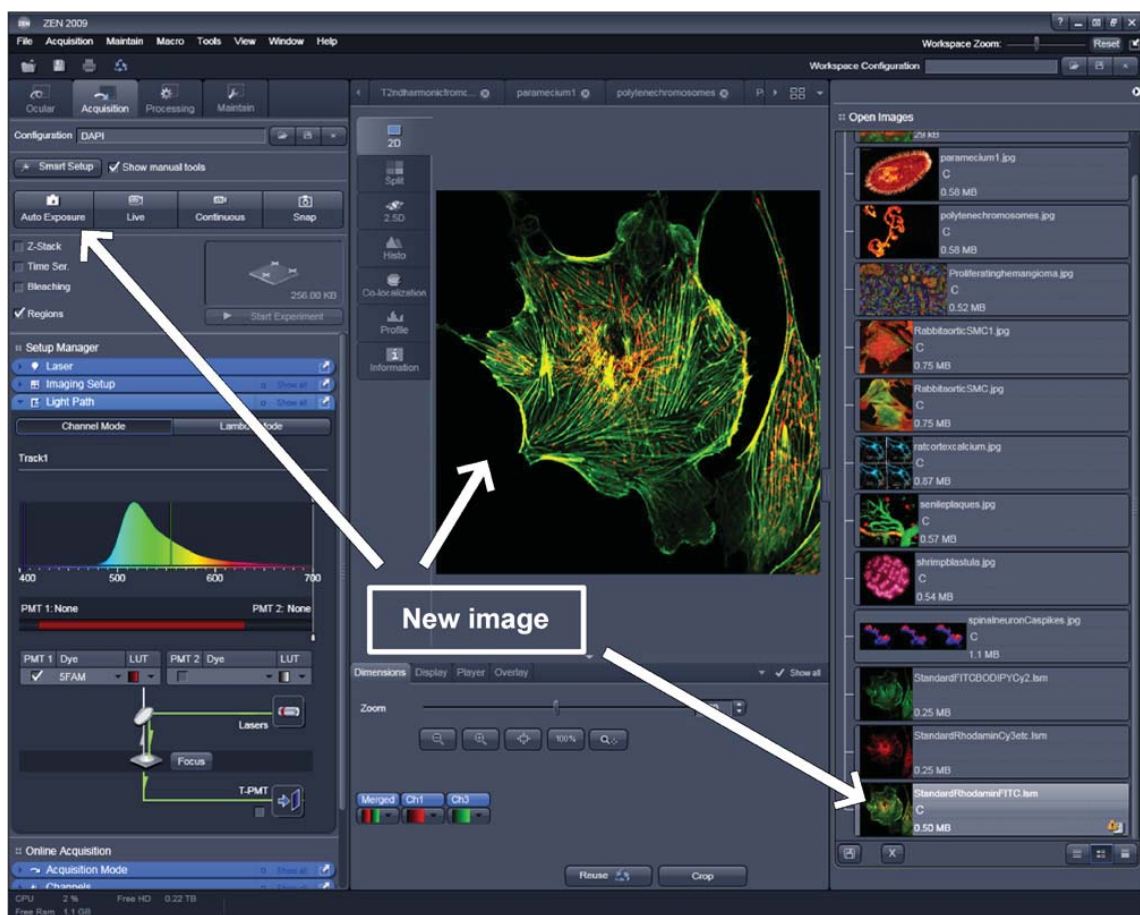
Fig. 8   New image document in the Open Images Ares

Advanced data browsing is available through the **ZEN File Browser** (Ctrl F or from the **File** menu). The File Browser can be used like the WINDOWS program file browser. Images can be opened by a double-click and image acquisition parameters are displayed with the thumbnails (Fig. 9). For more information on data browsing please refer to the detailed operating manual.
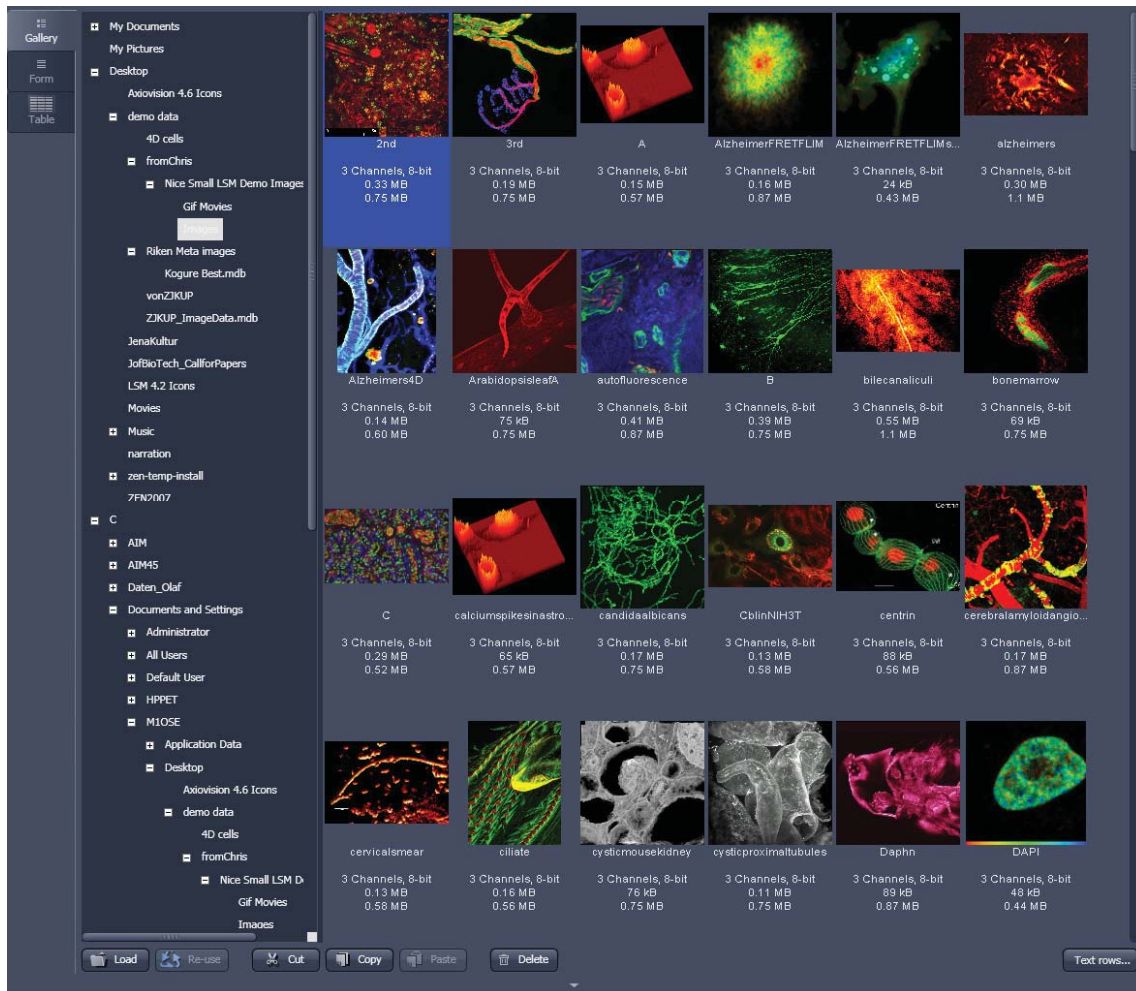
Fig. 9   File Browser

Turning on the lasers

- To manually switch lasers on or off open the **Laser** tool.
- All available lasers can be operated within this tool (Fig. 10).
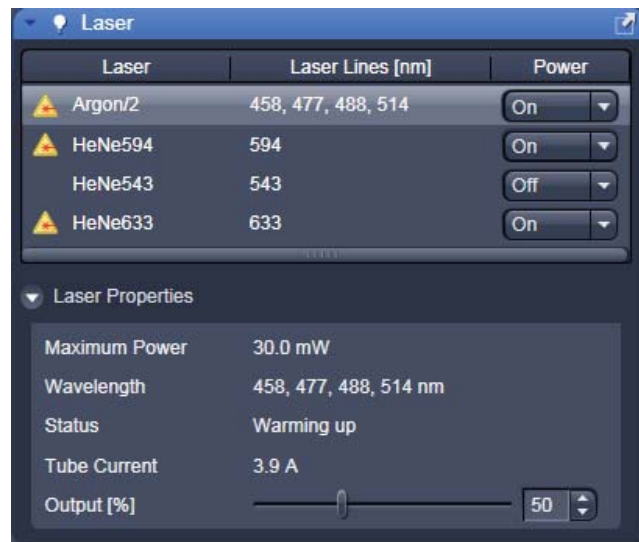
Fig. 10   **Laser Control tool**

Setting up the microscope

Changing between direct observation, camera detection and laser scanning mode

The **Ocular**, **Camera** and **LSM Acquisition** buttons switch between the use of the LSM and the microscope the beam path and indicate which beam path is currently in use for the microscope:

- Click on the **Ocular** button to change open the controls for the microscope beam path and for direct observation via the eyepieces of the binocular tube, lasers are blocked.

- To set the hardware in position for using the microscope, click **Online** if not yet active.

- To close the light shutters on the microscope click **Offline**.

- Click on the **LSM Acquisition** button to move back to the LSM system.

Fig. 11   Microscope Control window, e.g.: Axio Imager.Z1

Setting up the microscope and storing settings

Click on the **Ocular** tab for direct observation; press the **Online** button for your actions to take effect immediately. Then open the **Ocular** tool to configure the components of your microscope like filters, shutters or objectives (Fig. 11).

Selecting an objective

- Open the graphical pop-up menu by clicking on the **Objective** symbol and select the objective lens for your experiment (Fig. 11).

- The chosen objective lens will automatically move into the beam path.

Focusing the microscope for transmitted light

- Open the graphical pop-up menu by clicking on the **Transmitted Light** icon (Fig. 12).

- Click on the **On** button. Set the intensity of the Halogen lamp using the slider.

- Clicking outside the pop-up control closes it.

- Place specimen on microscope stage. The cover slip must be facing the objective lens. Remember the immersion medium if the objective chosen requires it!

- Use the focusing drive of the microscope to focus the object plane.

- Select specimen detail by moving the stage in X and Y using the XY stage fine motion control.

Setting the microscope for reflected light

- Click on the **Reflected Light** icon to open the X-Cite 120 controls and turn it on.

- Click on the **Reflected Light** shutter to open the shutter of the X-Cite 120 lamp / HBO100.

- Click on the **Reflector** button and select the desired filter set by clicking on it.

Storing the microscope settings

Microscope settings can be stored as configurations (Fig. 13) by typing a config name in the pull down selector and pressing the save button. Fast restoration of a saved config is achieved by selecting the config from the pull down list and pressing the load button. The current config can be deleted by pressing the delete button.

Fig. 12   **Microscope Control window with Transmitted Light pop-up menu**

These configurations can be assigned to buttons that are easier to press.

☞   Depending on the microscope configuration, settings must be done manually if necessary.

Fig. 13   **Configuration panel**

Configuring the beam path and lasers
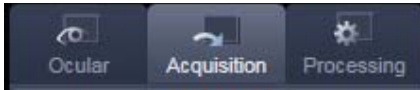
 • Click on the **Acquisition** button.

Setting up a configuration

Simultaneous scanning of single, double and triple labeling:
- – Advantage: faster image acquisition
- – Disadvantage: Eventual cross-talk between channels

Sequential scanning of double and triple labeling; line-by-line or frame-by-frame:
- – Advantage: Only one detector and one laser are switched on at any one time. This reduces cross-talk.
- – Disadvantage: slower image acquisition

• Open the **Imaging Setup** and the **Light Path** tool in the **Setup Manager** Tool group to access the hardware control window to set-up the beam path.

The open **Light Path** is shown in Fig. 14.
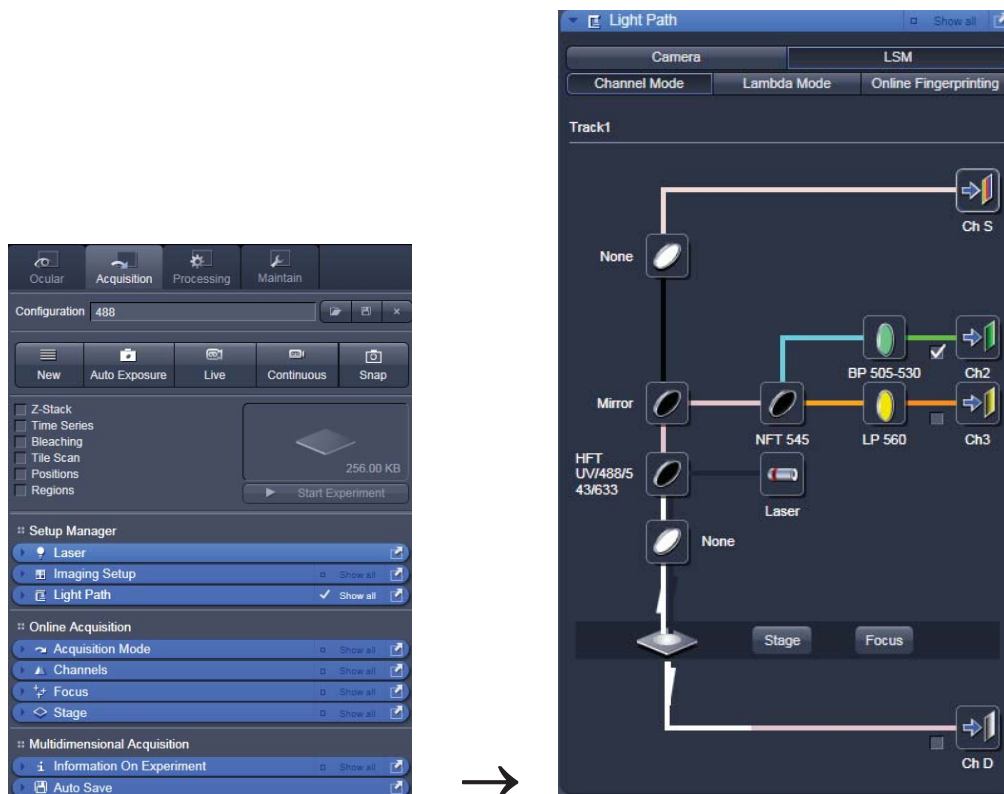
Fig. 14   **Light Path tool for a single track (LSM)**

Settings for track configuration in Channel Mode

- Select **Channel Mode** if necessary (Fig. 15).

The **Light Path** tool displays the selected track configuration which is used for the scan procedure.

- You can change the settings of this panel using the following function elements:
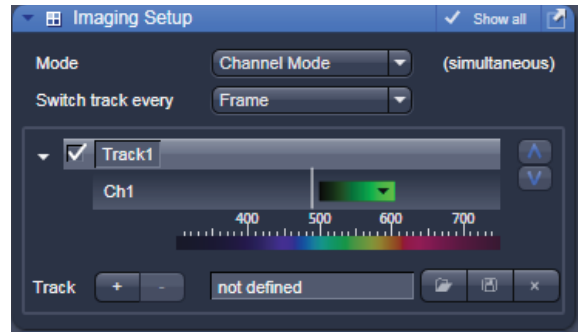
Fig. 15 **Imaging Setup tool for a single track (LSM)**

Activation / deactivation of the excitation wavelengths (check box) and setting of excitation intensities (slider). If necessary open the **Laser Control** tool (see above).

Selection of the main dichroic beam splitter (HFT) or secondary dichroic beam splitter (NFT) position through selection from the relevant list box.

Selection of an emission filter through selection from the relevant list box.

Activation / deactivation (via check box) of the selected channel (Ch 1-4, monitor diode ChM, META detectors ChS1-8, transmission ChD) for the scanning procedure and assigning a color to the channel.

- Select the appropriate filters and activate the channels.

- Click the **Laser** icon to select the laser lines and set the attenuation values (transmission in %) in the displayed window.

- For the configuration of the beam path, please refer to the application-specific configurations depending on the used dyes and markers and the existing instrument configuration.

- In the **Imaging Setup** tool the Detection Bands & Laser Lines are displayed in a spectral panel (Fig. 16) to visualize the activated laser lines for excitation (vertical lines) and activated detection channels (colored horizontal bars).
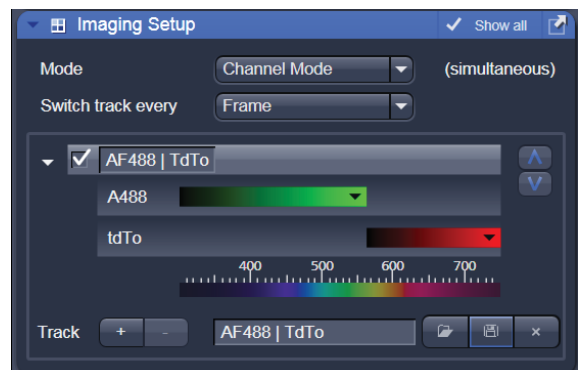
Fig. 16 **Detection Bands & Laser Lines display**
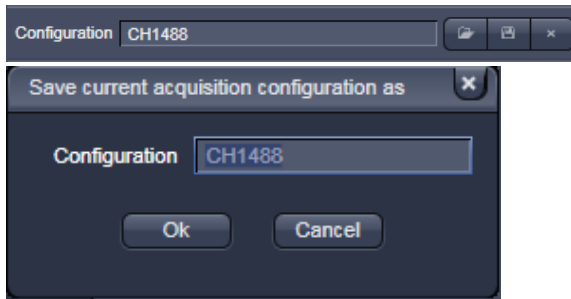
Fig. 17   **Track Configurations window**

- For storing a new configuration click and enter a desired name in the first line of the list box (Fig. 17), then click **Ok** to store the configuration.

- For loading an existing configuration click then select it from the list box.

- For deleting an existing configuration click then select it from the list box and confirm the deletion with **Ok**.

Settings for multiple track configurations in Channel Mode

Multiple track set-ups for sequential scanning can be defined as one configuration (**Channel Mode Configuration**), to be stored under any name, reloaded or deleted.

The maximum of four tracks with up to eight channels can be defined simultaneously and then scanned one after the other. Each track is a separate unit and can be configured independently from the other tracks with regard to channels, Acousto-Optical Tunable Filters (AOTF), emission filters and dichroic beam splitters.

The following functions are available in the **List of Tracks** panel in the **Imaging Setup Tool** (Fig. 15, Fig. 16 and Fig. 17).

Switch track every

Line	Tracks are switched during scanning line-by-line. The following settings can be changed between tracks: Laser line, laser intensity and channels.
Frame	Tracks are switched during scanning frame-by-frame. The following settings can be changed between tracks: Laser line and intensity, all filters and beam splitters, the channels incl. settings for gain and offset and the pinhole position and diameter.
Frame Fast	The scanning procedure can be made faster. Only the laser line intensity and the **Amplifier Offset** are switched, but no other hardware components. The tracks are all matched to the current track with regard to emission filter, dichroic beam splitter, setting of Detector Gain, pinhole position and diameter. When the **Line** button is selected, the same rules apply as for **Frame Fast**.

Add Track button

An additional track is added to the configuration list in the **Imaging Setup Tool**. The maximum of four tracks can be used. One track each with basic configuration is added, i.e.: Ch 1 channel is activated, all laser lines are switched off, emission filters and dichroic beam splitters are set in accordance with the last configuration used.

 Remove button

The track marked in the **List of Tracks** panel is deleted.

A click on this arrow button will move the selected track (highlighted in light grey) one position upwards in the list box.

A click on this arrow button will move the selected track (highlighted in light grey) one position downwards in the list box.

Scanning an image

Setting the parameters for scanning

- Select the **Acquisition Mode** tool from the **Left Tool Area** (Fig. 18).

- Select the **Frame Size** as predefined number of pixels or enter your own values (e.g. 300 x 600) in the **Acquisition Mode** tool. Click on the **Optimal** button for calculation of appropriate number of pixels depending on objective N.A. and λ.

The number of pixels influences the image resolution!
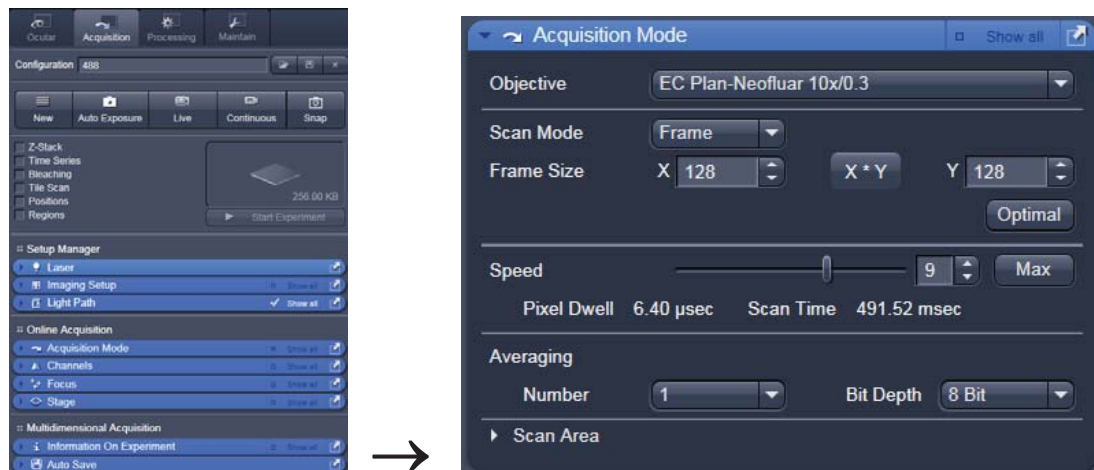
Fig. 18   Acquisition Mode tool

Adjusting scan speed

- Use the **Scan Speed** slider in the **Acquisition Mode** tool (Fig. 18) to adjust the scan speed.

A higher speed with averaging results in the best signal-to-noise ratio. Scan speed 8 usually produces good results. Use speed 6 or 7 for superior images.

Choosing the dynamic range

- Select the dynamic range 8 or 12 Bit (per pixel) in the **Bit Depth** pull down in the **Acquisition Mode** tool (Fig. 18).

8 Bit will give 256 gray levels; 12 Bit will give 4096 gray levels. Publication quality images should be acquired using 12 Bit data depth. 12 Bit is also recommended when doing quantitative measurements or when imaging low fluorescence intensities.

Setting scan averaging

Averaging improves the image by increasing the signal-to-noise ratio. Averaging scans can be carried out line-by-line or frame-by-frame. Frame averaging helps to reduce photo-bleaching, but does not give quite as smooth of an image.

- For averaging, select the **Line** or **Frame** mode in the **Acquisition Mode** tool.

- Select the number of lines or frames to average.

Adjusting pinhole size

- Select the **Channels** tool in the **Left Tool Area**.

- Set the **Pinhole** size to **1 AU** (Airy unit) for best compromise between depth discrimination and detection efficiency.

Pinhole adjustment changes the **Optical Slice thickness**. When collecting multi-channel images, adjust the pinholes so that each channel has the same **Optical Slice thickness**. This is important for colocalization studies.
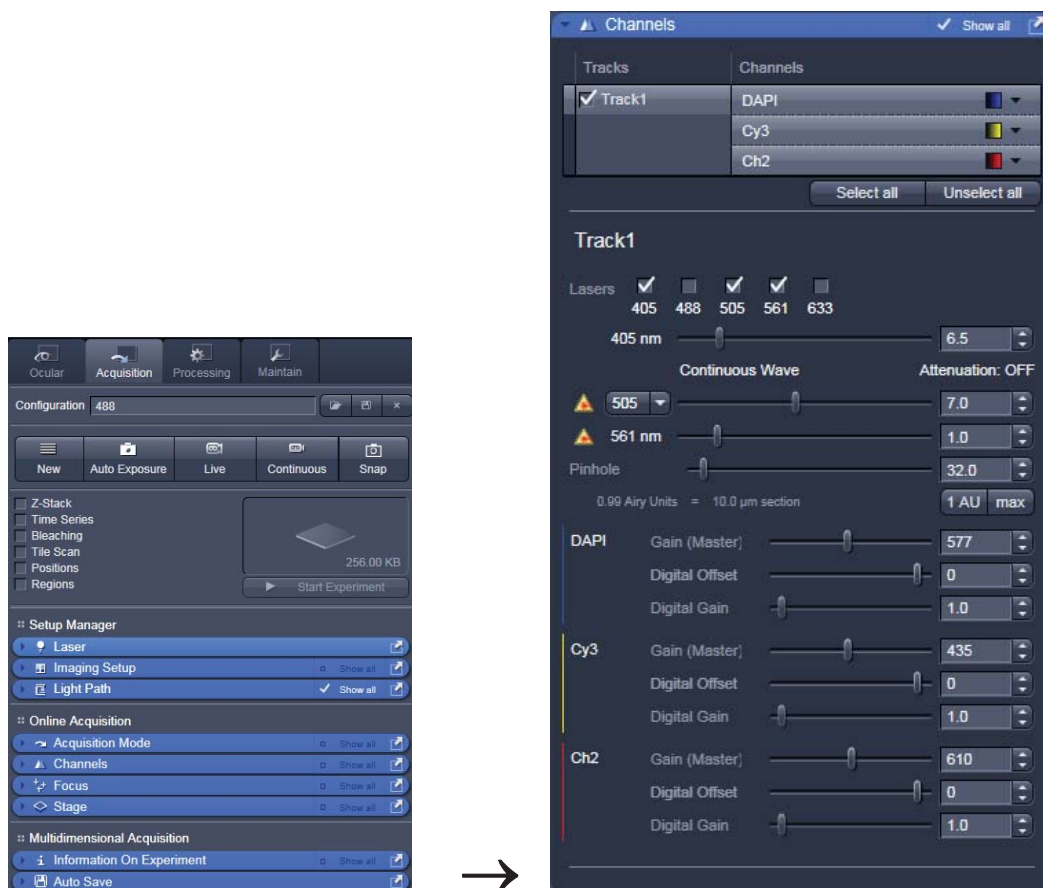
Fig. 19   **Channels tool**

Image acquisition

Once you have set up your parameter as defined in the above section, you can acquire a frame image of your specimen.

- Use one of the **Auto Exposure**, **Live**, **Continuous** or **Snap** buttons to start the scanning procedure to acquire an image.

- Scanned images are shown in separate windows.

- Click on the **Stop** button to stop the current scan procedure if necessary.

Fig. 20   Image Display
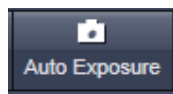
	Select **Auto Exposure** for automatic pre-adjustment of detector gain and offset.
	Select **Live** for continuous fast scanning – useful for finding and changing the focus.
	Select **Continuous** for continuous scanning with the selected scan speed.
	Select **Snap** for recording a single image.
	Select **Stop** for stopping the current scan procedure.
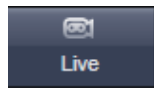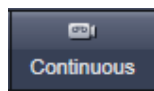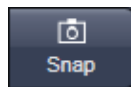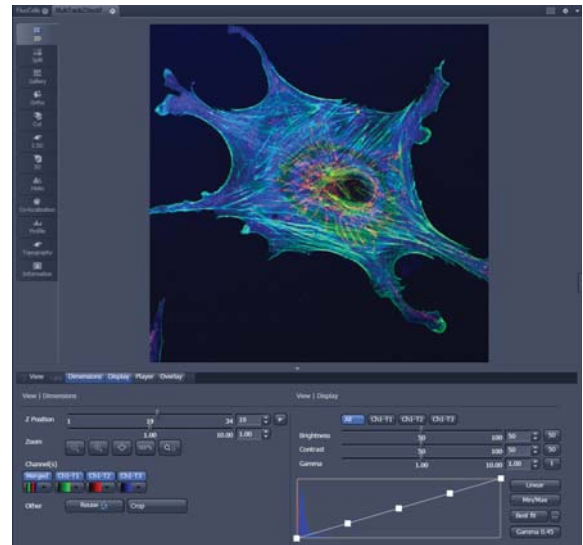
Image optimization

Choosing Range Indicator

- In the **View – Dimensions** View Option Control Block, click inside the color field in the button under the channel button (Fig. 21).

☞   Clicking on the right hand side of the button leads to a list of colors.

Fig. 21   View Dimensions Control Block
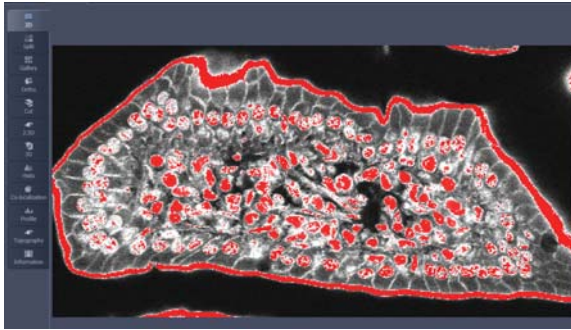
Fig. 22   Image Display

Fig. 23   Channels tool

The scanned image appears in a false-color presentation (Fig. 22).

If the image is too bright, it appears red on the screen. Red = saturation (maximum).

If the image is not bright enough, it appears blue on the screen. Blue = zero (minimum).

Adjusting the laser intensity

- Set the **Pinhole** to **1** Airy Unit (Fig. 23).

- Set the **Gain (Master)** high.

- When the image is saturated, reduce AOTF transmission in the **Laser** control section of the **Channels Tool** (Fig. 23) using the slider to reduce the intensity of the laser light to the specimen.
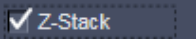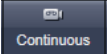
Adjusting gain and offset
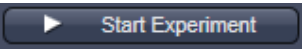
- Increase the **Digital Offset** until all blue pixels disappear, and then make it slightly positive (Fig. 23).

- Reduce the **Gain (Master)** until the red pixels only just disappear.

Scanning a Z-Stack

- Select **Z-Stack** ☑ Z-Stack in the main tools area.

- Open the **Z Stack** tool in the **Left Tool Area**.

- Select **Mode First/Last** on the top of the **Z-Stack** tool.

- Click on the [Continuous] button in the **Action Button** area.

A continuous XY-scan of the set focus position will be performed.

- Use the focus drive of the microscope to focus on the upper position of the specimen area where the Z-Stack is to start.

- Click on the **Set First** button to set the upper position of the Z-Stack.

Fig. 24   **Z-Stack tool**

- Then focus on the lower specimen area where the recording of the Z-Stack is to end.

- Click on the **Set Last** button to set this lower position.

- Click on the [Optimal 6.63 µm] button to set number of slices to match the optimal Z-interval for the given stack size, objective lens, and the pinhole diameter.

- Click on the [▶ Start Experiment] **Start Experiment** button to start the recording of the Z-Stack.

☞   When a multi-dimensional acquisition tool is not selected, the respective tool and its set parameters are not included in the multidimensional image acquisition. If no multidimensional tool is activated, the [▶ Start Experiment] **Start Experiment** button is grayed out and only single images can be scanned.
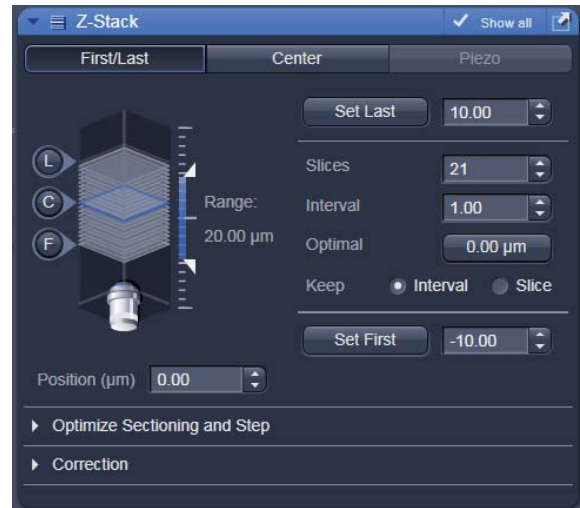
Storing and exporting image data

Fig. 25   **Save Image buttons in ZEN**

- To save your acquired or processed images, click on the **Save** or **Save As** button in **File Menu**, or click the ▣ button in the **Main Toolbar** (Fig. 25/**1**), or click on the ▣ button at the bottom of the **File Handling Area** (Fig. 25/**2**).
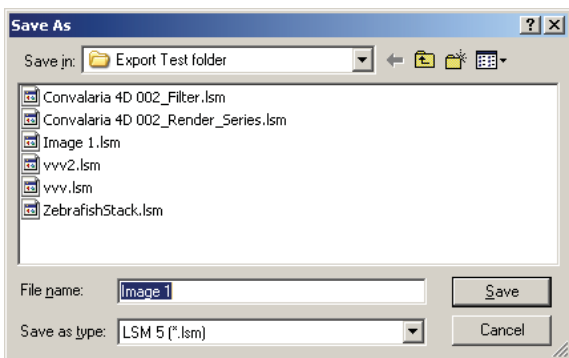
Fig. 26   **Save as window**

- The WINDOWS **Save As** window appears.
- Enter a file name and choose the appropriate image format. Note: the LSM 5 format is the native Carl Zeiss LSM image data format and contains all available extra information and hardware settings of your experiment.
- Click on the **SAVE** button.

If you close an image which has not been saved, a pop-up window will ask you if you want to save it. Choosing "yes" will lead you to the WINDOWS Save As window.

Fig. 27   **Export window**

To export image display data, a single optical section in raw data format or the contents of the image display window including analysis and overlays, choose **Export** from the **File Menu**. In the **Export** window you can select from a number of options and proceed to the WINDOWS Save As window to save the exported data to disk.

Switching off the system

- Click on the **File** button in the **Main Menu** bar and then click on the **Exit** button to leave the **ZEN 2009** software.

- If any lasers are still running you should shut them off now in the pop-up window indicating the lasers still in use.

- Shut down the computer.

- **Wait until the fan of the Argon laser has switched off.**

- On the REMOTE CONTROL turn off the **Components** switch and the **System/PC** switch (Fig. 1).

- Switch off the X-Cite 120 lamp or the HBO 100 mercury burner.

- Switch off the UV-Ar laser of by the toggle switch on the power supply (Fig. 2).

Principles

Confocal
Laser Scanning Microscopy
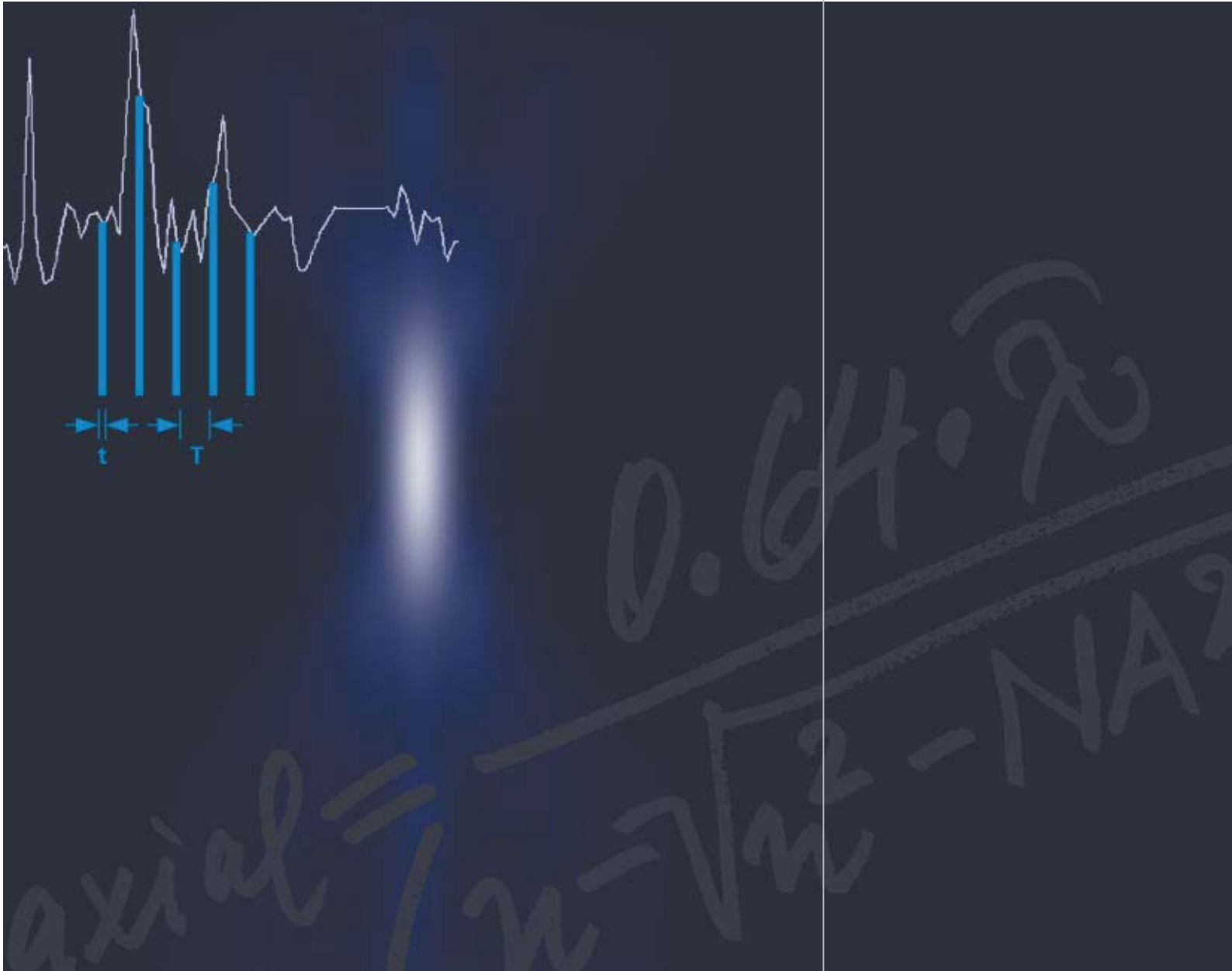
Optical Image Formation

Electronic Signal Processing

ZEISS

**Highlights of
Laser Scanning Microscopy**

1982
The first Laser Scanning Microscope from Carl Zeiss.
The prototype of the LSM 44 series is now on display in the
Deutsches Museum in Munich.

1988
The LSM 10 – a confocal system
with two fluorescence channels.

1991
The LSM 310 combines confocal laser
scanning microscopy with
state-of-the-art computer technology.

1992
The LSM 410 is the first inverted micro-
scope of the LSM family.

1997
The LSM 510 – the first system of the
LSM 5 family and a major breakthrough
in confocal imaging and analysis.
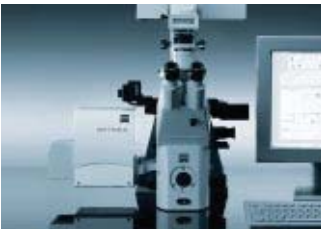
1998
The LSM 510 NLO is ready for multi-
photon microscopy.

1999
The LSM 5 PASCAL –
the personal confocal microscope.

2000
The LSM is combined with
the ConfoCor 2 Fluorescence
Correlation Spectroscope.

2001
The LSM 510 META –
featuring multispectral analysis.

Confocal Laser Scanning Microscopy

In recent years, the confocal Laser Scanning Microscope (LSM) has become widely established as a research instrument.

The present brochure aims at giving a scientifically sound survey of the special nature of image formation in a confocal LSM.

LSM applications in biology and medicine predominantly employ fluorescence, but it is also possible to use the transmission mode with conventional contrasting methods, such as differential interference contrast (DIC), as well as to overlay the transmission and confocal fluorescence images of the same specimen area.
Another important field of application is materials science, where the LSM is used mostly in the reflection mode and with such methods as polarization.

Confocal microscopes are even used in routine quality inspection in industry. Here, confocal images provide an efficient way to detect defects in semiconductor circuits.

Contents

Following a description of the fundamental differences between a conventional and a confocal microscope, this monograph will set out the special features of the confocal LSM and the capabilities resulting from them.

The conditions in fluorescence applications will be given priority treatment throughout.

Fig. 1 The quality of the image generated in a confocal LSM is not only influenced by the optics (as in a conventional microscope), but also, e.g., by the confocal aperture (pinhole) and by the digitization of the object information (pixel size). Another important factor is noise (laser noise, or the shot noise of the fluorescent light).
To minimize noise, signal-processing as well as optoelectronic and electronic devices need to be optimized.

Image generation

The complete generation of the two-dimensional object information from the focal plane (object plane) of a confocal LSM essentially comprises three process steps:

1. Line-by-line scanning of the specimen with a focused laser beam deflected in the X and Y directions by means of two galvanometric scanners.

2. Pixel-by-pixel detection of the fluorescence emitted by the scanned specimen details, by means of a photomultiplier tube (PMT).

3. Digitization of the object information contained in the electrical signal provided by the PMT (for presentation, the image data are displayed, pixel by pixel, from a digital matrix memory to a monitor screen).

Scanning process

In a conventional light microscope, object-to-image transformation takes place simultaneously and parallel for all object points. By contrast, the specimen in a confocal LSM is irradiated in a point-wise fashion, i.e. serially, and the physical interaction between the laser light and the specimen detail irradiated (e.g. fluorescence) is measured point by point. To obtain information about the entire specimen, it is necessary to guide the laser beam across the specimen, or to move the specimen relative to the laser beam, a process known as scanning. Accordingly, confocal systems are also known as point-probing scanners.

To obtain images of microscopic resolution from a confocal LSM, a computer and dedicated software are indispensable.

The descriptions below exclusively cover the point scanner principle as implemented, for example, in Carl Zeiss laser scanning microscopes. Configurations in which several object points are irradiated simultaneously are not considered.

Confocal beam path

The decisive design feature of a confocal LSM compared with a conventional microscope is the confocal aperture (usually called pinhole) arranged in a plane conjugate to the intermediate image plane and, thus, to the object plane of the microscope. As a result, the detector (PMT) can only detect light that has passed the pinhole. The pinhole diameter is variable; ideally, it is infinitely small, and thus the detector looks at a point (point detection).

As the laser beam is focused to a diffraction-limited spot, which illuminates only a point of the object at a time, the point illuminated and the point observed (i.e. image and object points) are situated in conjugate planes, i.e. they are focused onto each other. The result is what is called a confocal beam path (see figure 2).

Pinhole

Depending on the diameter of the pinhole, light coming from object points outside the focal plane is more or less obstructed and thus excluded from detection. As the corresponding object areas are invisible in the image, the confocal microscope can be understood as an inherently depth-discriminating optical system.

By varying the pinhole diameter, the degree of confocality can be adapted to practical requirements. With the aperture fully open, the image is nonconfocal. As an added advantage, the pinhole suppresses stray light, which improves image contrast.

Fig. 2 Beam path in a confocal LSM. A microscope objective is used to focus a laser beam onto the specimen, where it excites fluorescence, for example. The fluorescent radiation is collected by the objective and efficiently directed onto the detector via a dichroic beamsplitter. The interesting wavelength range of the fluorescence spectrum is selected by an emission filter, which also acts as a barrier blocking the excitation laser line. The pinhole is arranged in front of the detector, on a plane conjugate to the focal plane of the objective. Light coming from planes above or below the focal plane is out of focus when it hits the pinhole, so most of it cannot pass the pinhole and therefore does not contribute to forming the image.
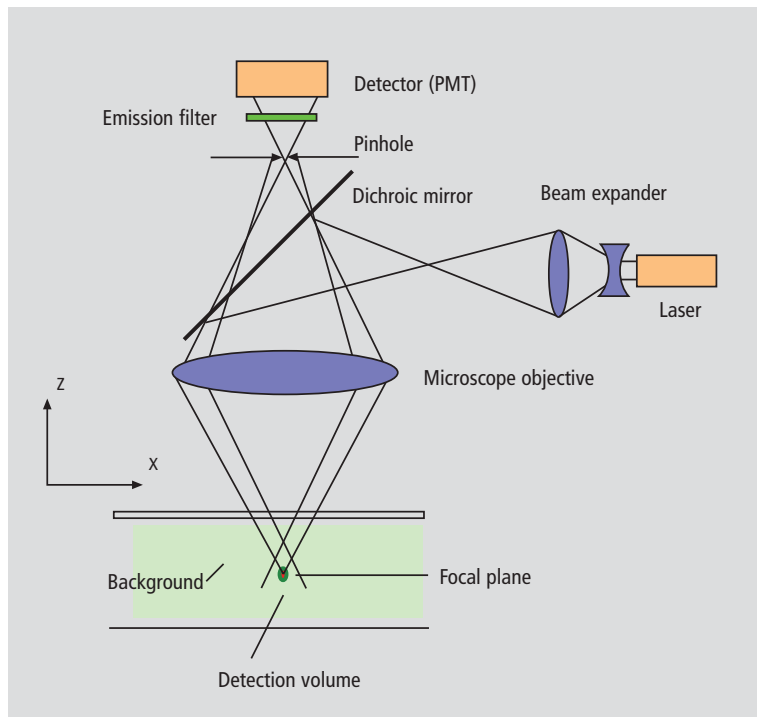
Optical slices

With a confocal LSM it is therefore possible to exclusively image a thin optical slice out of a thick specimen (typically, up to 100 μm), a method known as optical sectioning. Under suitable conditions, the thickness (Z dimension) of such a slice may be less than 500 nm.

The fundamental advantage of the confocal LSM over a conventional microscope is obvious: In conventional fluorescence microscopy, the image of a thick biological specimen will only be in focus if its Z dimension is not greater than the wave-optical depth of focus specified for the respective objective.

Unless this condition is satisfied, the in-focus image information from the object plane of interest is mixed with out-of focus image information from planes outside the focal plane. This reduces image contrast and increases the share of stray light detected. If multiple fluorescences are observed, there will in addition be a color mix of the image information obtained from the channels involved (figure 3, left).
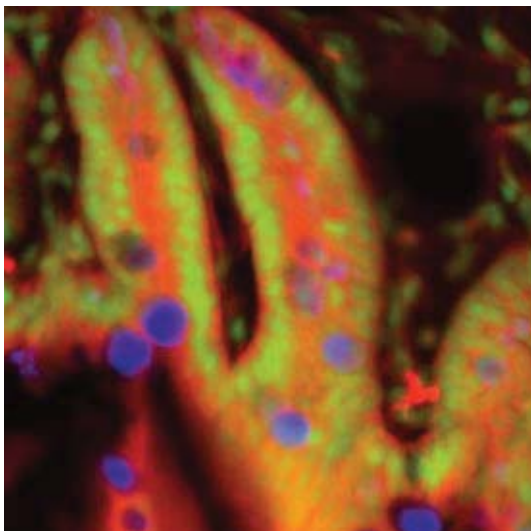
A confocal LSM can therefore be used to advantage especially where thick specimens (such as biological cells in tissue) have to be examined by fluorescence. The possibility of optical sectioning eliminates the drawbacks attached to the observation of such specimens by conventional fluorescence microscopy. With multicolor fluorescence, the various channels are satisfactorily separated and can be recorded simultaneously.

With regard to reflective specimens, the main application is the investigation of the topography of 3D surface textures.

Figure 3 demonstrates the capability of a confocal Laser Scanning Microscope.

Fig. 3   Non-confocal (left) and confocal (right) image of a triple-labeled cell aggregate (mouse intestine section). In the non-confocal image, specimen planes outside the focal plane degrade the information of interest from the focal plane, and differently stained specimen details appear in mixed color. In the confocal image (right), specimen details blurred in non-confocal imaging become distinctly visible, and the image throughout is greatly improved in contrast.
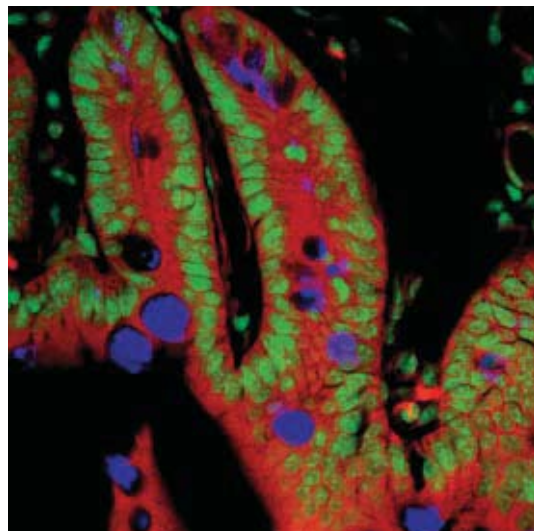
3rd dimension

In addition to the possibility to observe a single plane (or slice) of a thick specimen in good contrast, optical sectioning allows a great number of slices to be cut and recorded at different planes of the specimen, with the specimen being moved along the optical axis (Z) by controlled increments. The result is a 3D data set, which provides information about the spatial structure of the object. The quality and accuracy of this information depend on the thickness of the slice and on the spacing between successive slices (optimum scanning rate in Z direction = 0.5x the slice thickness). By computation, various aspects of the object can be generated from the 3D data set (3D reconstruction, sections of any spatial orientation, stereo pairs etc.). Figure 4 shows a 3D reconstruction computed from a 3D data set.

Time series

A field of growing importance is the investigation of living specimens that show dynamic changes even in the range of microseconds. Here, the acquisition of time-resolved confocal image series (known as time series) provides a possibility of visualizing and quantifying the changes.

The following section (Part 1, page 6 ff) deals with the purely optical conditions in a confocal LSM and the influence of the pinhole on image formation. From this, ideal values for resolution and optical slice thickness are derived.

Part 2, page 16 ff limits the idealized view, looking at the digitizing process and the noise introduced by the light as well as by the optoelectronic components of the system.

The table on page 15 provides a summary of the essential results of Part 1. A schematic overview of the entire content and its practical relevance is given on the poster inside this brochure.

Fig. 4   3D projection reconstructed from 108 optical slices of a three-dimensional data set of epithelium cells of a lacrimal gland. Actin filaments of myoepithelial cells marked with BODIPY-FL phallacidin (green), cytoplasm and nuclei of acinar cells with ethidium homodimer-1 (red).

Fig. 5   Gallery of a time series experiment with Kaede-transfected cells. By repeated activation of the Kaede marker (green-to-red color change) in a small cell region, the entire green fluorescence is converted step by step into the red fluorescence.
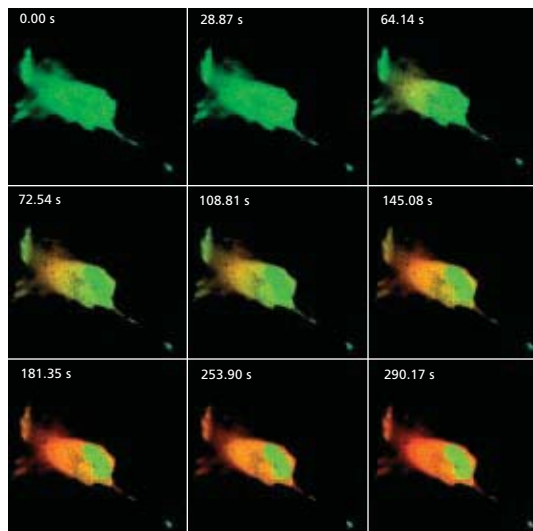
Point Spread Function

In order to understand the optical performance characteristics of a confocal LSM in detail, it is necessary to have a closer look at the fundamental optical phenomena resulting from the geometry of the confocal beam path. As mentioned before, what is most essential about a confocal LSM is that both illumination and observation (detection) are limited to a point.

Not even an optical system of diffraction-limited design can image a truly point-like object as a point. The image of an ideal point object will always be somewhat blurred, or "spread" corresponding to the imaging properties of the optical system. The image of a point can be described in quantitative terms by the point spread function (PSF), which maps the intensity distribution in the image space.

Where the three-dimensional imaging properties of a confocal LSM are concerned, it is necessary to consider the 3D image or the 3D-PSF.

In the ideal, diffraction-limited case (no optical aberrations, homogeneous illumination of the pupil – *see Details "Pupil Illumination"*), the 3D-PSF is of comet-like, rotationally symmetrical shape.

For illustration, Figure 6 shows two-dimensional sections (XZ and XY) through an ideal 3D-PSF.

From the illustration it is evident that the central maximum of the 3D-PSF, in which 86.5 % of the total energy available in the pupil are concentrated, can be described as an ellipsoid of rotation. For considerations of resolution and optical slice thickness it is useful to define the half-maximum area of the ellipsoid of rotation, i.e. the well-defined area in which the intensity of the 3D point image in axial and lateral directions has dropped to half of the central maximum.
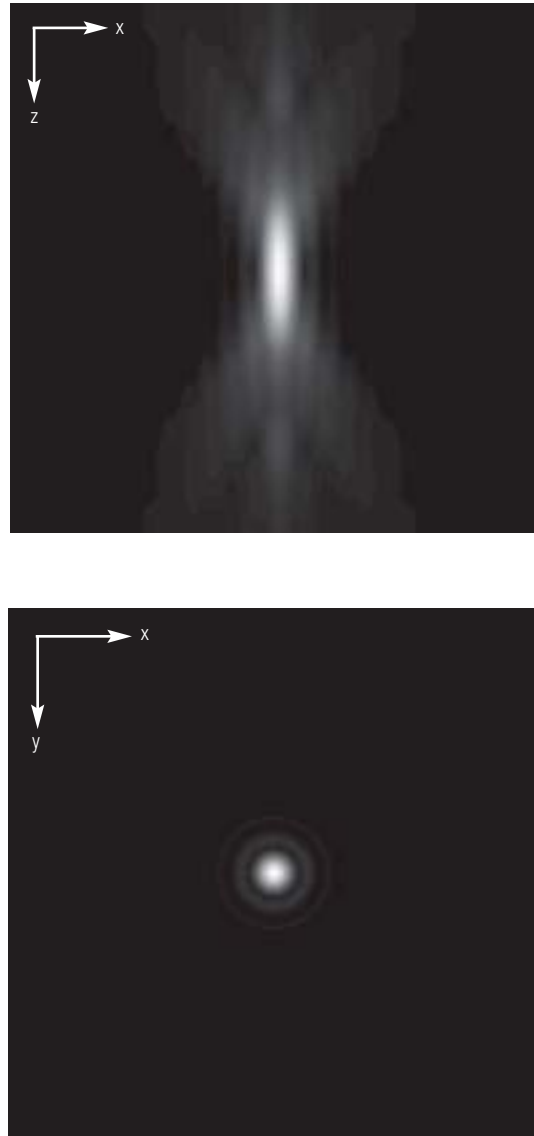
Fig. 6 Section through the 3D-PSF in Z direction – top, and in XY-direction – bottom (computed; dimensionless representation); the central, elliptical maximum is distinctly visible.

The central maximum in the bottom illustration is called Airy disk and is contained in the 3D-PSF as the greatest core diameter in lateral direction.

Any reference to the PSF in the following discussion exclusively refers to the half-maximum area. Quantitatively the half-maximum area is described in terms of the full width at half maximum (FWHM), a lateral or axial distance corresponding to a 50% drop in intensity.

The total PSF (PSF_{tot}) of a confocal microscope behind the pinhole is composed of the PSFs of the illuminating beam path (PSF_{ill}; point illumination) and the detection beam path (PSF_{det}; point detection). Accordingly, the confocal LSM system as a whole generates two point images: one by projecting a point light source into the object space, the other by projecting a point detail of the object into the image space. Mathematically, this relationship can be described as follows:

$$PSF_{tot}(x,y,z) = PSF_{ill}(x,y,z) \cdot PSF_{det}(x,y,z) \qquad (1)$$

PSF_{ill} corresponds to the light distribution of the laser spot that scans the object. Its size is mainly a function of the laser wavelength and the numerical aperture of the microscope objective. It is also influenced by diffraction at the objective pupil (as a function of pupil illumination) and the aberrations of all optical components integrated in the system. [Note: In general, these aberrations are low, having been minimized during system design].

Moreover, PSF_{ill} may get deformed if the laser focus enters thick and light-scattering specimens, especially if the refractive indices of immersion liquid and mounting medium are not matched and/or if the laser focus is at a great depth below the specimen surface (see Hell, S., et al., [9]).

PSF_{det} is also influenced by all these factors and, additionally, by the pinhole size. For reasons of beam path efficiency (see Part 2), the pinhole is never truly a point of infinitely small size and thus PSF_{det} is never smaller in dimension than PSF_{ill}. It is evident that the imaging properties of a confocal LSM are determined by the interaction between PSF_{ill} and PSF_{det}. As a consequence of the interaction process, $PSF_{tot} \leq PSF_{ill}$.

With the pinhole diameter being variable, the effects obtained with small and big pinhole diameters must be expected to differ.

In the following sections, various system states are treated in quantitative terms.

From the explanations made so far, it can also be derived that the optical slice is not a sharply delimited body. It does not start abruptly at a certain Z position, nor does it end abruptly at another. Because of the intensity distribution along the optical axis, there is a continuous transition from object information suppressed and such made visible.

Accordingly, the out-of-focus object information actually suppressed by the pinhole also depends on the correct setting of the image processing parameters (PMT high voltage, contrast setting). Signal overdrive or excessive offset should be avoided.

Resolution and Confocality

Wherever quantitative data on the resolving power and depth discrimination of a confocal LSM are specified, it is necessary to distinguish clearly whether the objects they refer to are point-like or extended, and whether they are reflective or fluorescent. These differences involve distinctly varying imaging properties. Fine structures in real biological specimens are mainly of a filiform or point-like fluorescent type, so that the explanations below are limited to point-like fluorescent objects. The statements made for this case are well applicable to practical assignments.

As already mentioned, the pinhole diameter plays a decisive role in resolution and depth discrimination. With a pinhole diameter greater than 1 AU (AU = Airy unit – *see Details "Optical Coordinates"*), the depth discriminating properties under consideration are essentially based on the law of geometric optics (geometric-optical confocality).

The smaller the pinhole diameter, the more PSF_{det} approaches the order of magnitude of PSF_{ill}. In the limit case (PH < 0.25 AU), both PSFs are approximately equal in size, and wave-optical image formation laws clearly dominate (wave-optical confocality).

Figure 7 illustrates these concepts. It is a schematic representation of the half-intensity areas of PSF_{ill} and PSF_{det} at selected pinhole diameters.

Depending on which kind of confocality dominates, the data and computation methods for resolution and depth discrimination differ. A comparison with image formation in conventional microscopes is interesting as well. The following sections deal with this in detail.

Fig. 7 Geometric-optical (a) and wave-optical confocality (c) [XZ view].
The pinhole diameter decreases from (a) to (c).
Accordingly, PSF_{det} shrinks until it approaches the order of magnitude of PSF_{ill} (c).

a) **PH~3.0 AU**

$FWHM_{ill, lateral}$

$FWHM_{ill, axial}$

$FWHM_{det, axial}$

$FWHM_{det, lateral}$

$PSF_{det} >> PSF_{ill}$

Geometric-optical confocality

b) **PH~1 AU**

$PSF_{det} > PSF_{ill}$

c) **PH~0,25 AU**

$PSF_{det} >= PSF_{ill}$

Wave-optical confocality
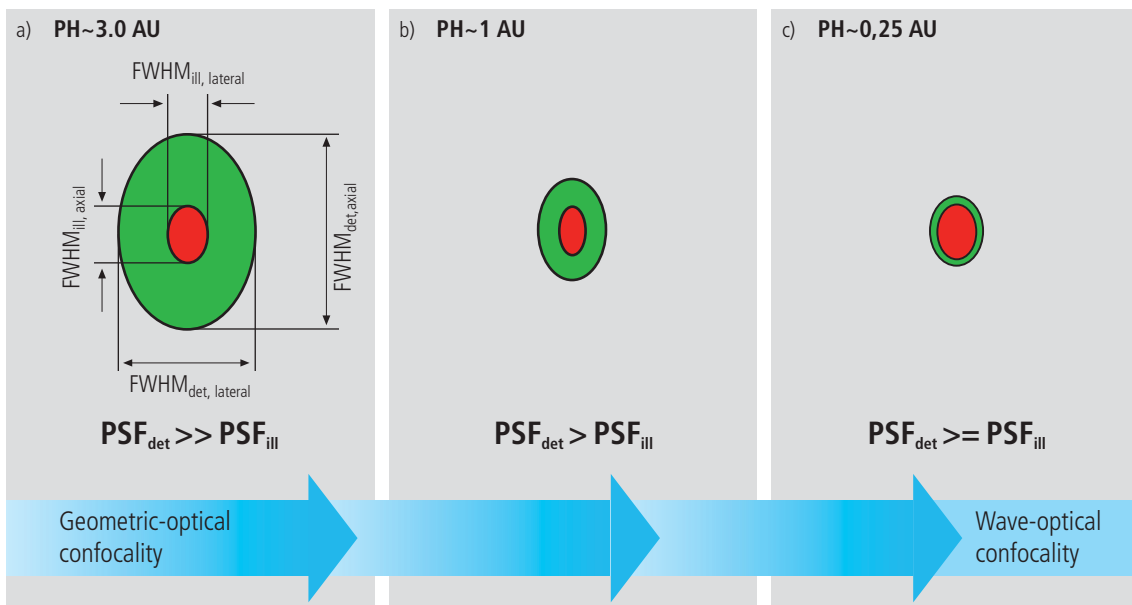
Resolution

Resolution, in case of large pinhole diameters (PH >1 AU), is meant to express the separate visibility, both laterally and axially, of points during the scanning process. Imagine an object consisting of individual points: all points spaced closer than the extension of PSF$_{ill}$ are blurred (spread), i.e. they are not resolved.

Quantitatively, resolution results from the axial and lateral extension of the scanning laser spot, or the elliptical half-intensity area of PSF$_{ill}$. On the assumption of homogeneous pupil illumination, the following equations apply:

Axial:

$$FWHM_{ill,axial} = \frac{0.88 \cdot \lambda_{exc}}{(n-\sqrt{n^2-NA^2})} \qquad (2)$$

n = refractive index of immersion liquid,
NA = numerical aperture of the microscope objective,
λ_{exc} = wavelength of the excitation light

If NA < 0.5, equation (2) can be approximated by:

$$\approx \frac{1.77 \cdot n \cdot \lambda_{exc}}{NA^2} \qquad (2a)$$

Lateral:

$$FWHM_{ill,lateral} = 0.51 \frac{\lambda_{exc}}{NA} \qquad (3)$$

At first glance, equations (2a) and (3) are not different from those known for conventional imaging (see Beyer, H., [3]). It is striking, however, that the resolving power in the confocal microscope depends only on the wavelength of the illuminating light, rather than exclusively on the emission wavelength as in the conventional case.

Compared to the conventional fluorescence microscope, confocal fluorescence with large pinhole diameters leads to a gain in resolution by the factor ($\lambda_{em}/\lambda_{exc}$) via the Stokes shift.
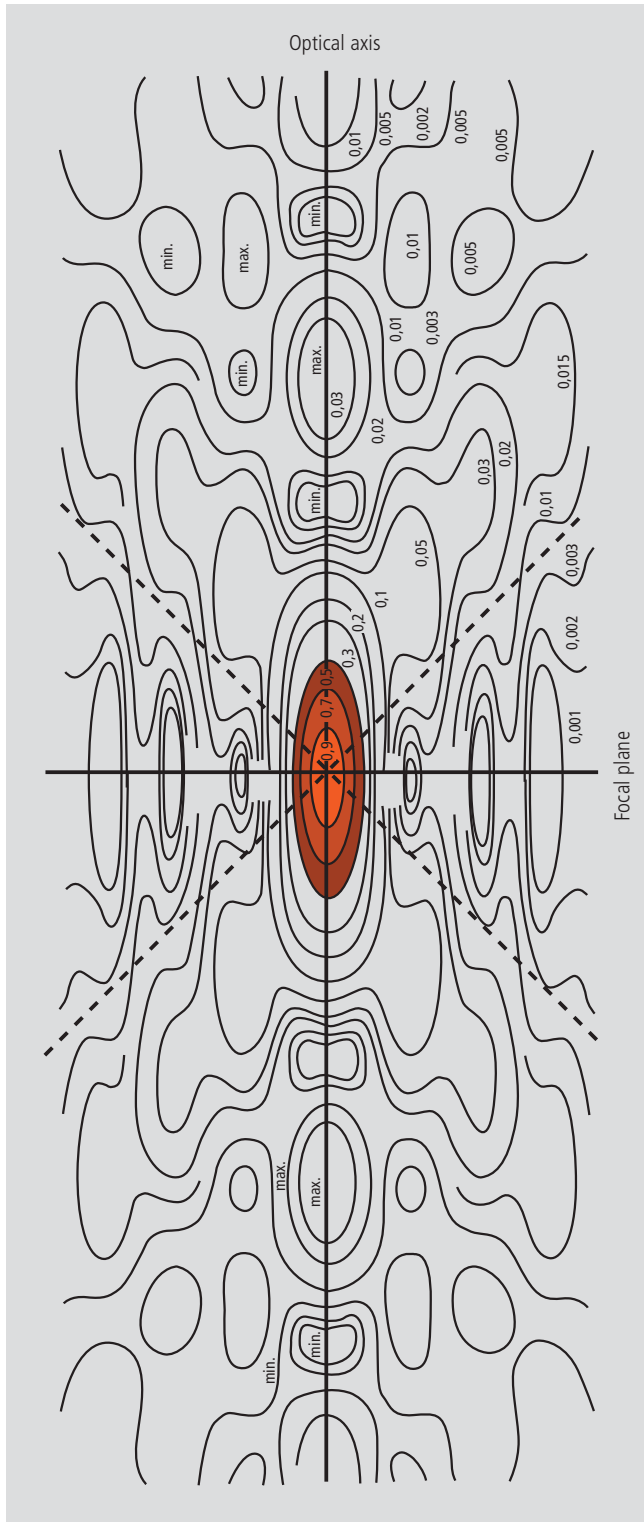
Let the statements made on PSF so far be further illustrated by the figure on the left. It shows a section through the resulting diffraction pattern surrounding the focus on the illumination side (PSF$_{ill}$). The lines include areas of equal brightness (isophote presentation). The center has a normalized intensity of 1. The real relationships result by rotation of the section about the vertical (Z) axis. Symmetry exists relative to the focal plane as well as to the optical axis. Local intensity maxima and minima are conspicuous. The dashed lines mark the range covered by the aperture angle of the microscope objective used.

For the considerations in this chapter, only the area inside the red line, i.e. the area at half maximum, is of interest.

Fig. 8 Isophote diagram of the intensity distribution around the illumination-side focus (PSF$_{ill}$). The intensity at the focus is normalized as 1. (Born & Wolf, Priniples of Optics, 6th edition 1988, Pergamon Press)

Optical Image Formation

Geometric optical confocality

Optical slice thickness (depth discrimination) and stray light suppression (contrast improvement) are basic properties of a confocal LSM, even if the pinhole diameter is not an ideal point (i.e. not infinitely small). In this case, both depth discrimination and stray light suppression are determined exclusively by PSF_{det}. This alone brings an improvement in the separate visibility of object details over the conventional microscope.

Hence, the diameter of the corresponding half-intensity area and thus the optical slice thickness is given by:

$$FWHM_{det,axial} = \sqrt{\left(\frac{0.88 \cdot \lambda_{em}}{n - \sqrt{n^2 - NA^2}}\right)^2 + \left(\frac{\sqrt{2} \cdot n \cdot PH}{NA}\right)^2} \quad (4)$$

λ_{em} = emission wavelength
PH = object-side pinhole diameter [μm]
n = refractive index of immersion liquid
NA = numerical aperture of the objective

Equation (4) shows that the optical slice thickness comprises a geometric-optical and a wave-optical term. The wave-optical term (first term under the root) is of constant value for a given objective and a given emission wavelength. The geometric-optical term (second term under the root) is dominant; for a given objective it is influenced exclusively by the pinhole diameter.

Likewise, in the case of geometric-optical confocality, there is a linear relationship between depth discrimination and pinhole diameter. As the pinhole diameter is constricted, depth discrimination improves (i.e. the optical slice thickness decreases). A graphical representation of equation (4) is illustrated in figure 9. The graph shows the geometric-optical term alone (blue line) and the curve resulting from eq. 4 (red line). The difference between the two curves is a consequence of the wave-optical term.

Above a pinhole diameter of 1 AU, the influence of diffraction effects is nearly constant and equation (4) is a good approximation to describe the depth discrimination. The interaction between PSF_{ill} and PSF_{det} becomes manifest only with pinhole diameters smaller than 1 AU.

Let it be emphasized that in case of geometric optical confocality the diameters of the half-intensity area of PSF_{det} allow no statement about the separate visibility of object details in axial and lateral direction.

In the region of the optical section ($FWHM_{det,axial}$), object details are resolved (imaged separately) only unless they are spaced not closer than described by equations (2) / (2a) / (3).

Fig.9 Optical slice thickness as a function of the pinhole diameter (red line). Parameters: NA = 0.6; n = 1; λ = 520 nm. The X axis is dimensioned in Airy units, the Y axis (slice thickness) in Rayleigh units (see also: Details "Optical Coordinates"). In addition, the geometric-optical term in equation 4 is shown separately (blue line).
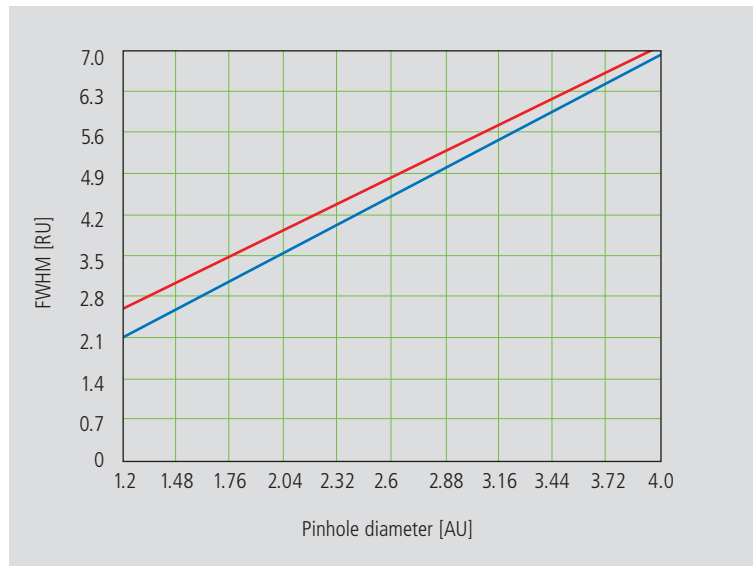
Wave-optical confocality

If the pinhole is closed down to a diameter of < 0.25 AU (virtually "infinitely small"), the character of the image changes. Additional diffraction effects at the pinhole have to be taken into account, and PSF_{det} (optical slice thickness) shrinks to the order of magnitude of PSF_{ill} (Z resolution) (see also figure 7c).

In order to achieve simple formulae for the range of smallest pinhole diameters, it is practical to regard the limit of PH = 0 at first, even though it is of no practical use. In this case, PSF_{det} and PSF_{ill} are identical.
The total PSF can be written as

$$PSF_{tot}(x,y,z) = \left(PSF_{ill}(x,y,z)\right)^2 \qquad (5)$$

In fluorescence applications it is furthermore necessary to consider both the excitation wavelength λ_{exc} and the emission wavelength λ_{em}. This is done by specifying a mean wavelength[1]:

$$\bar{\lambda} \approx \sqrt{2}\, \frac{\lambda_{em} \cdot \lambda_{exc}}{\sqrt{\lambda^2_{exc} + \lambda^2_{em}}} \qquad (6)$$

Thus, equations (2) and (3) for the widths of the axial and lateral half-intensity areas are transformed into:

Axial:

$$FWHM_{tot,axial} = \frac{0.64 \cdot \bar{\lambda}}{(n - \sqrt{n^2 - NA^2})} \qquad (7)$$

If NA < 0.5, equation (7) can be approximated by

$$\approx \frac{1.28 \cdot n \cdot \bar{\lambda}}{NA^2} \qquad (7a)$$

Lateral:

$$FWHM_{tot,lateral} = 0.37\, \frac{\bar{\lambda}}{NA} \qquad (8)$$

Note:
With the object being a mirror, the factor in equation 7 is 0.45 (instead of 0.64), and 0.88 (instead of 1.28) in equation 7a. For a fluorescent plane of finite thickness, a factor of 0.7 can be used in equation 7. This underlines that apart from the factors influencing the optical slice thickness, the type of specimen also affects the measurement result.

[1] For rough estimates, the expression $\bar{\lambda} \approx \sqrt{\lambda_{em} \cdot \lambda_{exc}}$ suffices.

From equations (7) and (7a) it is evident that depth resolution varies linearly with the refractive index n of the immersion liquid and with the square of the inverse value of the numerical aperture of the objective $\{NA = n \cdot \sin(\alpha)\}$.

To achieve high depth discrimination, it is important, above all, to use objectives with the highest possible numerical aperture.

As an NA > 1 can only be obtained with an immersion liquid, confocal fluorescence microscopy is usually performed with immersion objectives (see also figure 11).

A comparison of the results stated before shows that axial and lateral resolution in the limit of PH = 0 can be improved by a factor of 1.4. Furthermore it should be noted that, because of the wave-optical relationships discussed, the optical performance of a confocal LSM cannot be enhanced infinitely. Equations (7) and (8) supply the minimum possible slice thickness and the best possible resolution, respectively.

From the applications point of view, the case of strictly wave-optical confocality (PH = 0) is irrelevant (see also Part 2).

By merely changing the factors in equations (7) and (8) it is possible, though, to transfer the equations derived for PH = 0 to the pinhole diameter range up to 1 AU, to a good approximation. The factors applicable to particular pinhole diameters can be taken from figure 10.

It must also be noted that with PH <1 AU, a distinction between optical slice thickness and resolution can no longer be made. The thickness of the optical slice at the same time specifies the resolution properties of the system. That is why in the literature the term of depth resolution is frequently used as a synonym for depth discrimination or optical slice thickness. However, this is only correct for pinhole diameters smaller than 1 AU.
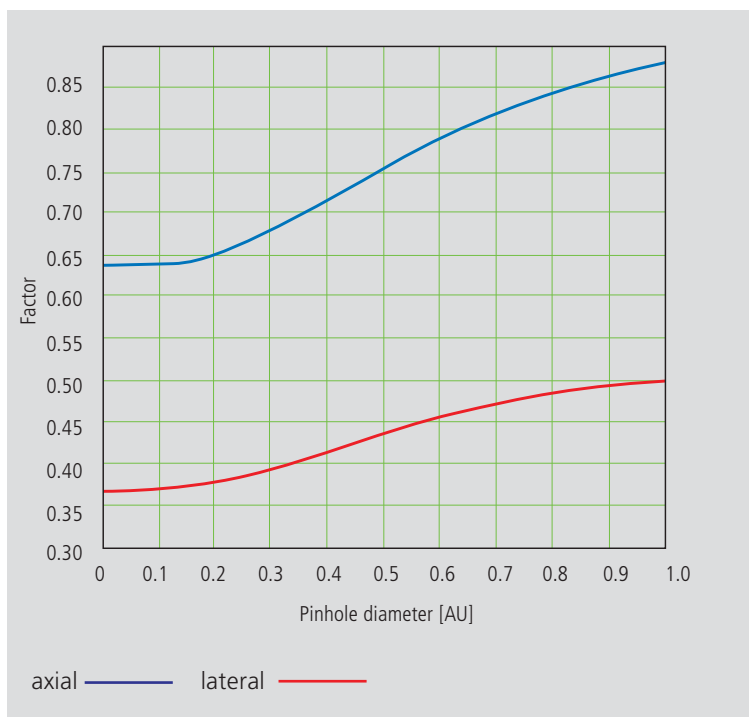
Fig. 10 Theoretical factors for equations (7) and (8), with pinhole diameters between 0 and 1 AU.

To conclude the observations about resolution and depth discrimination (or depth resolution), the table on page 15 provides an overview of the formulary relationships developed in Part 1. In addition, figure 11a shows the overall curve of optical slice thickness for a microscope objective of NA = 1.3 and n = 1.52 ($\bar{\lambda}$ = 496 nm).

In figure 11b-d, equation (7) is plotted for different objects and varied parameters (NA, $\bar{\lambda}$, n).

13

Optical slice
(NA = 1.3; n = 1.52;
$\bar{\lambda}$ = 496 nm)

FWHM [µm]

Pinhole diameter [AU]
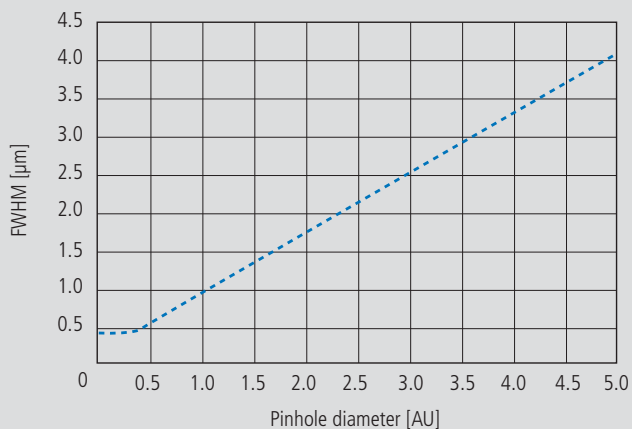
Fig. 11

a) Variation of
   pinhole diameter

Depth resolution
(PH = 0; n = 1.52;
$\bar{\lambda}$ = 496 nm)

FWHM [nm]

Numerical aperture
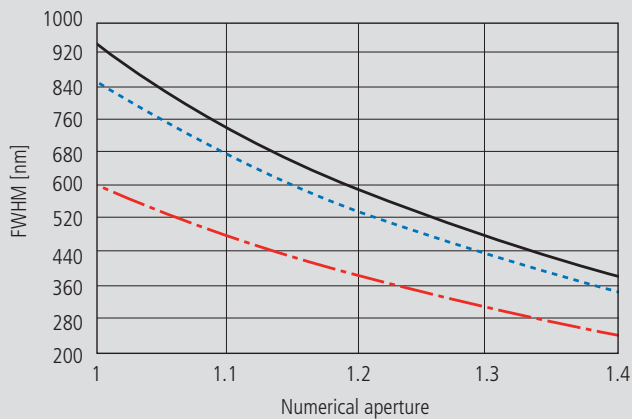
b) Variation of
   numerical aperture

Depth resolution
(PH = 0; NA = 1.3; n = 1.52)

FWHM [nm]
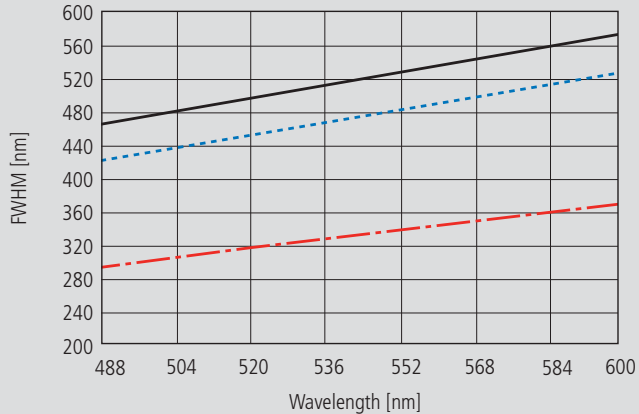
Wavelength [nm]

c) Variation of
   wavelength ($\bar{\lambda}$)

Depth resolution
(PH = 0; NA = 0.8;
$\bar{\lambda}$ = 496 nm)

FWHM [nm]

Refractive index of immersion liquid
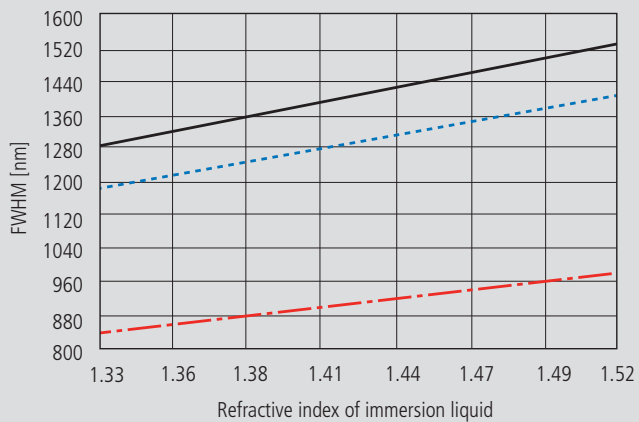
d) Variation of
   refractive index

——— fluorescent plane
········· fluorescent point
–·–·– reflecting plane (mirror)

14

Optical Image Formation

Overview

Conventional microscopy	Confocal microscopy 1 AU < PH <∞	Confocal microscopy PH < 0.25 AU
1. Optical slice thickness not definable With a conventional microscope, unlike in confocal microscopy, sharply defined images of "thick" biological specimens can only be obtained if their Z dimension is not greater than the wave-optical depth of field specified for the objective used. Depending on specimen thickness, object information from the focal plane is mixed with blurred information from out-of-focus object zones. Optical sectioning is not possible; consequently, no formula for optical slice thickness can be given.	1. Optical slice thickness[1] $$\sqrt{\left(\frac{0.88 \cdot \lambda_{em}}{\left(n-\sqrt{n^2 - NA^2}\right)}\right)^2 + \left(\frac{\sqrt{2} \cdot n \cdot PH}{NA}\right)^2}$$ Corresponds to the FWHM of the intensity distribution behind the pinhole (PSF_{det}). The FWHM results from the emission-side diffraction pattern and the geometric-optical effect of the pinhole. Here, PH is the variable object-side pinhole diameter in μm.	1. Optical slice thickness $$\frac{0.64 \cdot \bar{\lambda}}{\left(n-\sqrt{n^2 - NA^2}\right)}$$ The term results as the FWHM of the total PSF – the pinhole acts according to wave optics. $\bar{\lambda}$ stands for a mean wavelength – see the text body above for the exact definition. The factor 0.64 applies only to a fluorescent point object.
2. Axial resolution (wave-optical depth of field) $$\frac{n \cdot \lambda_{em}}{NA^2}$$ Corresponds to the width of the emission-side diffraction pattern at 80% of the maximum intensity, referred to the object plane. In the literature, the wave-optical depth of field in a conventional microscope is sometimes termed depth resolution. However, a clear distinction should be made between the terms resolution and depth resolution.	2. Axial resolution $$\frac{0.88 \cdot \lambda_{exc}}{\left(n-\sqrt{n^2 - NA^2}\right)}$$ FWHM of PSF_{ill} (intensity distribution at the focus of the microscope objective) in Z direction. No influence by the pinhole.	2. Axial resolution $$\frac{0.64 \cdot \bar{\lambda}}{\left(n-\sqrt{n^2 - NA^2}\right)}$$ FWHM of total PSF in Z direction As optical slice thickness and resolution are identical in this case, depth resolution is often used as a synonym.
3. For comparison: FWHM of PSF in the intermediate image (Z direction) – referred to the object plane. $$\frac{1.77 \cdot n \cdot \lambda_{em}}{NA^2}$$	3. Approximation to 2. for NA < 0.5 $$\frac{1.77 \cdot n \cdot \lambda_{exc}}{NA^2}$$	3. Approximation to 2. for NA < 0.5 $$\frac{1.28 \cdot n \cdot \bar{\lambda}}{NA^2}$$
4. Lateral resolution $$\frac{0.51 \cdot \lambda_{em}}{NA}$$ FWHM of the diffraction pattern in the intermediate image – referred to the object plane) in X/Y direction.	4. Lateral resolution $$\frac{0.51 \cdot \lambda_{em}}{NA}$$ FWHM of PSF_{ill} (intensity distribution at the focus of the microscope objective) in X/Y direction plus contrast-enhancing effect of the pinhole because of stray light suppression.	4. Lateral resolution $$\frac{0,37 \cdot \bar{\lambda}}{NA}$$ FWHM of total PSF in X/Y direction plus contrast-enhancing effect of the pinhole because of stray light suppression.

All data in the table refer to quantities in the object space and apply to a fluorescent point object.
1) PH <∞ is meant to express a pinhole diameter of < 4–5 AU.

Sampling and Digitization

After the optical phenomena have been discussed in Part 1, Part 2 takes a closer look at how the digitizing process and system-inherent sources of noise limit the performance of the system .

As stated in Part 1, a confocal LSM scans the specimen surface point by point. This means that an image of the total specimen is not formed simultaneously, with all points imaged in parallel (as, for example, in a CCD camera), but consecutively as a series of point images. The resolution obtainable depends on the number of points probed in a feature to be resolved.

Confocal microscopy, especially in the fluorescence mode, is affected by noise of light. In many applications, the number of light quanta (photons) contributing to image formation is extremely small. This is due to the efficiency of the system as a whole and the influencing factors involved, such as quantum yield, bleaching and saturation of fluorochromes, the transmittance of optical elements etc. (*see Details "Fluorescence"*). An additional influence factor is the energy loss connected with the reduction of the pinhole diameter.

In the following passages, the influences of scanning and noise on resolution are illustrated by practical examples and with the help of a two-point object. This is meant to be an object consisting of two self-luminous points spaced at 0.5 AU (*see Details "Optical Coordinates"*). The diffraction patterns generated of the two points are superimposed in the image space, with the maximum of one pattern coinciding with the first minimum of the other. The separate visibility of the points (resolution) depends on the existence of a dip between the two maxima (see figure 12).

As a rule, object information is detected by a photomultiplier tube (PMT). The PMT registers the spatial changes of object properties I(x) as a temporal intensity fluctuation I(t). Spatial and temporal coordinates are related to each other by the speed of the scanning process (x=t·v_{scan}). The PMT converts optical information into electrical information. The continuous electric signal is periodically sampled by an analog-to-digital (A/D) converter and thus transformed into a discrete, equidistant succession of measured data (pixels) (figure 12).

Types of A/D conversion

The quality of the image scanned depends on the type of A/D conversion which is employed. Two types can be distinguished:

• Sampling: The time (t) for signal detection (measurement) is small compared to the time (T) per cycle (pixel time) (see figure 12).

• Integration: The signal detection time has the same order of magnitude as the pixel time.

Integration is equivalent to an averaging of intensities over a certain percentage of the pixel time known as pixel dwell time. To avoid signal distortion (and thus to prevent a loss of resolution), the integration time must be shorter than the pixel time. The highest resolution is attained with point sampling (the sampling time is infinitesimally short, so that a maximum density of sampling points can be obtained). By signal integration, a greater share of the light emitted by the specimen contributes to the image signal. Where signals are weak (e.g. fluorescence), this is a decisive advantage over point sampling with regard to the signal-to-noise ratio (SNR). Therefore, Carl Zeiss confocal LSM systems operate in the integration mode, as a rule. The absolute integration time can be modified by varying the scanning speed, which also means a change of the pixel time.

Fig. 12 Pointwise sampling of a continuous signal
T = spacing of two consecutive sampling points
t = time of signal detection (t<<T)
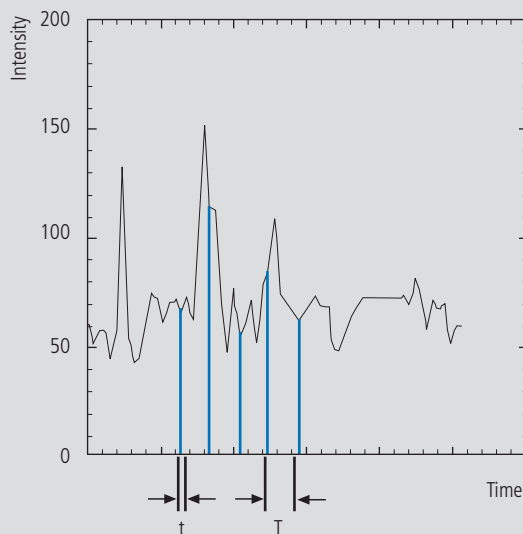
Nyquist theorem

It is known from Part 1 that the information content of the signal is limited by the resolving power of the microscope optics. A realistic estimate for the resolving power is the full width at half maximum intensity ($FWHM_{lat}$) of a point image (see equation 3).

To avoid a loss of information during the scanning process, it is necessary to stick to the Nyquist theorem. The optimal pixel spacing in scanning a periodic signal, as defined by the Nyquist theorem, is half the period of the feature spacing to be resolved, or two pixels per resolvable structure detail. Together with the resolving power defined above, this results in a maximum pixel spacing of $d_{pix} = 0.5 \times FWHM_{lat}$.

With a two-point object (see explanation on page 17), the pixel spacing needed to separate the two Airy discs in the digitized image is 0.25 AU (figure 13).

If the number of sampling points per feature size is smaller than that given by the Nyquist theorem (undersampling), part of the information will be lost. This is evident in Figure 14c especially by the unresolved fine features.

A greater number of sampling points per feature size (oversampling) means a greater number of readings without a gain in information; simultaneously, the time per pixel becomes shorter. Thus, the volume of data to be processed is inflated, and the noise of the measurement signal increases (see page 20)

Under unfavorable conditions, also artefacts may result out of the digitization process (aliasing). As a rule, this is the case if the feature spacing in the specimen is equal, or nearly equal, to the pixel spacing.

Fig. 13 The graph illustrates the scanning of a two-point object with the minimum number of sampling points needed to avoid a loss of resolution (spacing of sampling points 0.25 AU).

Fig. 14 Oversampling, correct sampling and undersampling of a continous signal.
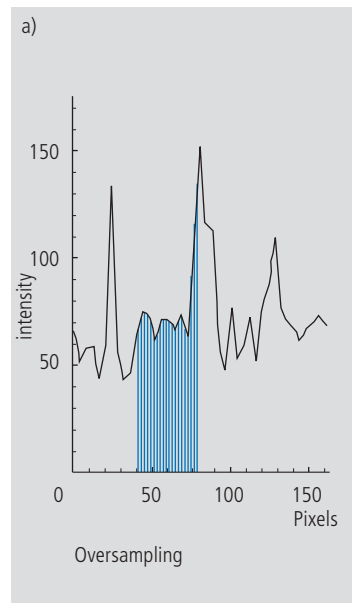
Pixel size

A quantity of decisive importance in this connection is the maximum scanning angle set via the scanning zoom. By varying the scanning angle, it is possible to directly influence the edge length of the scanned field in the intermediate image plane (or object plane), and thus the pixel size (at a given number of pixels per line). The smaller the scanning angle, the smaller is the edge length of the scanned field, and the smaller is the pixel (see the example below).

In this way, the user of a Carl Zeiss confocal LSM can control the sampling rate (pixel size). For setting the suitable scanning zoom for correct Nyquist sampling, the pixel size d_{Pix} in the object plane is important.

For a Carl Zeiss confocal LSM, there is a simple formula, based on the edge length of the scanned field in the intermediate image:

$$d_{pix} = \frac{system\ constant}{number\ of\ pixels \cdot zoomfactor \cdot magnification_{obj}}$$

Number of pixels = number of pixels per line
Zoom factor (*Z*) = scanning zoom set in the software (Example: Zoom factor 2 reduces the edge length of the scanned field by a factor of 2)
Magnification$_{obj}$ = objective magnification
System constant = 8.94 mm with LSM 510, LSM 5 Pascal (minimum zoom factor = 0.7); 12.77 mm with LSM 310, LSM 410 (minimum zoom factor=1)

The minimum scanning zoom needed to fullfill the Nyquist theorem can therefore be calculated as follows:

$$Z \geq \frac{3.92 \cdot NA \cdot system\ constant}{number\ of\ pixels \cdot magnification_{obj} \cdot \lambda_{exc}}$$

NA = numerical aperture of objective
λ_{exc} = excitation wavelength
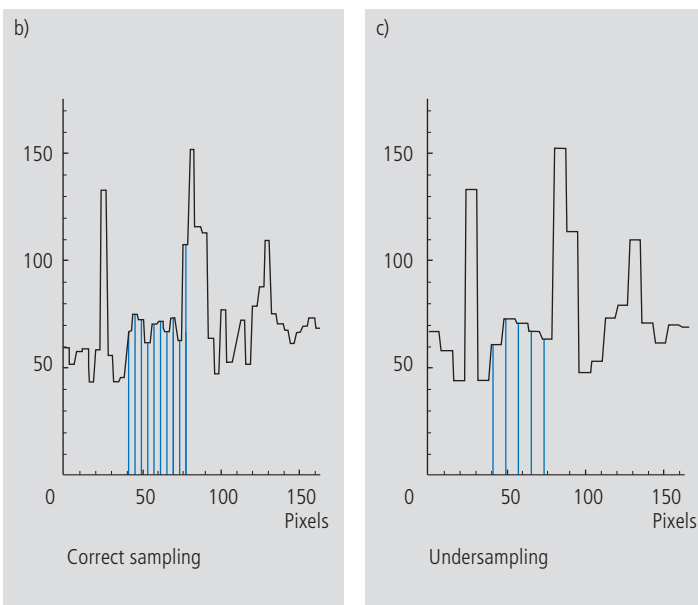
For example, with a 40x objective (NA = 1.3), 512 pixels per scan line and a wavelength of 488 nm, the full resolving power (correct sampling) is achieved with a scanning zoom of 4.56 as a minimum; the corresponding pixel size is 95.8 nm. With lower factors of the scanning zoom the pixel size itself will be the limiting factor for resolution (pixel resolution). Higher factors will cause oversampling. Hence, the zoom factor influences not only the total magnification but also the resolution properties of the system.

With the more recent LSM systems of Carl Zeiss, the number of sampling points can also be influenced by an increase in the number of pixels per scan line.

(The number of pixels (X/Y) per image can be freely selected between 4 x 2 and 2048 x 2048).

Correct sampling

Undersampling

Noise

The main types of noise important in a confocal LSM system are detector noise (dark noise, secondary emission noise), laser noise, and shot noise of the light (*see Details "Sources of Noise"*). As a rule, these sources of noise are of a statistical nature. Periodic noise rarely occurs, and if it does, it tends to correlate with defective devices or mechanical vibration in the setup; therefore it has been left out of consideration here.

As the graphs in figure 15 show, the number of photons hitting the PMT depends not only on the intensity of the fluorescence signal (*see Details "Fluorescence"*), but also on the diameter of the pinhole. The graph shows the intensity distribution of a two-point object resulting behind the pinhole, in normalized (left) and non-normalized form (right). The pinhole diameter was varied between 2 AU and 0.05 AU. At a diameter of 1 AU the pinhole just equals the size of the Airy disk, so that there is only a slight loss in intensity. The gain in resolution, is minimum in this case.

With a pinhole diameter <1AU, resolution improves (better point separation thanks to a deeper dip), which is penalized by a drastic loss in energy.

Moreover, it should be considered that it depends on the signal level wich noise source dominates. With high-amplitude signals (number of detected photons > 10,000), laser noise is the dominating effect, whereas the quality of low signals (number of detected photons < 1000) is limited by the shot noise of the light.

Therefore, laser noise tends to be the decisive noise factor in observations in the reflection mode, while shot noise dominates in the fluorescence mode. With recent PMT models (e.g., from Hamamatsu), detector dark noise is extremely low, same as secondary emission noise, and both can be neglected in most practical applications (*see Details "Sources of Noise"*).

Therefore, the explanations below are focused on the influence of shot noise on lateral resolution.

Fig. 15   As shown in Part 1, small pinhole diameters lead to improved resolution (smaller FWHM, deeper dip – see normalized graph on the left). The graph on the right shows, however, that constricting the pinhole is connected with a drastic reduction in signal level. The drop in intensity is significant from PH <1 AU.
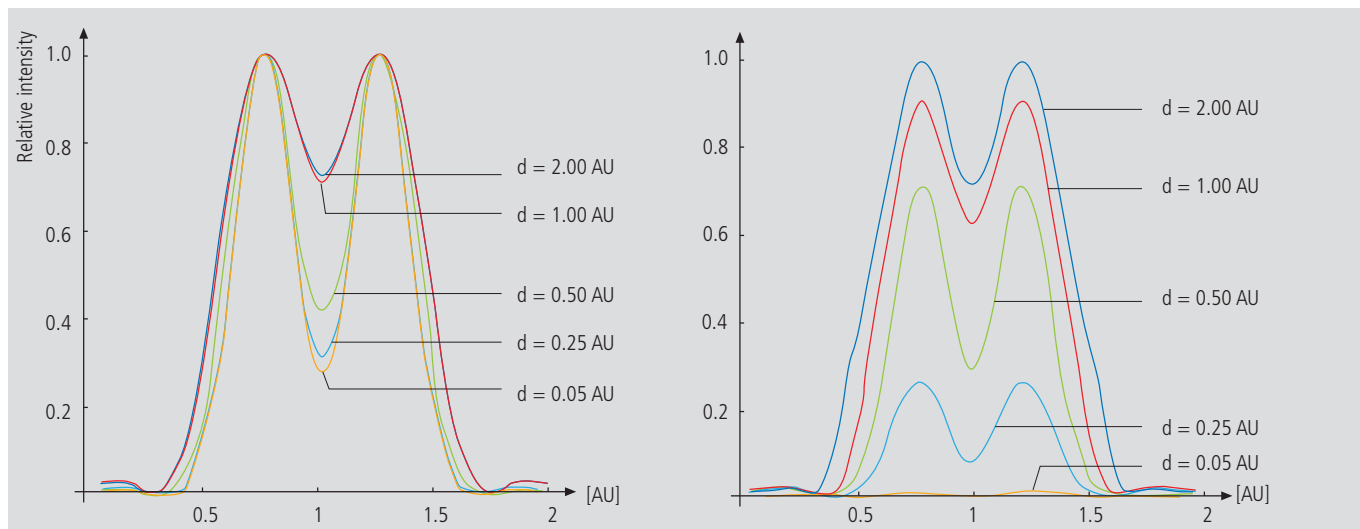
Resolution and shot noise – resolution probability

If the number of photons detected (N) is below 1000, fluorescence emission should be treated as a stochastic rather than a continuous process; it is necessary, via the shot noise, to take the quantum nature of light into account (the light flux is regarded as a photon flux, with a photon having the energy $E = h \cdot \nu$). Resolution becomes contingent on random events (the random incidence of photons on the detector), and the gain in resolution obtainable by pinhole constriction is determined by the given noise level. Figure 16 will help to understand the quantum nature of light.

As a possible consequence of the shot noise of the detected light, it may happen, for example, that noise patterns that change because of photon statistics, degrade normally resolvable object details in such a way that they are not resolved every time in repeated measurements. On the other hand, objects just outside optical resolvability may appear resolved because of noise patterns modulated on them. Resolution of the "correct" object structure is the more probable the less noise is involved, i.e. the more photons contribute to the formation of the image.

Therefore, it makes sense to talk of resolution probability rather than of resolution. Consider a model which combines the purely optical understanding of image formation in the confocal microscope (PSF) with the influences of shot noise of the detected light and the scanning and digitization of the object. The essential criterion is the discernability of object details.

Figure 17 (page 22) shows the dependence of the resolution probability on signal level and pinhole diameter by the example of a two-point object and for different numbers of photoelectrons per point object. [As the image of a point object is covered by a raster of pixels, a normalization based on pixels does not appear sensible.]

Thus, a number of 100 photoelectrons/point object means that the point object emits as many photons within the sampling time as to result in 100 photoelectrons behind the light-sensitive detector target (PMT cathode). The number of photoelectrons obtained from a point object in this case is about twice the number of photoelectrons at the maximum pixel (pixel at the center of the Airy disk). With photoelectrons as a unit, the model is independent of the sensitivity and noise of the detector and of detection techniques (absolute integration time / point sampling / signal averaging). The only quantity looked at is the number of detected photons.

Fig. 16   The quantum nature of light can be made visible in two ways:
• by reducing the intensity down to the order of single photons and
• by shortening the observation time at constant intensity, illustrated in the graph below: The individual photons of the light flux can be resolved in their irregular (statistical) succession.
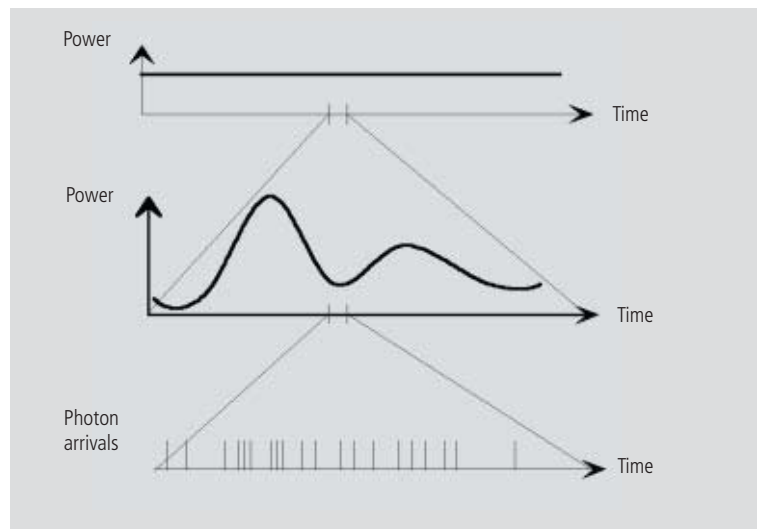
A resolution probability of 90% is considered necessary for resolving the two point images. Accordingly, the two-point object defined above can only be resolved if each point produces at least about 25 photoelectrons. With pinhole diameters smaller than 0.25 AU, the drastic increase in shot noise (decreasing intensity of the detected light) will in any case lead to a manifest drop in resolution probability, down to the level of indeterminateness (≤ 50% probability) at PH = 0.

As another consequence of shot noise, the curve maximum shifts toward greater pinhole diameters as the number of photoelectrons drops.

The general slight reduction of resolution probability towards greater pinhole diameters is caused by the decreasing effectiveness of the pinhole (with regard to suppression of out-of-focus object regions, see Part 1).

The pinhole diameter selected in practice will therefore always be a trade-off between two quality parameters: noise (SNR as a function of the intensity of the detected light) and resolution (or depth discrimination). The pinhole always needs a certain minimum aperture to allow a minimum of radiation (depending on the intensity of fluorescence) to pass to the detector.

Where fluorescence intensities are low, it may be sensible to accept less than optimum depth discrimination so as to obtain a higher signal level (higher intensity of detected light = less noise, better SNR). For most fluorescent applications a pinhole diameter of about 1 AU has turned out to be the best compromise.
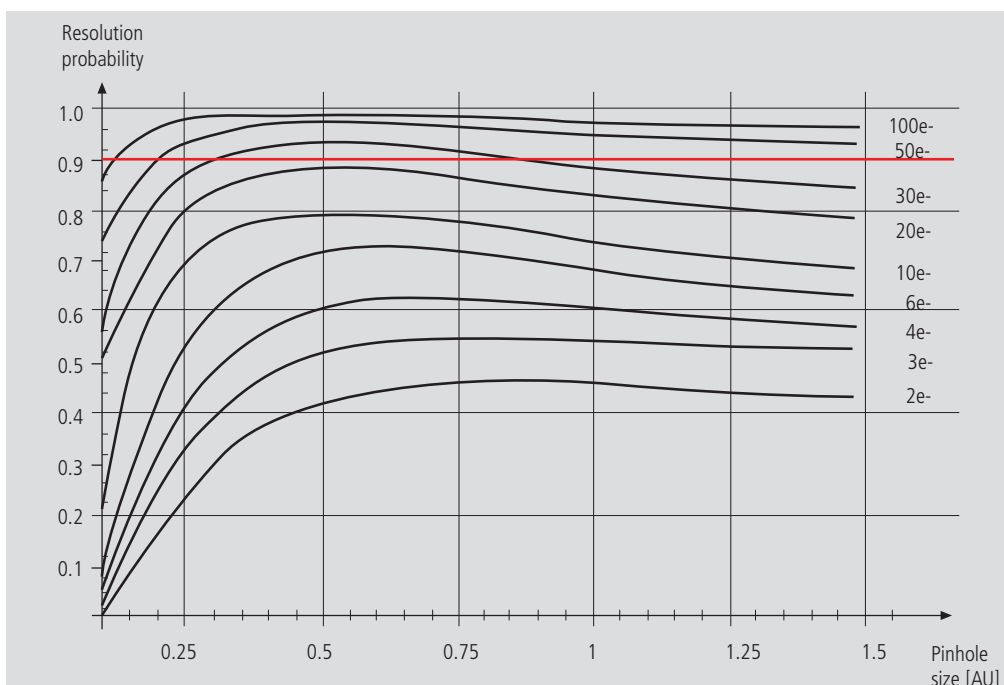
Fig. 17   The graph shows the computed resolution probability of two self-luminous points (fluorescence objects) spaced at 1/2 AU, as a function of pinhole size and for various photoelectron counts per point object (e-). The image raster conforms to the Nyquist theorem (critical raster spacing = 0.25 AU); the rasterized image is subjected to interpolation. The photoelectron count per point object is approximately twice that per pixel (referred to the pixel at the center of the Airy disk). Each curve has been fitted to a fixed number of discrete values, with each value computed from 200 experiments.

The resolution probability is the quotient between successful experiments (resolved) and the total number of experiments. A resolution probability of 70% means that 7 out of 10 experiments lead to resolved structures. A probability > 90 % is imperative for lending certainty to the assumption that the features are resolved. If we assume a point-like fluorescence object containing 8 FITC fluorescence molecules (fluorochrome concentration of about 1 nMol) a laser power of 100 µW in the pupil and an objective NA of 1.2 (n = 1.33), the result is about 45 photoelectrons / point object on the detection side.

Possibilities to improve SNR

Pinhole diameters providing a resolution proba-
bility below 90% may still yield useful images if
one uses a longer pixel time or employs the signal
averaging function. In the former case, additional
photons are collected at each pixel; in the latter
case, each line of the image, or the image as a
whole, is scanned repeatedly, with the intensities
being accumulated or averaged. The influence of
shot noise on image quality decreases as the num-
ber of photons detected increases. As fluores-
cence images in a confocal LSM tend to be shot-
noise-limited, the increase in image quality by the
methods described is obvious.

Furthermore, detector noise, same as laser noise at
high signal levels, is reduced. The figures on the
right show the influence of pixel time (figure 18)
and the influence of the number of signal acquisi-
tions (figure 19) on SNR in [dB]. The linearity
apparent in the semilogarithmic plot applies
to shot-noise-limited signals only. (As a rule,
signals are shot-noise-limited if the PMT high
voltage needed for signal amplification is greater
than 500 V).

A doubling of pixel time, same as a doubling of
the number of signal acquisitions, improves SNR
by a factor of $\sqrt{2}$ (3 dB). The advantage of the

averaging method is the lower load on the speci-
men, as the exposure time per pixel remains con-
stant. Photon statistics are improved by the addi-
tion of photons from several scanning runs (SNR =
$\sqrt{n \cdot N}$; N = const., n = number of scans averaged).
By comparison, a longer pixel time directly
improves the photon statistics by a greater num-
ber N of photons detected per pixel (SNR = $\sqrt{N}$,
N = variable), but there is a greater probability of
photobleaching or saturation effects of the fluo-
rophores.
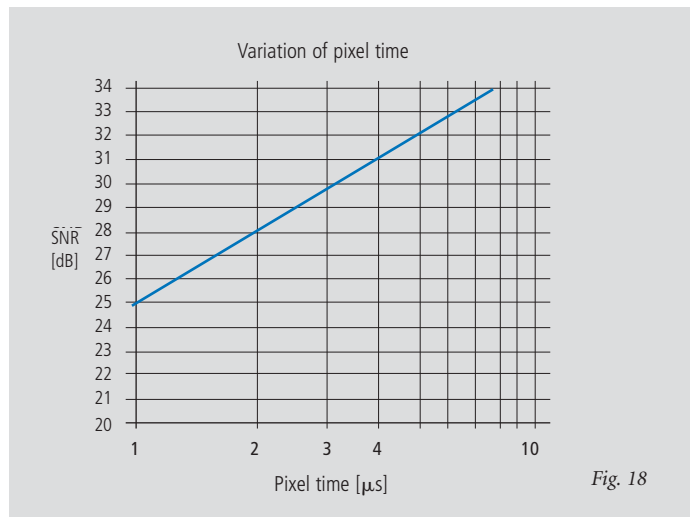
Fig. 18
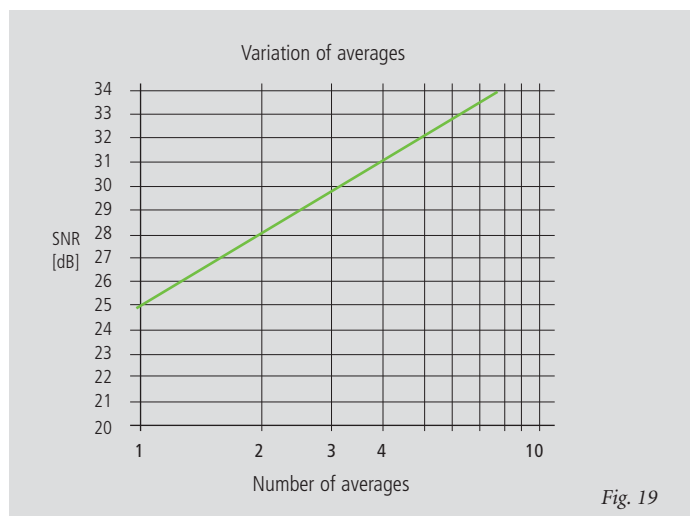
*Figures 18 and 19   Improvement of the signal-
to-noise ratio. In figure 18 (top), pixel time is
varied, while the number of signal acquisitions
(scans averaged) is constant.
In figure 19 (bottom), pixel time is constant,
while the number of signal acquisitions is varied.
The ordinate indicates SNR in [dB], the abscissa
the free parameter (pixel time, scans averaged).*

Fig. 19

23

a)

b)

Fig. 20 Three confocal images of the same fluorescence specimen (mouse kidney section, glomeruli labeled with Alexa488 in green and actin labelled with Alexa 564 phalloidin in red).
All images were recorded with the same parameters, except pixel time and average. The respective pixel times were 0.8 μs in a), 6.4 μs (no averaging) in b), and 6.4 μs plus 4 times line-wise averaging in c).

c)

The pictures on the left demonstrate the influence of pixel time and averaging on SNR; object details can be made out much better if the pixel time increases or averaging is employed.

Another sizeable factor influencing the SNR of an image is the efficiency of the detection beam path. This can be directly influenced by the user through the selection of appropriate filters and dichroic beamsplitters. The SNR of a FITC fluorescence image, for example, can be improved by a factor of about 4 (6 dB) if the element separating the excitation and emission beam paths is not a neutral 80/20 beamsplitter[1] but a dichroic beamsplitter optimized for the particular fluorescence.

[1] An 80/20 beamsplitter reflects 20% of the laser light onto the specimen and transmits 80% of the emitted fluorescence to the detector.

Summary

This monograph comprehensively deals with the quality parameters of resolution, depth discrimination, noise and digitization, as well as their mutual interaction. The set of equations presented allows in-depth theoretical investigations into the feasibility of carrying out intended experiments with a confocal LSM.

The difficult problem of quantifying the interaction between resolution and noise in a confocal LSM is solved by way of the concept of resolution probability; i.e. the unrestricted validity of the findings described in Part 1 is always dependent on a sufficient number of photons reaching the detector.

Therefore, most applications of confocal fluorescence microscopy tend to demand pinhole diameters greater than 0.25 AU; a diameter of 1 AU is a typical setting.

Glossary

α	Aperture angle of a microscope objective
AU	Airy unit (diameter of Airy disc)
dpix	Pixel size in the object plane
FWHM	Full width at half maximum of an intensity distribution (e.g. optical slice)
n	Refractive index of an immersion liquid
NA	Numerical aperture of a microscope objective
PH	Pinhole; diaphragm of variable size arranged in the beam path to achieve optical sections
PMT	Photomultiplier tube (detector used in LSM)
PSF	Point spread function
RU	Rayleigh unit
SNR	Signal-to-noise ratio

To give some further insight into Laser Scanning Microscopy, the following pages treat several aspects of particular importance for practical work with a Laser Scanning Microscope.

Pupil Illumination

Optical Coordinates

Fluorescence

Sources of Noise

Pupil Illumination

All descriptions in this monograph suggest a confocal LSM with a ray geometry providing homogeneous illumination at all lens cross sections. The focus generated in the object has an Airy distribution, being a Fourier transform of the intensity distribution in the objective's pupil plane. However, the truncation of the illuminating beam cross-section needed for an Airy distribution causes a certain energy loss (a decrease in efficiency). [In Carl Zeiss microscope objectives, the pupil diameter is implemented by a physical aperture close to the mounting surface].

The Airy distribution is characterized by a smaller width at half maximum and a higher resolving power. Figure 21 (left) shows the intensity distribution at the focus as a function of the truncation factor T (the ratio of laser beam diameter $(1/e^2)$ and pupil diameter).

The graph presents the relative intensity distributions at the focus (each normalized to 1) for different truncation factors. (The red curve results at a homogeneous pupil illumination with T > 5.2, while the blue one is obtained at a Gaussian pupil illumination with T ≤ 0.5; the green curve

corresponds to a truncation factor T = 1.3). The lateral coordinate is normalized in Airy units (AU). From T = 3, the Airy character is predominating to a degree that a further increase in the truncation factor no longer produces a gain in resolution. (Because of the symmetry of the point image in case of diffraction-limited imaging, the graph only shows the intensity curve in the +X direction). Figure 21 (right) shows the percentage efficiency as a function of pupil diameter in millimeter, with constant laser beam expansion. The smaller the pupil diameter, the higher the T-factor, and the higher the energy loss (i.e. the smaller the efficiency). Example: If the objective utilizes 50 % of the illuminating energy supplied, this means about 8 % resolution loss compared to the ideal Airy distribution. Reducing the resolution loss to 5 % is penalized by a loss of 70 % of the illuminating energy. In practice, the aim is to reach an optimal approximation to a homogeneous pupil illumination; this is one reason for the fact that the efficiency of the excitation beam path in a confocal LSM is less than 10 %.

Fig. 21

Intensity distribution at the focus — Relative intensity vs Lateral distance [AU]: T < 0.3 (Gauss); T = 1.3; T > 5,2 (Airy)

Efficiency — Relative efficiency vs Pupil diameter [mm]
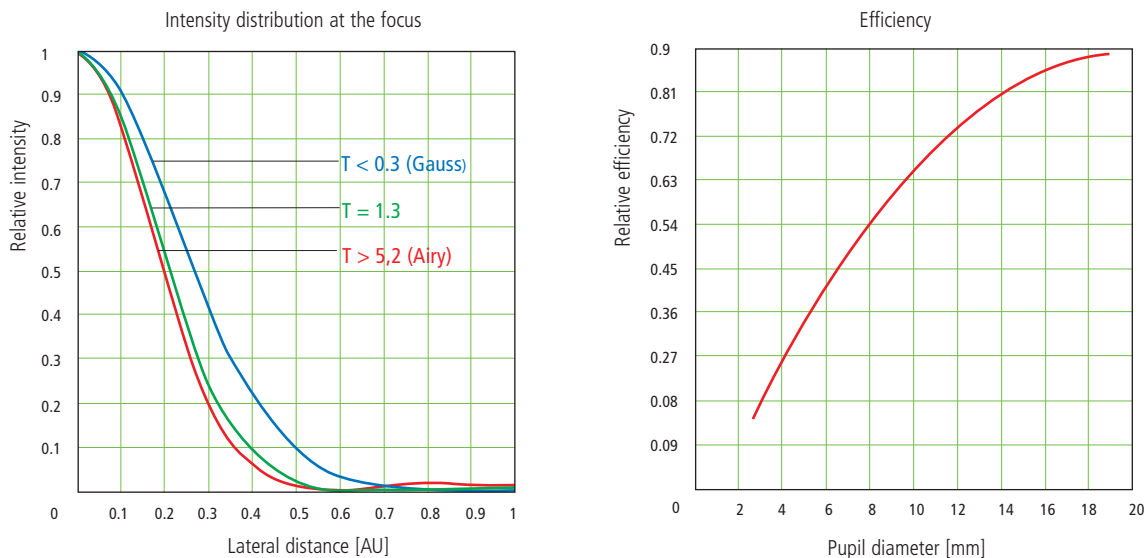
The trunction factor T is defined as the ratio of laser beam and pupil diameter of the objective lens used:

$$T = \frac{d_{laser}}{d_{pupille}} \; ; \quad \text{the resulting efficiency is defined as} \quad \eta = 1 - e^{\left(\frac{-2}{T^2}\right)}$$

The full width at half maximum of the intensity distribution at the focal plane is definied as

$$FWHM = 0.71 \cdot \frac{\lambda}{NA} \cdot \omega \; , \quad \text{with} \quad \omega = \sqrt{0.51 + 0.14 \cdot \ln\left(\frac{1}{1-n}\right)}$$

With T< 0.6, the Gaussian character, and with T>1 the Airy character predominates the resulting intensity distribution.

Optical Coordinates

In order to enable a representation of lateral and axial quantities independent of the objective used, let us introduce optical coordinates oriented to microscopic imaging.

Given the imaging conditions in a confocal microscope, it suggests itself to express all lateral sizes as multiples of the Airy disk diameter. Accordingly, the Airy unit (AU) is defined as:

$$1AU = \frac{1.22 \cdot \bar{\lambda}}{NA}$$

NA = numerical aperture of the objective
$\bar{\lambda}$ = wavelength of the illuminating laser light
with NA = 1.3 and $\bar{\lambda}$ = 496 nm → 1 AU = 0.465 µm

The **AU** is primarily used for normalizing the pinhole diameter.

Thus, when converting a given pinhole diameter into AUs, we need to consider the system's total magnification; which means that the Airy disk is projected onto the plane of the pinhole (or vice versa).
Analogously, a sensible way of normalization in the axial direction is in terms of multiples of the wave-optical depth of field. Proceeding from the Rayleigh criterion, the following expression is known as Rayleigh unit (RU):

$$1RU = \frac{1.22 \cdot \bar{\lambda}}{NA^2}$$

n = refractive index of immersion liquid
with NA = 1.3, $\bar{\lambda}$ = 496 nm and n = 1.52 → 1 RU = 0.446 µm

The **RU** is used primarily for a generally valid representation of the optical slice thickness in a confocal LSM.

Fluorescence

Fluorescence is one of the most important contrasting methods in biological confocal microscopy.

Cellular structures can be specifically labeled with dyes (fluorescent dyes = fluorochromes or fluorophores) in various ways. Let the mechanisms involved in confocal fluorescence microscopy be explained by taking fluorescein as an example of a fluorochrome. Fluorescein has its absorption maximum at 490 nm. It is common to equip a confocal LSM with an argon laser with an output of 15 – 20 mW at the 488 nm line. Let the system be adjusted to provide a laser power of 500 µW in the pupil of the microscope objective. Let us assume that the microscope objective has the ideal transmittance of 100 %.

With a C-Apochromat 63x/1.2W, the power density at the focus, referred to the diameter of the Airy disk, then is $2.58 \cdot 10^5$ W/cm^2. This corresponds to an excitation photon flux of $6.34 \cdot 10^{23}$ photons/cm^2 sec. In conventional fluorescence microscopy, with the same objective, comparable lighting power (xenon lamp with 2 mW at 488 nm) and a visual field diameter of 20 mm, the excitation photon flux is only $2.48 \cdot 10^{18}$ photons/cm^2 sec, i.e. lower by about five powers of ten.

This is understandable by the fact that the laser beam in a confocal LSM is focused into the specimen, whereas the specimen in a conventional microscope is illuminated by parallel light.

The point of main interest, however, is the fluorescence (F) emitted.

The emission from a single molecule (F) depends on the molecular cross-section (σ), the fluorescence quantum yield (Qe) and the excitation photon flux (I) as follows:

$$F = \sigma \cdot Qe \cdot I \; \text{[photons/sec]}$$

In principle, the number of photons emitted increases with the intensity of excitation. However, the limiting parameter is the maximum emission rate of the fluorochrome molecule, i.e. the number of photons emittable per unit of time. The maximum emission rate is determined by the lifetime (= radiation time) of the excited state. For fluorescein this is about 4.4 nsec (subject to variation according to the ambient conditions). On average, the maximum emission rate of fluorescein is $2.27 \cdot 10^8$ photons/sec. This corresponds to an excitation photon flux of $1.26 \cdot 10^{24}$ photons/cm^2 sec.

At rates greater than $1.26 \cdot 10^{24}$ photons/cm^2 sec, the fluorescein molecule becomes saturated. An increase in the excitation photon flux will then no longer cause an increase in the emission rate; the number of photons absorbed remains constant. In our example, this case occurs if the laser power in the pupil is increased from 500 µW to roughly 1 mW. Figure 22 (top) shows the relationship between the excitation photon flux and the laser power in the pupil of the stated objective for a wavelength of 488 nm. Figure 22 (bottom) illustrates the excited-state saturation of fluorescein molecules. The number of photons absorbed is approximately proportional to the number of photons emitted (logarithmic scaling).

The table below lists the characteristics of some important fluorochromes:

	Absorpt. max. (nm)	$\sigma/10^{-16}$	Qe	$\sigma^*Q/10^{-16}$
Rhodamine	554	3.25	0.78	0.91
Fluorescein	490	2.55	0.71	1.81
Texas Red	596	3.3	0.51	1.68
Cy 3.18	550	4.97	0.14	0.69
Cy 5.18	650	7.66	0.18	1.37

Source:
Handbook of Biological Confocal Microscopy, p. 268/Waggoner
In the example chosen,
$F = 1.15 \cdot 10^8$ photons/sec or 115 photons/µsec

What has been said so far is valid only as long as the molecule is not affected by photobleaching. In an oxygen-rich environment, fluorescein bleaches with a quantum efficiency of about $2.7 \cdot 10^{-5}$. Therefore, a fluorescence molecule can, on average, be excited n = 26,000 times (n = Q/Q_b) before it disintegrates.

With $t = \dfrac{n}{F_{max}}$, and referred to the maximum emission rate, this corresponds to a lifetime of the fluorescein molecule of about 115 µs.

It becomes obvious that an increase in excitation power can bring about only a very limited gain in the emission rate. While the power provided by the laser is useful for FRAP (fluorescence recovery after photobleaching) experiments, it is definitely too high for normal fluorescence applications. Therefore it is highly important that the excitation power can be controlled to fine increments in the low-intensity range.

A rise in the emission rate through an increased fluorophore concentration is not sensible either, except within certain limits. As soon as a certain molecule packing density is exceeded, other effects (e.g. quenching) drastically reduce the quantum yield despite higher dye concentration. Another problem to be considered is the system's detection sensitivity. As the fluorescence radiated by the molecule goes to every spatial direction with the same probability, about 80% of the photons will not be captured by the objective aperture (NA = 1.2).

With the reflectance and transmittance properties of the subsequent optical elements and the quantum efficiency of the PMT taken into account, less than 10% of the photons emitted are detected and converted into photoelectrons (photoelectron = detected photon).

In case of fluorescein (NA = 1.2, 100 µW excitation power, λ = 488 nm), a photon flux of F ~ 23 photons/µsec results. In combination with a sampling time of 4 µsec/pixel this means 3 – 4 photoelectrons/molecule and pixel.

In practice, however, the object observed will be a labeled cell. As a rule, the cell volume is distinctly greater than the volume of the sampling point. What is really interesting,
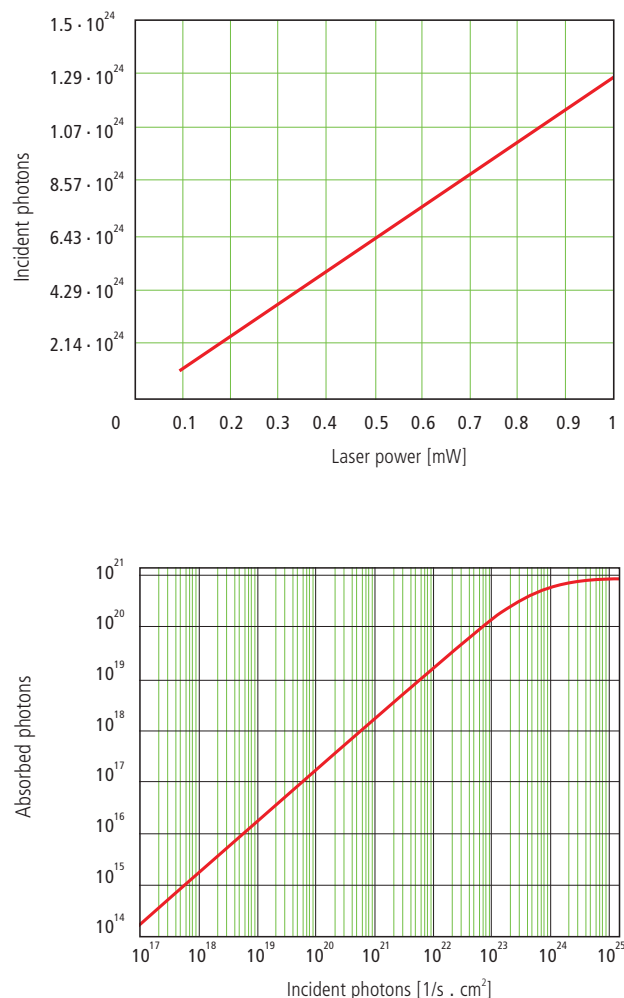
therefore, is the number of dye molecules contained in the sampling volume at a particular dye concentration. In the following considerations, diffusion processes of fluorophore molecules are neglected. The computed numbers of photoelectrons are based on the parameters listed above.

With λ = 488 nm and NA = 1.2 the sampling volume can be calculated to be V = $12.7 \cdot 10^{-18}$ l. Assuming a dye concentration of 0.01 µMol/l, the sampling volume contains about 80 dye molecules. This corresponds to a number of about 260 photoelectrons/pixel. With the concentration reduced to 1 nMol/l, the number of dye molecules drops to 8 and the number of photoelectrons to 26/pixel.

Finally it can be said that the number of photons to be expected in many applications of confocal fluorescence microscopy is rather small (<1000). If measures are taken to increase the number of photons, dye-specific properties such as photobleaching have to be taken into account.

Sources of Noise

Sources of noise effective in the LSM exist everywhere in the signal chain – from the laser unit right up to A/D conversion. Essentially, four sources of noise can be distinguished:

Laser noise q

Laser noise is caused by random fluctuations in the filling of excited states in the laser medium. Laser noise is proportional to the signal amplitude N and therefore significant where a great number of photons (N < 10000) are detected.

Shot noise (Poisson noise)

This is caused by the quantum nature of light. Photons with the energy $h \cdot \upsilon$ hit the sensor at randomly distributed time intervals. The effective random distribution is known as Poisson distribution. Hence,

$$SNR \approx \Delta N_{Poisson} = \sqrt{N}$$

where N = number of photons detected per pixel time (= photoelectrons = electrons released from the PMT cathode by incident photons). With low photoelectron numbers (N < 1000), the number N of photons incident on the sensor can only be determined with a certainty of $\pm \sqrt{N}$.

N can be computed as

$$N = \frac{photons}{QE_{(\lambda)} \cdot pixel\ time}$$

where QE (λ) = quantum yield of the sensor at wavelength λ; 1 photon = $h \cdot c/\lambda$; c = light velocity; h = Planck's constant

Secondary emission noise

Caused by the random variation of photoelectron multiplication at the dynodes of a PMT. The amplitude of secondary emission noise is a factor between 1.1 and 1.25, depending on the dynode system and the high voltage applied (gain). Generally, the higher the PMT voltage, the lower the secondary emission noise; a higher voltage across the dynodes improves the collecting efficiency and reduces the statistical behavior of multiplication.

Dark noise

Dark noise is due to the generation of thermal dark electrons N_d, irrespective of whether the sensor is irradiated. N_d statistically fluctuates about $\sqrt{N_d}$. Dark noise is specified for a PMT voltage of 1000 V; with lower voltages it progressively loses significance.

Dark noise can be reduced by cooling the sensor. However, the reduction is significant only if $N \leq N_d$, e.g. in object-free areas of a fluorescence specimen. In addition, the dark noise must be the dominating noise source in order that cooling effects a signal improvement; in most applications, this will not be the case.

Additional sources of noise to be considered are amplifier noise in sensor diodes and readout noise in CCD sensors. In the present context, these are left out of consideration.

The mean square deviation ΔN from the average $(N + N_d)$ of the photoelectrons and dark electrons registered,

$$\Delta N = se \cdot \sqrt{(N+N_d)\ (1+q^2)}$$

so that the total signal-to-noise ratio can be given as

$$SNR = \sqrt{\frac{N^2}{se^2\ (N+N_d)\ (1+q^2)}}$$

where
N = number of photoelectrons per pixel time (sampling time)
se = multiplication noise factor of secondary emission
q = peak-to-peak noise factor of the laser
N_d = number of dark electrons in the pixel or sampling time

Example:

For N = 1000, N_d = 100, se = 1.2, and q = 0.05

$$SNR = \sqrt{\frac{1000^2}{1.2^2\ (1000+100)\ (1+0.05^2)}} = 25.1$$

LITERATURE

1. Barton, D.L., Tangyunyong, P.,
Scanning Fluorescent Microthermal Imaging,
Proceedings of 23rd Int Symposium for Testing and
Failure Analysis (10/1997), Santa Clara, California

2. Barton, D.L., Tangyunyong, P.,
Infrared Light Emission from Semiconductor
Devices, ISTFA, pp. 9-17, (1996)

3. Beyer, H.,
Handbuch der Mikroskopie, 2nd Edition,
VEB Verlag Technik Berlin, (1985)

4. Born & Wolf,
Priniples of Optics,
6th edition 1988, Pergamon Press

5. Brismar, H., Trepte, O., Ulfhake, B.:
Spectra and Fluorescence Lifetimes of Lissamine,
Rhodamine etc....: Influences of Some
Environmental Factors Recorded with a Confocal
Laser Scanning Microscope, The Journal of
*Histochemistry and Cytochemistry, Vol. **43**,*
pp. 699-707, (7/1995)

6. Keller, H.E.,
Objective Lens for Confocal Microscopy,
Handbook of Biological Confocal Microscopy,
pp. 111-125, Plenum Press, 2nd Edition (1995)

7. Lackmann, F., et. al.,
An Automated Latch-up Measurement System
Using a Laser Scanning Microscope,
*SPIE Vol **1028** Scanning Imaging, (1988)*

8. Gröbler, B.,
Untersuchungen zur Bildübertragung in ab-
tastenden Mikroskopen unter besonderer
Berücksichtigung der 3D-Abbildung, PhD thesis,
University of Potsdam, (1995)

9. Hell, S., et al.,
Aberrations in confocal fluorescence microscopy
induced by mismatches in refractive index,
*Journal of Microscopy, Vol. **169**, pp. 391-405*
(3/1993)

10. Nitschke, R.,Wilhelm, S., et al.,
A modified confocal laser scanning microscope
allows fast ultraviolet ratio imaging of intracellular
Ca^{2+} activity using Fura 2, Euro. J. Physiologie,
*Vol. **433**: pp. 653-663, (1997)*

11. Oldenbourg, R. et al.,
Image sharpness and contrast transfer in coherent
confocal microscopy, Journal of Microscopy
*Vol.**172**, pp. 31-39, (10/1993)*

12. Pawley, J.,
Handbook of Biological Confocal Microscopy,
Plenum Press, 2nd Edition (1995)

13. Stelzer, E.H.K.,
The intermediate optical system of laser scanning
confocal microscopes; Handbook of Biological
Confocal Microscopy, pp. 139-154,
Plenum Press, 2nd Edition (1995)

14. Stelzer, E.H.K., et. al.,
Nondestructive sectioning of fixed and living
specimens using a confocal scanning laser
fluorescence microscope: Microtomoscopy; SPIE,
*Vol. **809**, pp. 130-136, (1987)*

15. Tanke, H.J., van Oostveldt, P., et al.,
A parameter for the distribution of fluorophores in
cells derived from measurements of inner filter effect
and reabsorption phenomenon,
*Cytometry Vol. **2**, pp. 359-369 (6/1982)*

16. Tsien, R.Y., Waggoner, A.,
Fluorophores for Confocal Microscopy,
Handbook of Biological Confocal Microscopy,
pp. 267-277, Plenum Press, 2nd Edition (1995)

17. Webb, R.H., Dorey, C.K.,
The Pixelated Image, Handbook of Biological Confocal
Microscopy, pp. 55-66, Plenum Press, 2nd Edition (1995)

18. Wilhelm, S.,
Über die 3-D Abbildungsqualität eines konfokalen
Laser Scan Mikroskops,
Dissertation, Fachhochschule Köln, (1994)

19. Wilson, T., Carlini, A.R.,
Three dimensional imaging in confocal imaging
systems with finite sized detectors; Journal of
*Microscopy, Vol. **149**, pp. 51-66, (1/1988)*

20. Wilson, T., Carlini, A.R.,
Size of detector in confocal imaging systems;
*Optical Letters Vol.**12**, pp. 227-229, (4/1987)*

21. Wilson, T., Sheppard, C.J.R.,
Theory and Practice of Scanning Optical Microscopy,
Academic Press, 2nd Edition (1985)

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
We gratefully acknowledge the assistance of many other
staff members who contributed to this brochure.

Laser

- **Light source** – projected into specimen
- Laser power: adjustable via attenuation device (AOTF, AOM, MOTF) and tube current setting (Ar)
- Lifetime Ar: prolonged by using lower tube current; but laser noise will be increased (8 A = minimum noise)
- Stand-by mode: prolongs laser lifetime; not suitable for image acquisition
- Laser line: can be chosen via selection device (AOTF, MOTF) dependent on fluorescent dye. Generally: the shorter the wavelength, the higher the resolution
- Application goals: (1) Protect specimen (reduction of dye bleaching and phototoxicity) by reduction of laser power. (2) Maximize fluorescence signal (higher SNR) by longer pixel dwell times or averaging

Scanning Mirrors

- **Scanning unit** – moves focused laser beam across specimen line by line
- Scanning speed: defines frame rate (frames/sec) and pixel time, i.e. time the specimen is illuminated
- Pixel time: influences SNR of image; the longer the pixel time, the more photons per pixel, the less noise in the picture; but bleaching of fluorochromes may increase
- Pixel resolution: maximum resolution can be achieved if pixel size is set correctly (at least 4 x 4 pixels (x, y) per smallest detail) → directly adjustable via scan zoom
- x/y frame size: variable from 4 x 2 up to 2048 x 2048 pixels; maximum frame rate with 512 x 512 pixels 5 frames/sec (bidirectional scan ⇄); unidirectional scan ➡ : slower by factor 2

Z-Motor

- **Focusing the specimen** – acquisition of image stacks or x-z sections
- z-interval: distance between two optical slices (step size of z-motor: min. 25 nm)
- Optimum z-motor step size: 0.5 x optical slice thickness (compare: min. slice thickness about 340 nm for NA = 1.4, n = 1.52, λ = 488 nm)
- Optional: fast z-scanning stage (HRZ) fast piezo objectiv focus

Detector
Emission filter
Confocal pinhole
Laser source
Main dichroic beamsplitter
Collimator
Scanning mirrors
Objective
Specimen
Focal plane
Z-motor
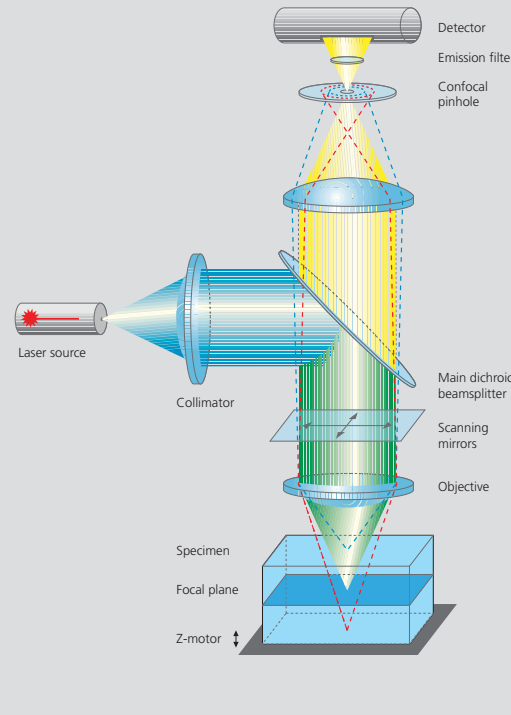
Photomultiplier (PMT)

- **Detector** – pixelwise detection of photons emitted / reflected by the respective specimen detail
- Parameters: "Detector Gain" = PMT high voltage, "Amplifier Offset" = black level setting, "Amplifier Gain" = electronic post-amplification
- Calibration: "Amplifier Offset" on image background (object-free area), "Detector Gain" according to scanned image (object); setting aid = "Range Indicator" (→ "Palette"). Goal: least number of overmodulated (red, Gain) and undermodulated (blue, Offset) pixels
- Signal amplification: First exploit "Detector Gain" slider before "Amplifier Gain" > 1

Confocal Pinhole

- **Depth discrimination** – confocal aperture to prevent detection of out-of-focus light (optical sectioning)
- Diameter: determines thickness of optical slice; optimum diameter: 1 Airy unit = best trade-off between depth discrimination capability and efficiency
- x/y position: factory-adjusted for all beam path configurations; can be modified manually (→ "Maintain-Pinhole")

Beam Splitter

- **Fluorescence beam path** – definable by combination of main (HFT) and secondary (NFT) dichroic mirrors and emission filters (BP = bandpass, LP = longpass, KP = shortpass) (→ "Acquire" – "Config")
- HFT: separates excitation and emission light
- NFT: effects spectral division of (different) fluorescence emissions (e.g. NFT 545: reflects light of λ < 545nm and transmits light of λ > 545nm)
- BP, LP, KP: determines bandwidth of fluorescence emission for the respective channel (e.g. LP 505: $\lambda \geq$ 505 nm → detection)

Objective Lens

- **Optical image formation** – determines properties of image quality such as resolution (x, y, z)
- Numerical Aperture (N.A.): determines imaged spot size (jointly with wavelength), and substantially influences the minimum optical slice thickness achievable
- Refractive index (n): match $n_{immersion\ liquid}$ with $n_{specimen\ mounting\ medium}$ for better image quality.
- Best confocal multifluorescence images (VIS, UV): use water immersion objectives with apochromatic correction (C- Apochromat)

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3 Steps to Get a Confocal Image

1 View specimen in VIS mode

Focus the specimen in epi-fluorescence mode using the binocular and center the part of interest; select fluorescence filter cube according to application (e.g. FITC or Cy3) via SW (window "Microscope Control"); match the field of view: change to appropriate objective magnification (consider use of correct immersion medium).

2 Load an LSM configuration

Activate LSM mode (operate manual tube slider or button "LSM"). Open window "Configuration control", and select a predefined configuration from list (Single Track).
A click on "Apply" automatically sets up the system: laser lines, attenuation, emission filters, beam splitters (HFT, NFT), pinhole diameter, detector settings (channels, gain, offset). Or: Click on "Reuse" button (stored image/image database window) to restore settings of a previous experiment.

3 Scan an image

Click on "Find" button (right row in window "Scan Control") => System automatically opens image window, optimizes detector settings (matches PMT gain and offset to dynamic range of 8 or 12 bit), and scans an image.
See operating manual for scanning a stack of slices, time series etc.

How to Enhance Image Quality

More signal!

- Change to longer pixel dwell times by reducing scanning speed
- Use "Average" method: Calculation of "Sum" or "Mean" value of pixels of consecutive "Line" or "Frame" scans.
- Increase bandwidth of emission filter (e.g. LP instead of BP).
- Enlarge pinhole diameter; Note: optical slice thickness increases accordingly.
- Increase excitation energy (laser power); but pay attention to bleaching, saturation and phototoxic effects.

More details!

- Use objective with higher numerical aperture (NA); x/y-resolution ~ 1/NA, z-resolution ~ 1/NA2.
- Increase "FrameSize" = number of pixels per line + lines per frame, e.g. 1024 x 1024 or 2048 x 2048 (min. 4 x 2).
- Optimize scan zoom (Z), i.e. pixel size ≤ 0.25 x diameter of Airy disk (e.g.: Objective 40x, NA 1.3, l = 488 nm => Z = 4.56).
- Increase dynamic range (change from 8 to 12 bit per pixel).

More reliability!

- Use Multitracking: very fast switching of excitation wavelengths; prevents crosstalk of signals between channels; predefined configurations available.
- Use ROI (Region Of Interest) function: significantly reduces excited area of specimen and increases acquisition rate at constant SNR; several ROIs of any shape can be defined and used simultaneously.

The Confocal Laser Scanning Microscope

ZEISS

We make it visible.

Methods

Confocal
Laser Scanning Microscopy

Applications in Research and Teaching.
Design, Functions, Methods.

ZEISS

We make it visible.

**Confocal
Laser Scanning Microscopy**

Contents

Having decoded the human genome, biomedical research today is focused on exploring the interaction between cellular components. Scientists want to find out which protein is where, and at what time, and what other structural and functional modules it interacts with.

In the search for answers to these questions, imaging systems based on the classical light microscope have come to play an unprecedented role, thanks to many technical innovations and a high degree of automation. Many experiments have only become possible because of the new functions provided by modern microscopes.

*Triple staining of a primary culture of a rat's cortical neurons.
Nucleus: blue (DAPI),
Nestin: green (Cy2),
Doublecortin: red (Cy3).
Specimen: Dr. H. Braun, FAN GmbH, Magdeburg, Germany.*

High Resolution in Space and Time

Confocal laser scanning microscopes (LSMs) are distinguished by their high spatial and temporal resolving power. They clearly outperform classical light microscopes especially by their axial resolution – a quality that enables users to acquire optical sections (slices) of a specimen. An object can thus be imaged completely in three dimensions and subsequently visualized as a 3D computer image. In another group of applications, exactly defined areas of a specimen can be selectively illuminated by laser light. This functionality is essential for quantitative investigations of dynamic processes in living cells using techniques such as FRAP (fluorescence recovery after photobleaching), FRET (fluorescence resonance energy transfer), photoactivation and photoconversion.

New acquisition methods on the LSM permit the detection of additional properties of the emitted light including spectral signatures and fluorescence lifetimes. With such information it is possible to increase the number of fluorescent labels used in an experiment, or to use fluorochrome combinations unthinkable with conventional detection methods. The advantages are obvious: the more components in a cellular process that are observed simultaneously, the greater the yield of information.

Because of its versatility, laser scanning microscopy has become an established mainstream method in biomedical research – a tool permitting scientists to follow innovative experimental paths. This article will show which basic functions and applications of laser scanning microscopy can be taught in academic tuition. The modern method of confocal laser scanning microscopy can be taught on the basis of classical light microscopy, an established part of fundamental biomedical teaching.

Triple-labeled tissue section of mouse intestine

In the non-confocal image, the interesting information of the focal plane mixes with unwanted information from extrafocal specimen planes; differently stained details result in a color mix.
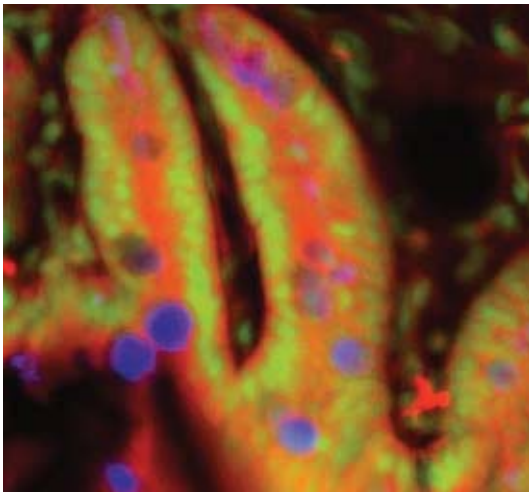
In the confocal image, object details blurred in the non-confocal image are visible clearly and in greater contrast.
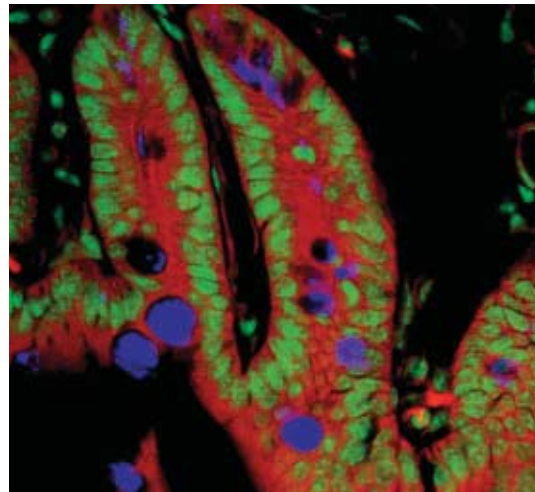
The Confocal Principle

In this chapter, the mode of operation of an LSM will be explained using a fluorescence-labeled specimen as an example. Fluorescent dyes, also known as fluorochromes, are used as markers in most biomedical applications to make the structures of interest visible. But laser scanning microscopes can just as well be combined with other microscopic contrast techniques such as reflected light or polarization.

An LSM can be easily understood as a modified light microscope supplemented by a laser module that serves as a light source, and a scanning head (attached to the microscope stand) that is used to detect the signal. Signal processing is effected by an electronic system contained in a box. The whole system is controlled by a computer.

To generate a confocal LSM image, let us first excite the fluorescence marker in a defined specimen area with a laser. For this purpose, monochromatic light from the laser module is coupled into the scanning head via a fiber optic. In the scanning head, the beam is made parallel by means of a collimator, and reflected into the microscope's light path by the principal dichroic beam splitter. The objective focuses the excitation beam onto a small three-dimensional specimen region called the excitation volume. The spatial extension of this volume is directly related to the system's resolving power. The greater the numerical aperture of the objective, the smaller the focal volume, and the higher the resolution. The position of the excitation volume can be shifted laterally (in X and Y) by means of two scanning mirrors, and vertically (in Z) with the microscope's focusing knob. The current Z position marks the system's focusing plane.
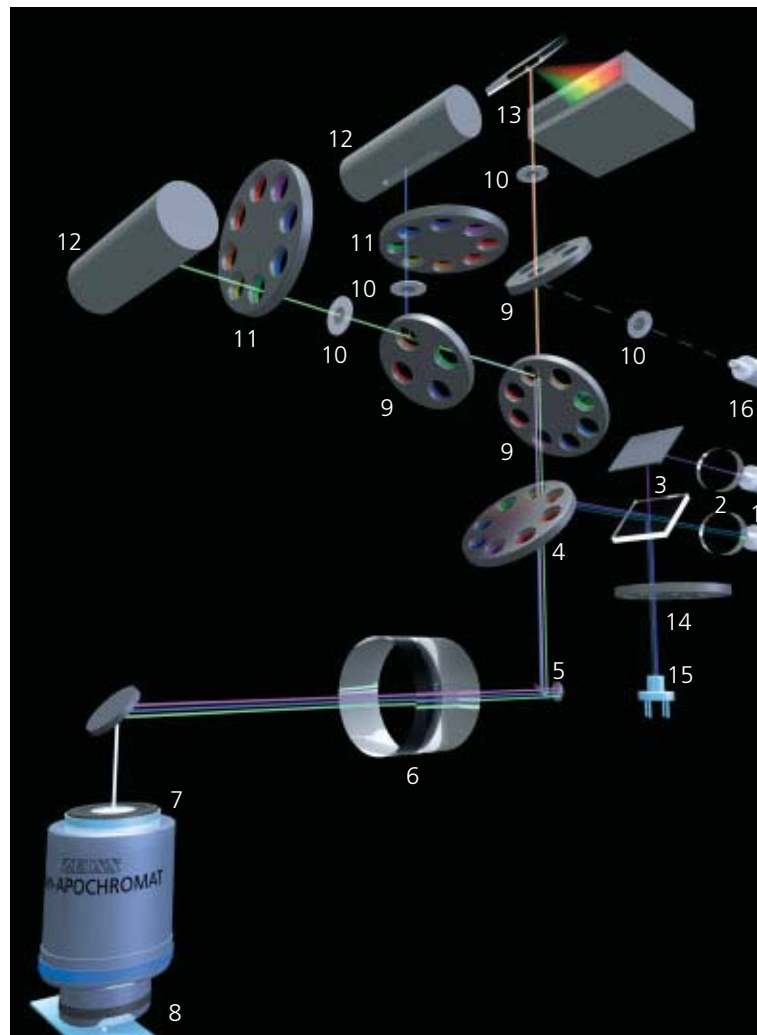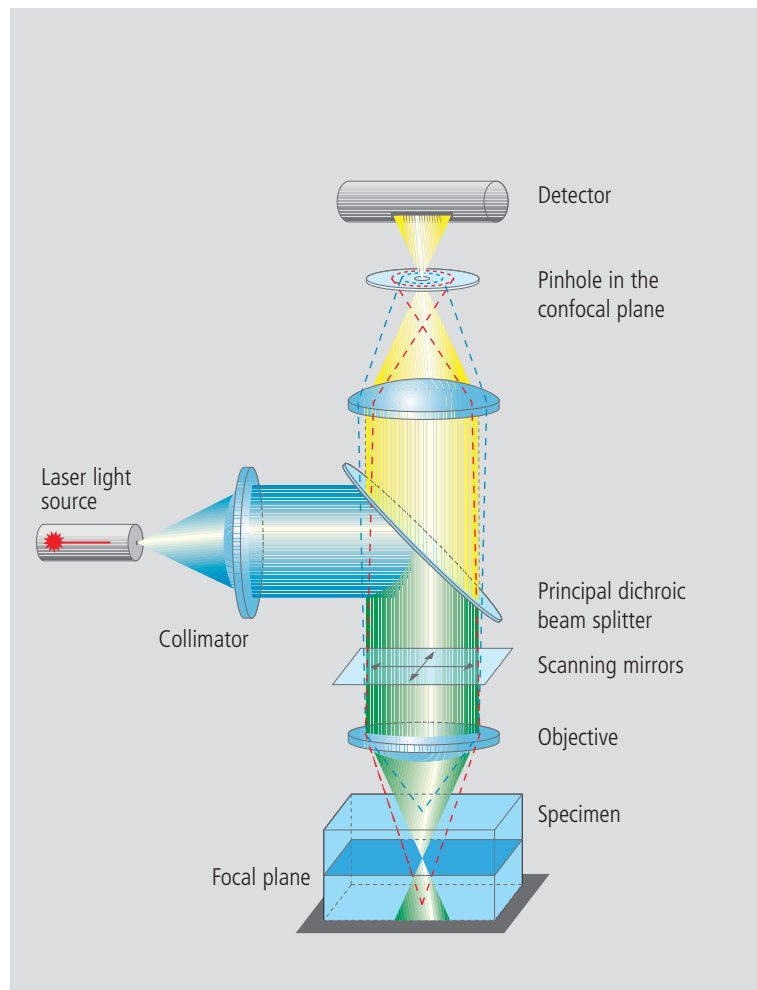
Excitation light path

The laser focused through the objective forms a double cone of excitation light inside the specimen. While the excitation intensity is strongest at the center of the double cone (in the focal plane), it is sufficiently high in the planes above and below the focus to excite fluorescence there, too.
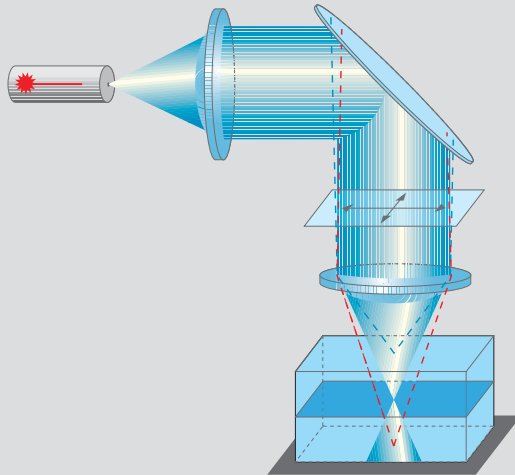
Detection light path

The only fluorescence that reaches the detector is that emitted in the focal plane. Light originating from other planes is blocked by a pinhole diaphragm.
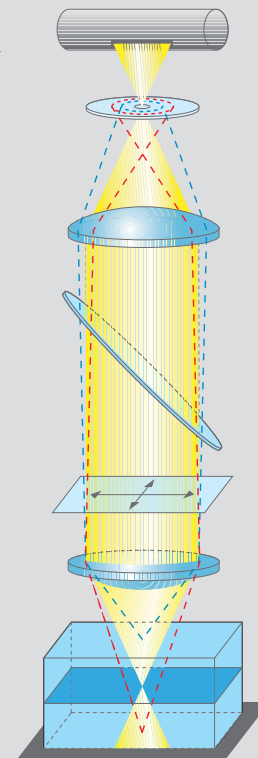
The laser light illuminating a particular detail of the specimen in order to excite fluorescence is focused by the objective into the focal plane. Fluorescence excitation and emission are most efficient within the focal volume. Therefore, the fluorescence from labeled structures in the focal plane forms a sharp image. The laser light, whilst less efficient, is still intensive enough to also excite fluorescently labeled structures above and below the focal plane. Light emitted there would be superimposed onto the sharp focal plane image and blur it. This is prevented by a pinhole diaphragm arranged in the ray path, which only permits light emitted in the focal plane to reach the detector.

The pinhole is essential to the generation of sharp images and for the optical sectioning capability. The very designation of confocal laser scanning microscopy refers to the pinhole, as this is in a plane conjugated to that of the focal plane (confocal plane). The thickness or Z dimension of an optical section can be set by motor-driven adjustment of the pinhole diameter. Fluorescence light from the focal plane, having passed the pinhole, is then detected by a photomultiplier. As an LSM image is formed sequentially, i.e. pixel by pixel, the detector does not require any spatial resolution. It merely measures the fluorescence intensity as a function of time. The image proper is formed only when the intensity measured by the detector is assigned to the corresponding site of the laser focus in the specimen. The laser beam is directed by the two independent scanning mirrors to scan the specimen in a line-by-line mode. The result of the scanning process is an XY image that represents a two-dimensional optical section of the specimen.

1   Fiber (from laser source)
2   Motor-driven collimators
3   Beam combiner
4   Primary dichroic beam splitter
5   Scanning mirrors
6   Scanning lens
7   Objective
8   Specimen
9   Secondary dichroic beam splitters
10 Pinholes
11 Emission filters
12 Photomultipliers
13 META detector
14 Gray filter
15 Monitor diode
16 Fiber output

Two-Dimensional Images

For examining flat specimens such as cell culture monolayers, it is usually sufficient to acquire one XY image to obtain the desired information. The same applies if the specimen is a three-dimensional tissue section of which a single optical section is representative.

The thickness of the optical section (slice) and the focal position are selected so that the structures of interest are contained in the slice. The lateral resolution of a 2D image is defined by the pixel size in X and Y. The pixel size, in turn, varies with the objective used, the number of pixels per scan field, and the zoom factor. Pixels that are too large degrade resolution, whereas pixels too small require longer scanning times, bleach the specimen and generate superfluous data volumes. The optimum pixel size for a given objective and a given zoom factor can be set by selecting the number of pixels with a mouse click.

The procedure for a two-dimensional image

1 Position and focus on the specimen in the *Vis*(ual) mode

2 Select the configuration to match the fluorochromes used

3 Define pixel resolution, scanning speed and, where required, *Average* Mode

4 Set the optical slice thickness by means of the pinhole diameter

5 Adapt the dynamic range to the specimen; automatically via *Find*, or manually via *Gain* and *Offset*

6 Adapt the scanning field to specimen substructures, using the *Crop* function
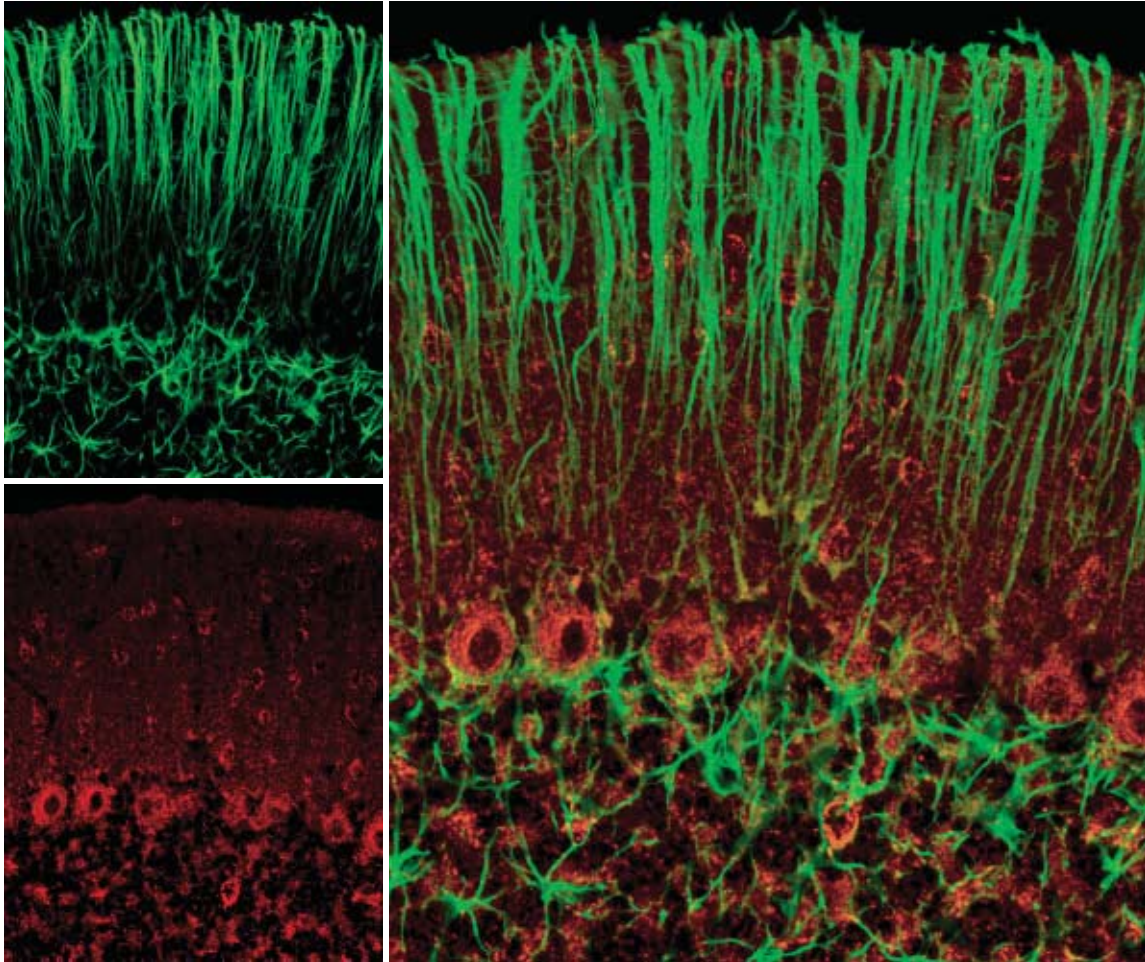
Confocal section through the cerebellum of a rat.
Green: astroglia cells (GFAP labeling); red: superoxide dismutase in neurons.
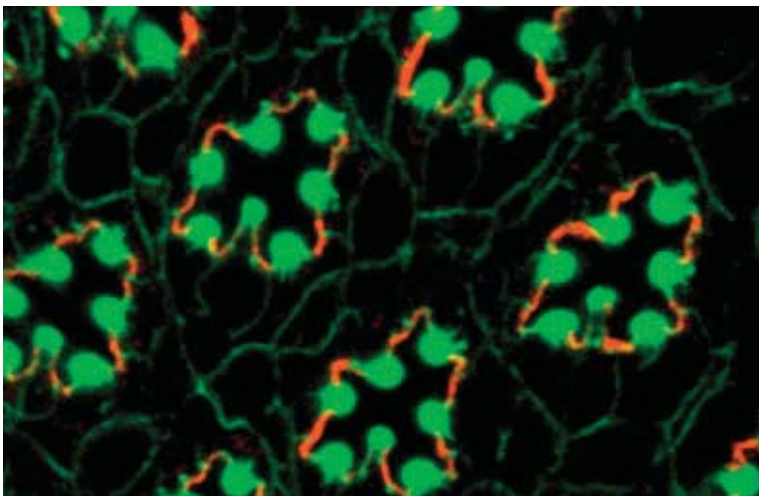
Double labeling of a Drosophila retina.
Green: actin; red: Crumbs.
Specimen: Dr. O. Baumann, University of Potsdam, Germany.

Three-Dimensional Images

To record the three-dimensional structure of a specimen, several two-dimensional optical sections are made in different focal planes. The result is an XYZ image stack, which can be visualized, processed and analyzed.

The optical section is selected by shifting the position of the focus in the specimen. This can be effected by moving either the objective or the specimen stage along the Z axis, according to the microscope stand design. Whether the image acquisition exhausts the resolving power given by the objective's numerical aperture depends on the thickness of the optical slice and on the spacing of two successive sections (the Z interval). According to the Nyquist criterion, the optimum Z interval is equal to half the optical slice thickness. If the pinhole diameter is selected to equal one Airy unit (1 AU), an optimum compromise between contrast and intensity is achieved for the XY image. The respective settings can be made by a mouse click in the software.

If the sample to be examined is labeled with more than one fluorochrome, it is necessary to adjust the optical slice thicknesses of the various image channels. The slice thickness is a function of the numerical aperture of the objective, the wavelength used, and the pinhole diameter. It differs for channels detecting light of different wavelengths. In the systems of the Zeiss LSM 510 family, every detector is equipped with a separate pinhole. This makes it easy to equalize the optical slice thicknesses in the software – an important condition for 3D colocalization analyses or for reconstructing 3D images.

Once a 3D stack of images has been recorded, the user has various presentation options. The data may be displayed as a gallery of depth-coded images or as orthogonal projections of the XY, XZ and YZ planes. To create a 3D impression on a 2D monitor, animations of different viewing angles versus time, shadow projections, and surface rendering techniques are possible.

The procedure for a three-dimensional image

1 Optimize the recording conditions for an XY image at the center
   of the three-dimensional specimen (see box for 2D images)

2 Define the *Start* and *Stop* stack limits in the *Z Setting* menu

3 Define the optimum Z interval in the *Z Slice* menu

4 Acquire the Z stack

5 Display and analyze the stack in one of the *Gallery, Ortho* or *Cut* display modes

6 With multiple-labeled specimens, equalize the optical slice thicknesses in the *Z Slice* menu

Three-dimensional specimen

0.00 µm	0.88 µm	1.75 µm	2.63 µm
3.51 µm	4.39 µm	5.26 µm	6.14 µm
7.02 µm	7.89 µm	8.77 µm	9.65 µm
10.53 µm	11.40 µm	12.28 µm	13.16 µm
14.03 µm	14.91 µm	15.79 µm	16.66 µm
17.54 µm	18.42 µm	19.30 µm	20.17 µm
21.05 µm	21.93 µm	22.80 µm	23.68 µm
24.56 µm	25.44 µm	26.31 µm	27.19 µm
28.07 µm	28.94 µm	29.82 µm	30.70 µm
31.58 µm	32.45 µm	33.33 µm	34.21 µm
35.08 µm	35.96 µm	36.84 µm	
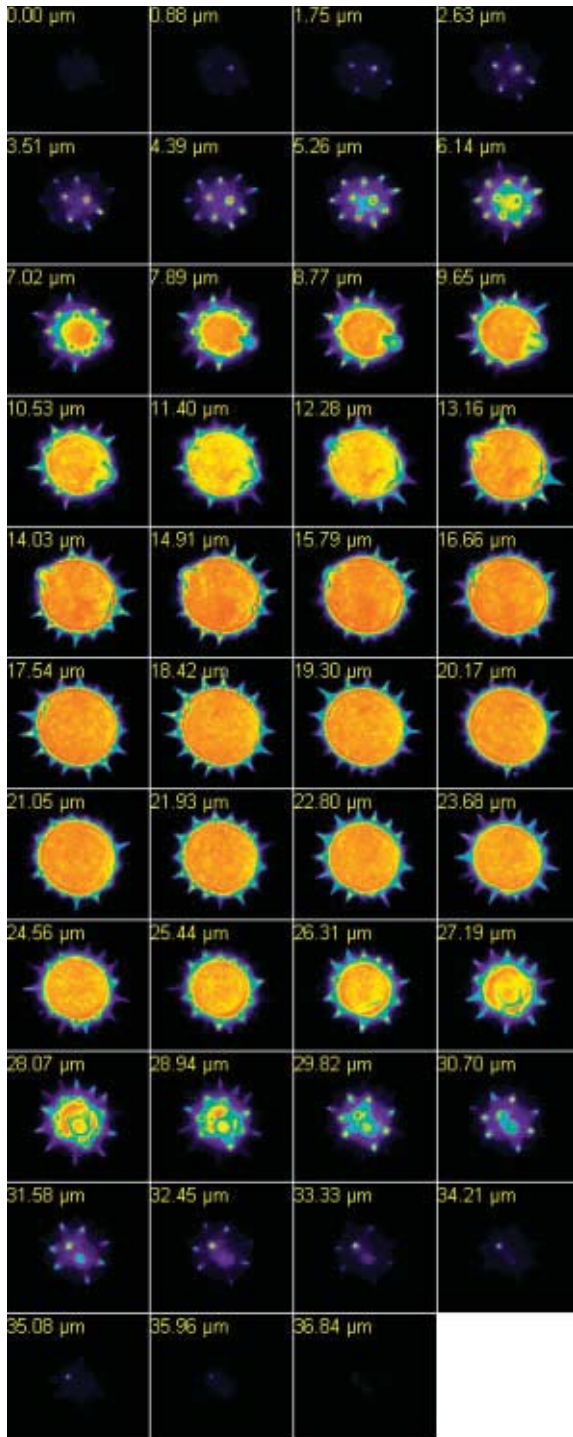
XYZ image stack of a pollen grain. A series of XY images acquired in different focus positions represents the Z dimension of the specimen.

10µm
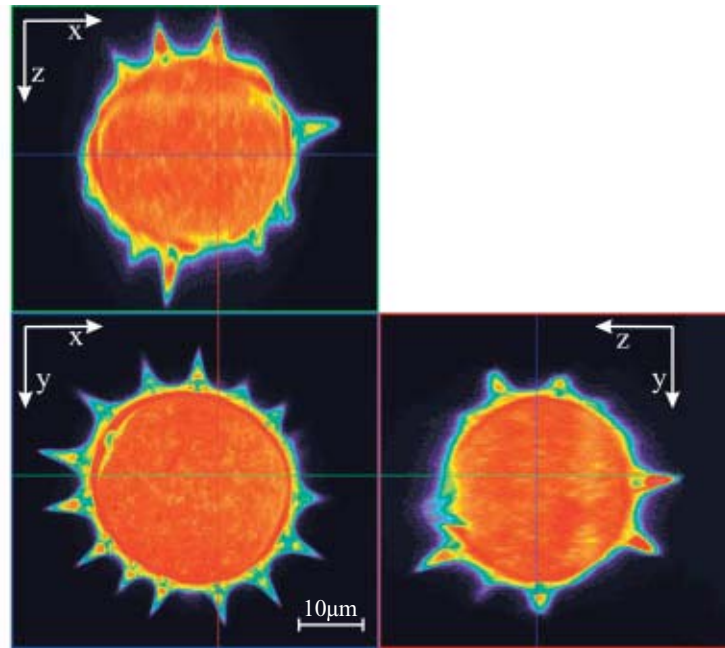
The orthogonal projection of the three-dimensional image data stack permits the raw data stack to be sectioned anywhere in any of the three mutually perpendicular planes.

Bottom left (above): Horizontal section through the center of the pollen grain (XY image).

Top: Projection of a vertical section along the horizontal axis in the XY image.

Bottom right: Vertical section along the vertical axis in the XY image.

Surface-rendered projection of the pollen grain.
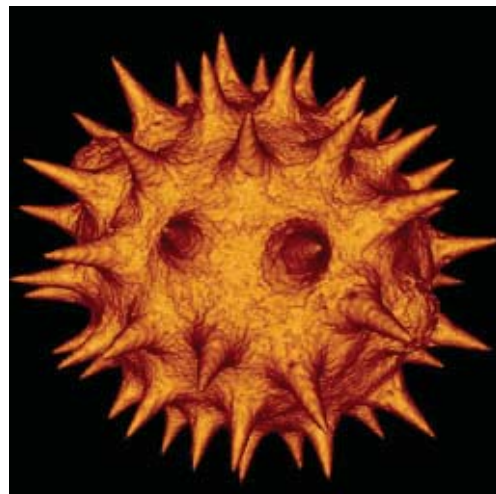
Time Series

Dynamic processes in living specimens can be recorded by means of time series. Data thus acquired can be analyzed "off-line", i.e. after image acquisition, or "on-line", i.e. right during the experiment, for example in the Online Ratio mode.

Time series are defined by a start time and the time interval between two successive images. The series can be started by a mouse click, automatically at a preselected time, or by some external trigger. To analyze a time series, the Physiology software option allows fluorescence intensity changes to be quantified in defined regions of interest (ROIs).

Within a time series, the LSM 510 permits selective, point-accurate illumination of ROIs with laser light.

This function is useful for generating a photobleaching routine within a FRAP experiment (fluorescence recovery after photobleaching), for analyzing dynamic processes, and for the photoactivation or photoconversion of suitable fluorochromes. Complex time series experiments, with different images to be taken at different sites within a specimen according to a defined time pattern, can be defined by means of a special software option.
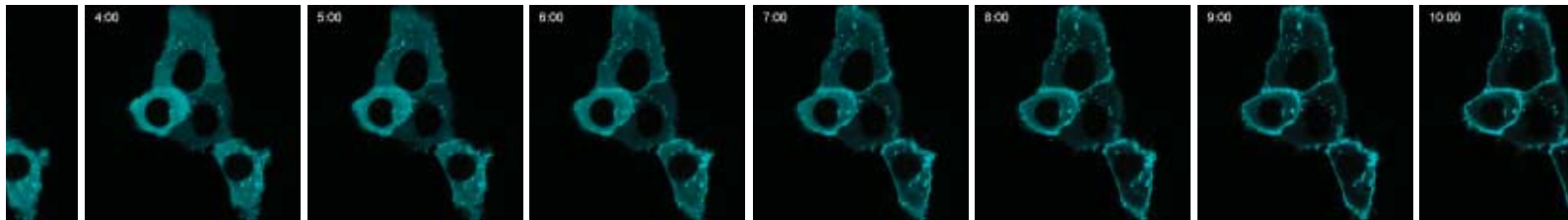
The procedure for a time series

1 Define the image dimensions to be recorded versus time *(XY* image, *Z stack, λ stack)*

2 Optimize the recording conditions at minimum laser output to avoid or minimize bleaching

3 Define the number of images to be taken and the time interval between two successive images *(Time Interval* or *Time Delay*)

4 Combine with a photobleaching routine if required: define the region to be bleached, the laser line and its power, the number of bleaching actions, and the bleaching start time within the series

5 Start the time series with the Start button, at a preselected time, or by an external trigger

Evaluation of the experiment
Selection of ROIs
within the specimen.
ROI 1: Cytosol
ROI 2: Cytoplasmic membrane
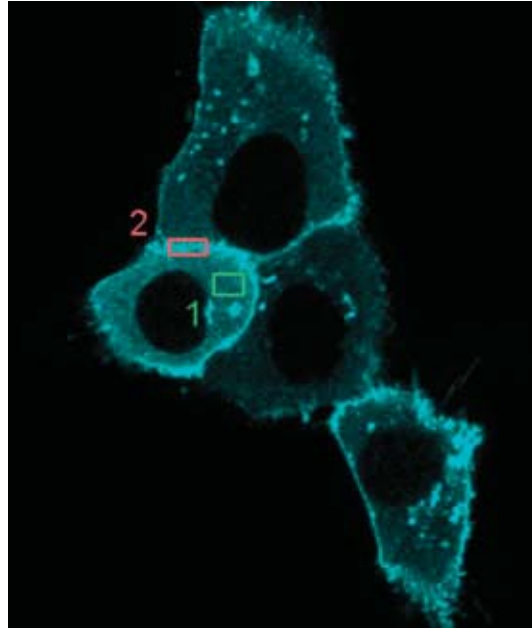
Investigation of protein movements

Time series of PKC-GFP transfected HeLa cells.
The stimulation of the cells with PMA at the time t=1 min
leads to a redistribution of PKC from the cytosol to the
cytoplasmic membrane (times in minutes).
Specimen: Dr. S. Yamamoto,
Medical University of Hamamatsu, Japan.
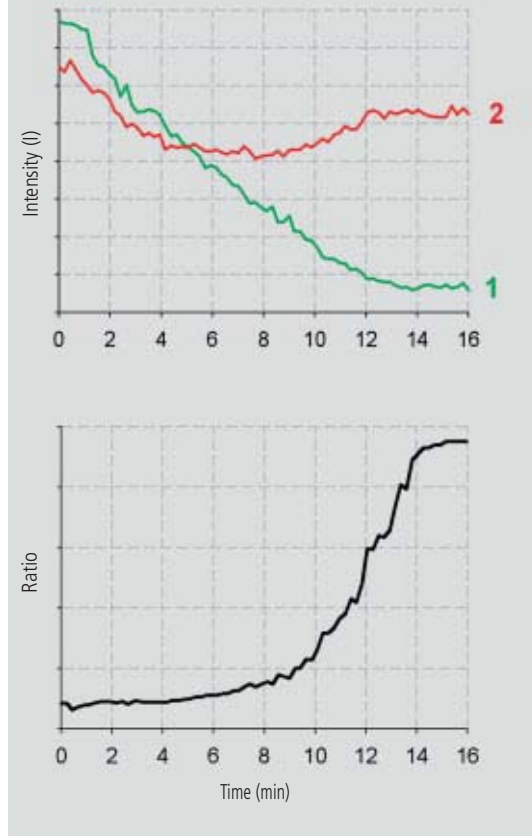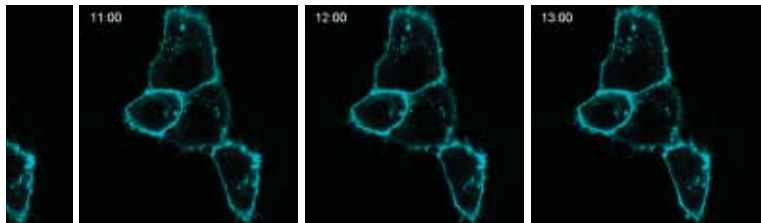
The individual
intensities (upper
graph) and the ratio
of intensities in the
two ROIs marked in
the top picture (lower
graph) illustrate PKC
redistribution.
Colors are assigned
correspondingly.

11

Multifluorescence –
The Crosstalk Problem and Its Solution

If a specimen is labeled with more than one fluorochrome, each image channel should only show the emission signal of one of them.

If, in a specimen labeled red and green, part of the green light is detected in the red channel, the phenomenon is known as crosstalk or bleed-through. This may lead to misleading results, especially in colocalization experiments.

One can distinguish between two kinds of crosstalk: emission and excitation crosstalk.

In a pure emission crosstalk between two fluorochromes A and B, the two emission spectra will overlap, but the laser lines will excite the dyes independently of each other; i.e. there is no overlap of the excitations.
Excitation crosstalk would occur if the laser that excites fluorochrome A also partially excited fluorochrome B.

The problem of emission crosstalk can be solved by sequential excitation and detection (Multitracking) of the fluorochromes. In case of a combination of excitation and emission crosstalk, additional spectral information is needed for separating the emission signals.

Emission crosstalk

Section through a mouse kidney, double-labeled with Alexa 488 (wheat germ agglutinin) and Alexa 568 (phalloidin). Simultaneous excitation with 488 and 543 nm. The emission of Alexa 488 is detected in both the green (BP 505-530 nm) and red (BP 560-615 nm) channels. Because of this bleed-through, the areas labeled with Alexa 488 appear yellow in the superposition and could be misinterpreted as colocalization with the Alexa 568.
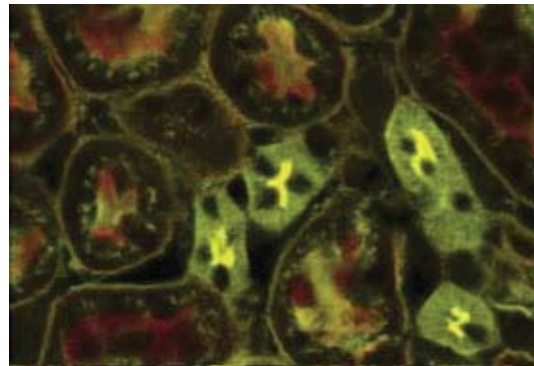
Elimination of emission crosstalk by Multitracking

*If Alexa 488 and 568 are excited and detected sequentially, no green signal is detected in the red channel.
Structures labeled with Alexa 488 appear green in channel superposition.*
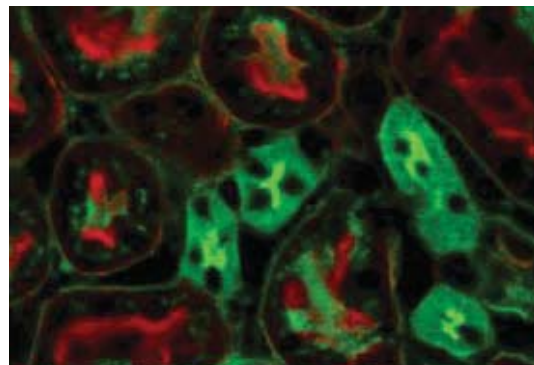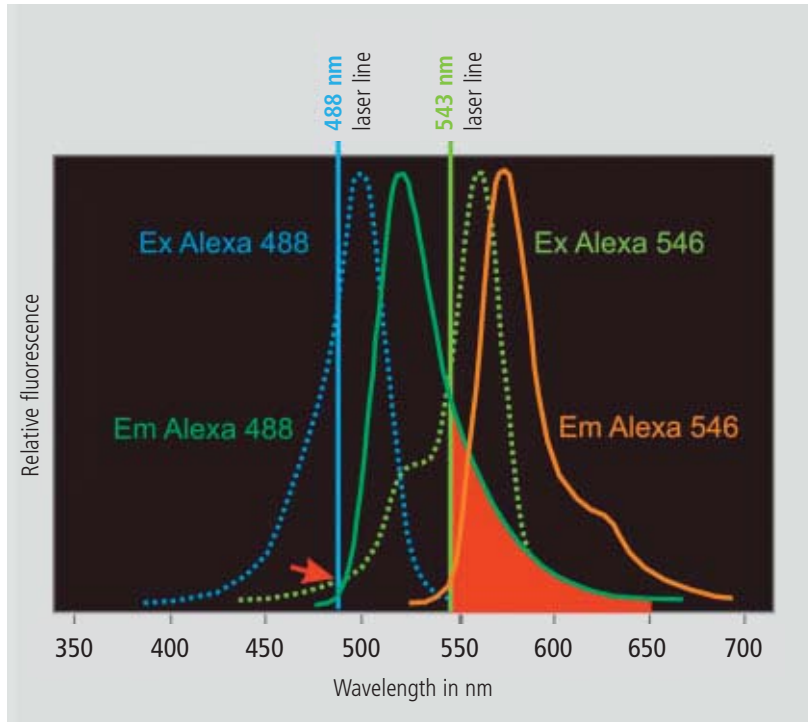
Emission crosstalk of Alexa Fluor 488 and 546

The excitation efficiency of the two fluorochromes is determined by the point of intersection between the laser line used and the excitation spectrum (dotted line).

Accordingly, Alexa Fluor 488 is excited to about 80 %, Alexa Fluor 546 to about 60 %.

At a level of about 5%, the excitation spectrum of Alexa Fluor 546 is also intersected by the 488 nm laser line (arrow). Theoretically, this constitutes excitation crosstalk, as one line excites both markers, but it is inefficient enough to be negligible.

Contrary to this, the emission spectra of the two dyes overlap significantly.

The red area marks the emission crosstalk of Alexa Fluor 488 occurring if Alexa Fluor 546 is detected to the right of the 543 nm laser line.
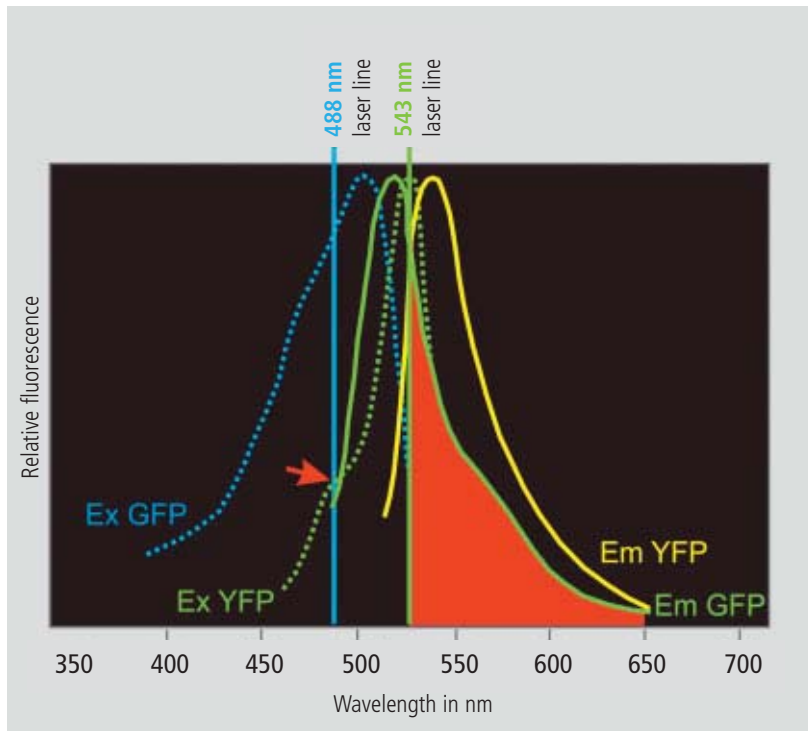
Combined excitation and emission crosstalk

If GFP is used together with YFP, the emission spectra will overlap considerably.

The red area marks the emission crosstalk between GFP and YFP occurring if YFP is detected to the right of the 514 nm laser line.

In addition, there will be a pronounced excitation crosstalk. The 488 nm line excites not only GFP but also YFP to an efficiency of about 30 % (arrow).

Source: http://home.ncifcrf.gov/ccr/flowcore/welcome.htm; modified

13

Spectral Imaging

The acquisition of spectral data becomes necessary where the overlapping emission signals of multiple-labeled specimens have to be separated, or where the cellular parameter to be measured is coded by changes of the emission spectrum (e.g., FRET and ratio imaging of ion concentrations).

The LSM 510 META is a system for the fast acquisition of images of high spectral resolution. The hardware enabling this functionality consists of a spectrally dispersive element, a photomultiplier (PMT) with 32 parallel detection channels (META Detector), and special electronic circuitry for detector control and signal amplification.

Whereas the beam paths for conventional and META detection are identical on the excitation side, the emission beam for spectral detection, after having passed the pinhole, hits a reflective grating. The grating spreads the beam into a spectrum and projects it onto the surface of the linear detector array. Each of the 32 PMT elements in that array thus registers a different part of the spectrum, each part having a width of 10 nm. The result is a lambda stack of XY images in which each image represents a different spectral window.

META Detector
Part of the beam path in the LSM 510 META scanning head

1 Confocal pinhole
2 Reflective grating for spectral dispersion
3 META Detector with 32 separate PMT elements
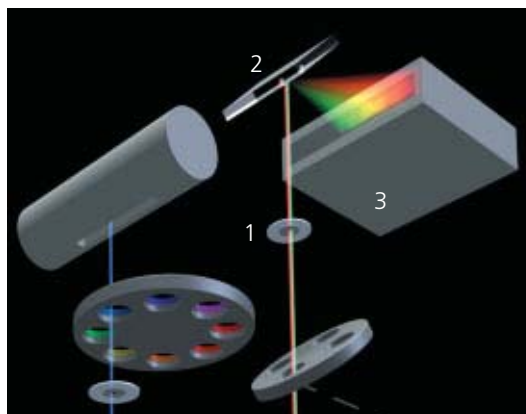
The procedure for a lambda stack

1 Select the spectral range in the *Lambda-Mode*

2 Activate the excitation laser lines

3 Carefully control the dynamic range to avoid over- and underexposed pixels *(Range Indicator)*

4 In multiple-labeled specimens, vary the power of the respective laser lines instead of the *Amplifier Gain,* in order to match the signal intensities of the fluorochromes

5 Record the lambda stack

6 Display the data in one of the modes: *Gallery, Single, Slice, Max* or *λ-coded*

Lambda stack
All images show the same area, but different spectral windows of the specimen.
The marker dyes are represented by different parts of the stack depending on the emission spectrum.
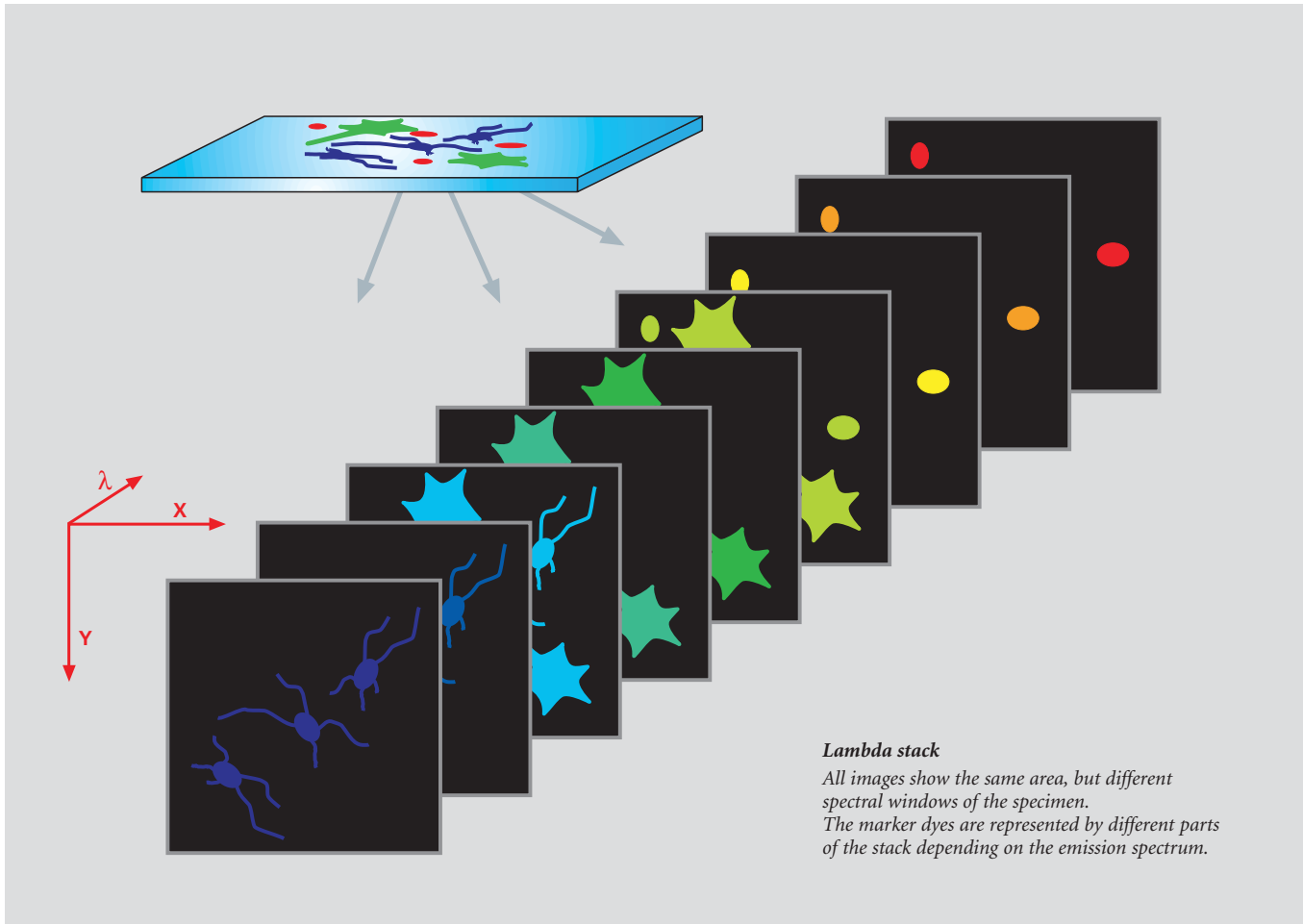
By connecting adjacent detector elements (binning), the spectral width of the images can be extended. From a lambda stack, the intensity of the signal for each pixel of the image can be extracted as a function of wavelength. These spectral "fingerprints" can easily be obtained for any image area by means of the Mean of *ROI* function. Lambda stacks can be recorded as time series, Z stacks, or as Z stacks versus time. In the last-named case, the result would be a five-dimensional image file with the coordinates, X, Y, Z, lambda and time.

The META Detector is good not only for recording lambda stacks, but also as a channel detector in the conventional mode. By binning the respective detector elements in this mode, the optimum spectral bandwidth can be adjusted for any fluorescent dye.

Spectral Imaging

Emission Fingerprinting

Emission Fingerprinting is a method for the complete separation (unmixing) of overlapping emission spectra. It is used with specimens labeled with more than one fluorescent dye, exhibiting excitation and emission crosstalk.

The typical raw data for Emission Fingerprinting are lambda stacks. The previous chapter described how they are recorded by means of the META Detector. The second step is to define reference spectra for all spectral components contained in the specimen. As a rule, these are dyes internationally used for labeling the specimen. Other possible components are autofluorescent and highly reflecting structures. Autofluorescences, in particular, often have rather broad emission spectra that overlap with the fluorescent markers; this makes them an added source of "impurities" degrading the signals in conventional laser scanning microscopy.

With Emission Fingerprinting, autofluorescences are simply included in the unmixing process. The user can subsequently decide between switching the autofluorescence channel off and using it to obtain structural information possibly contained in the specimen.

The reference spectra can either be loaded from a spectra database, or directly extracted from the lambda stack. For the latter version, the user has two options. One is to define spectra via ROIs. The other uses a statistical method, Automatic Component Extraction (ACE), to find the reference spectra. In either case, the images of the lambda stack must contain structures marked with a single fluorochrome only.

The third step of Emission Fingerprinting is Linear Unmixing, which converts the lambda stack into a multichannel image. Each spectral components of the specimen is then displayed in one channel only. The accuracy of the technique allows the complete unmixing even of such dyes whose spectra have almost identical emission maxima.

Linear Unmixing

Linear mathematical algorithm for spectral unmixing.
If we regard a pixel of a lambda stack that represents a locus in the specimen
where three fluorescent dyes A, B and C with their spectra $S(\lambda)_{dye\ A,\ B\ and\ C}$ overlap,
the cumulative spectrum $\Sigma S(\lambda)$ measured can be expressed as

$$\Sigma S(\lambda) = [intensity \cdot S(\lambda)]_{dye\ A} + [intensity \cdot S(\lambda)]_{dye\ B} + [intensity \cdot S(\lambda)]_{dye\ C}$$

By means of known reference spectra $S(\lambda)_{dye\ A,\ B\ and\ C}$, the equation can be solved for the intensities of the dyes A, B and C, which yields the intensity shares of the three dyes for this pixel. If this calculation is made for each pixel, a quantitatively correct 3-channel image results, in which each channel represents a single dye.

Intensity

GFP   YFP

Emission wavelength in nm
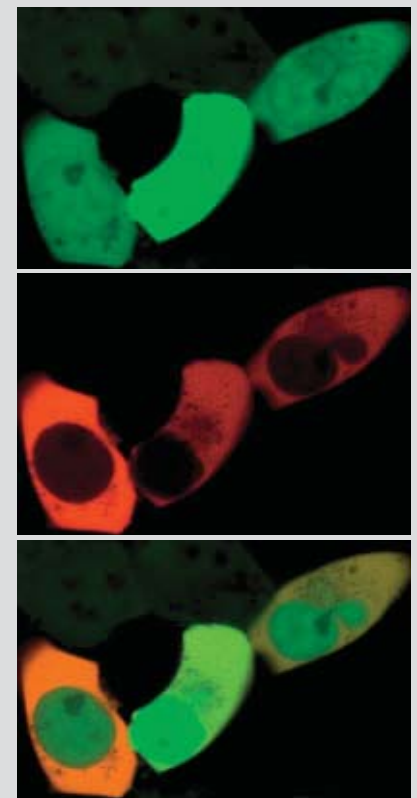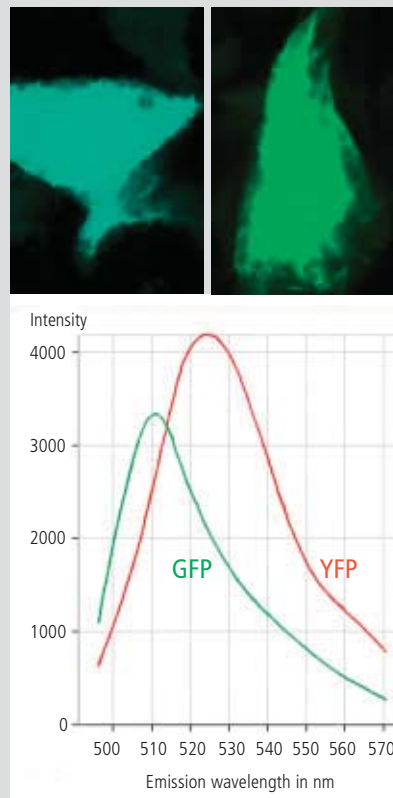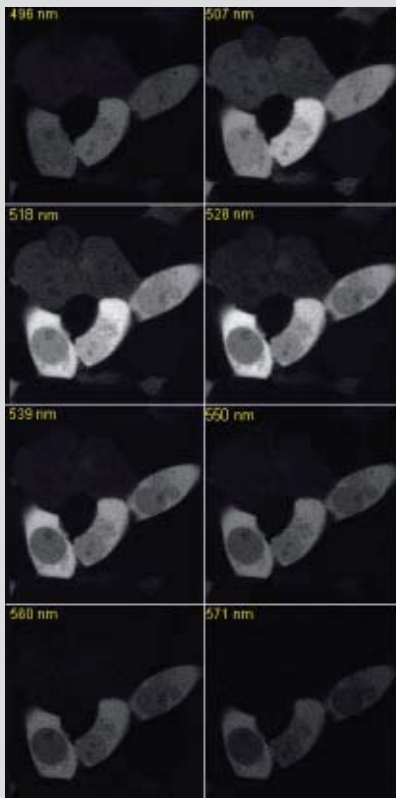
The 3 Steps of Emission Fingerprinting

1 Recording of a lambda stack

The illustration shows an 8-channel image of a cell culture transfected with GFP and YFP. Each image shows the mean wavelength of the channel.

2 Definition of reference spectra

The reference spectra were obtained by means of lambda stacks of cells single-marked with GFP and YFP, respectively. Top: Lambda-coded projections of a cell marked with GFP (left) or YFP (right). Bottom: Reference spectra for GFP (green) and YFP (red).

3 Linear Unmixing

Using the reference spectra from the lambda stack, the *Linear Unmixing* function generates a two-channel image, in which each channel represents only one of the two fluorochromes.
Top: GFP
Center: YFP
Bottom: Both channels superimposed

17

Spectral Imaging

Channel Unmixing

If the emission spectra of fluorescent markers overlap only slightly, the signals can be separated with the *Channel Unmixing* function.

As raw data for unmixing in this case, it is sufficient to have multichannel images in which one of the marker dyes dominates in each channel. Such images can be acquired without the META Detector, i.e. with an LSM PASCAL, LSM 510 or a CCD camera.

Channel Unmixing also allows unmixing based on the excitation behavior of dyes, if the raw data are multichannel images in which the channels differ only by their excitation wavelength.

Double labeling of the nervous system of a zebra fish embryo

Two-channel single-track images with emission crosstalk. The nerve labeled with Alexa 488 can be discerned (arrows) in the green (top) and, faintly, in the red channel (center). Bottom: Superposition of the two channels.
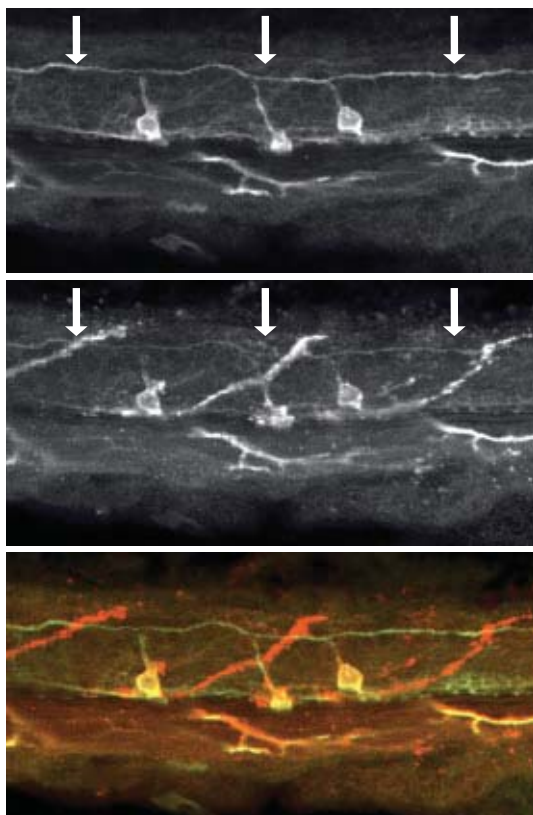
The same images after Channel Unmixing. The Alexa 488-positive nerve is visible in the green channel (top) only but vanished from the red one (Center). Bottom: Superposition of the two channels.
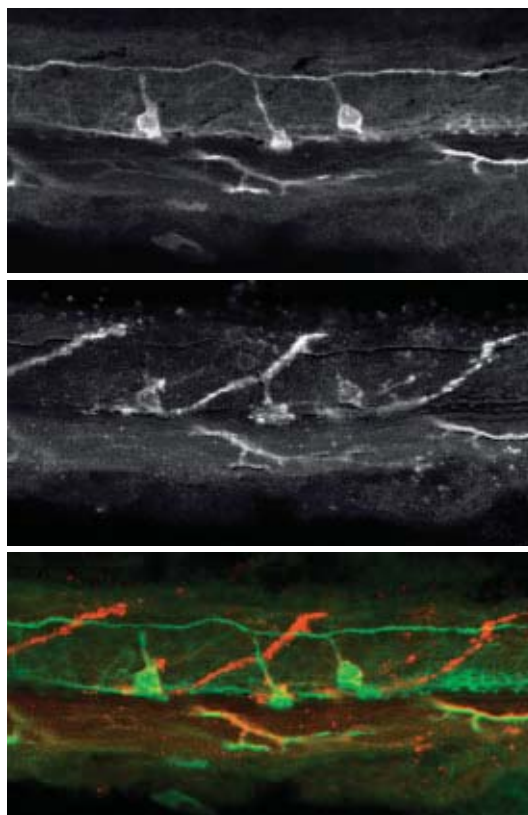
Specimen: Prof. M. Bastmeyer, Friedrich Schiller University of Jena, Germany.

Online Fingerprinting

The functionality of *Online Fingerprinting* can be used to separate overlapping emissions even while a time series is being recorded. This may be of decisive importance where dynamic processes are investigated.

Here, a reference spectrum is assigned to each image channel before image acquisition starts. During the experiment proper, lambda stacks are acquired and immediately unmixed in a background operation. The user sees the unmixed multichannel image during the acquisition of the time series. *Online Fingerprinting* is of advantage especially in spectral FRET experiments and in studies of dynamic processes with fluorescent proteins.

Visualization of FRET by means of acceptor photobleaching

Expression of a FRET-positive protein construct (CFP linker citrin) in HEK 293 cells.
Recording conditions: Simultaneous excitation with 458 and 514 nm.
Spectral detection from 462 to 655 nm in Lambda Mode.
Online Fingerprinting and simultaneous display of the two-channel image (CFP blue, citrin green).
During the combined time-&-bleaching series (bleaching region marked), citrin (green channel) as a FRET partner is destroyed by intensive irradiation with 514 nm. The decrease in FRET is visible as an increase in CFP fluorescence (blue channel).
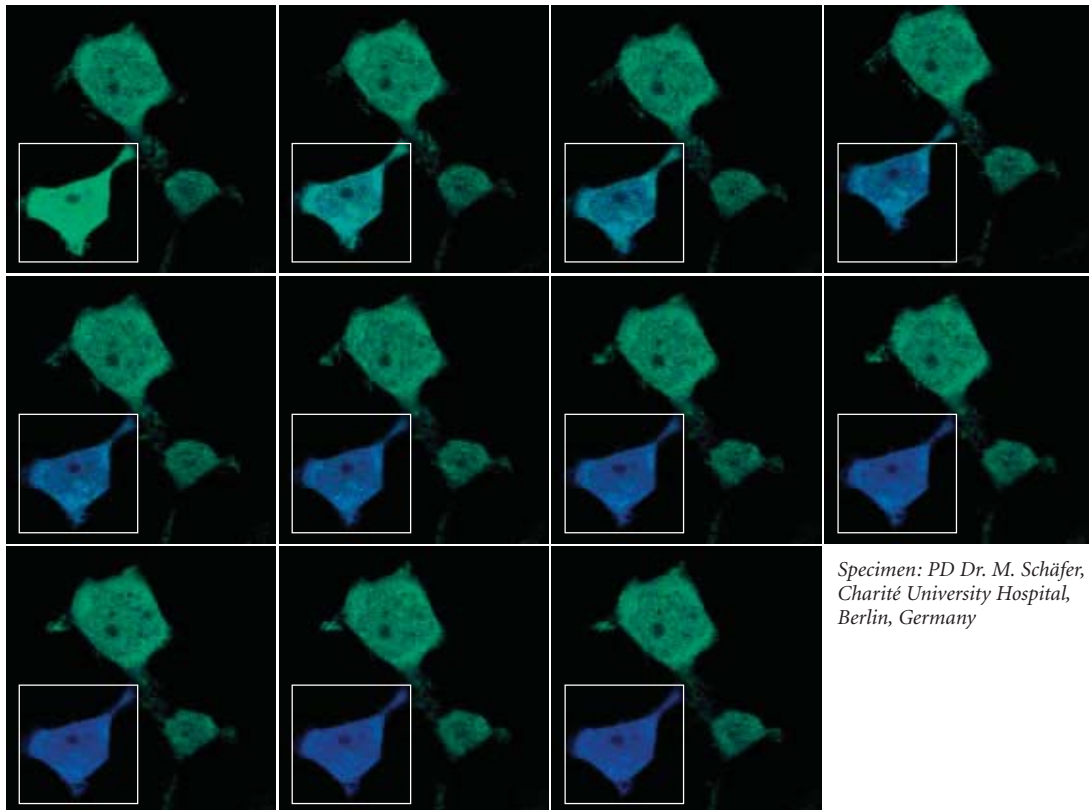
Specimen: PD Dr. M. Schäfer, Charité University Hospital, Berlin, Germany

Spectral Imaging

Excitation Fingerprinting

By means of tunable excitation lasers such as those used in multiphoton systems, it is possible to detect also the excitation spectra of fluorochromes. These can be used for unmixing as an alternative to emission spectra.

Multiphoton systems are a special class of confocal laser scanning microscopes, distinguished from classical one-photon systems essentially by an additional light source, known as a multiphoton or NLO (non-linear optics) laser.

The infrared (IR) light emitted by such lasers can penetrate tissues to greater depths than visible light can. Due to its low phototoxicity, IR light is suitable for long-time observation of live samples. Usually, the emission wavelength of these lasers can be varied continuously to excite the respective fluorochrome used in the multiphoton mode.

In *Excitation Fingerprinting,* this property is used for the acquisition of excitation lambda stacks. For that purpose, the multiphoton laser is controlled by the LSM software to shift its excitation wavelength by a defined interval before every new image. The image stacks thus recorded can be used for the unmixing of spectral components differing by their excitation properties, analogously to the (emission) lambda stacks described before. For more information on multiphoton microscopy, refer to the literature cited on the rear cover.

The procedure for Excitation Fingerprinting

1 Define an excitation lambda stack (wavelength range and interval size) in the *Excitation Fingerprinting* macro

2 Record the excitation lambda stack

3 Define the reference spectra via single-labeled specimen regions, single-labeled reference samples, or by using the *ACE* function

4 Run *Linear Unmixing*

*Excitation Fingerprinting separates
widely overlapping emission signals
by their excitation spectra.*

Ready, 1024 x 1024 , 8 channels , 12 bit , Intensity (985,445,6) 38
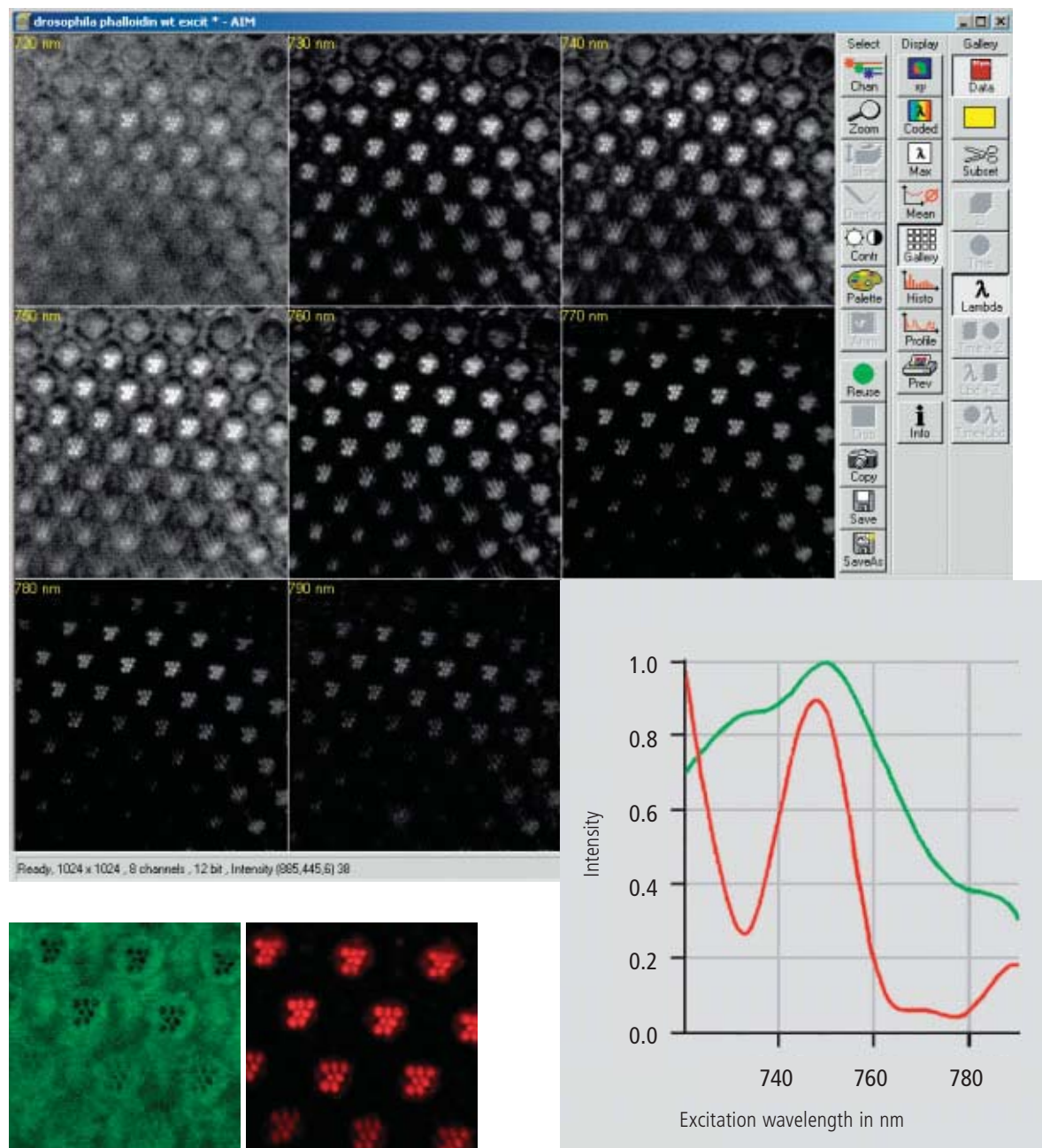
*Retina of a Drosophila fly, labeled for actin
(Alexa Fluor 586 phalloidin);
autofluorescence and emission signal can be cleanly separated
by Emission Fingerprinting.*

Specimen: PD Dr. O. Baumann, University of Potsdam, Germany

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Laser scanning microscopy has become a mainstream technique in biomedical research.
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Summary

Modern laser scanning microscopes are versatile tools for visualizing cellular structures and analyzing dynamic processes in biomedical research.

Apart from mere imaging, Carl Zeiss laser scanning microscopes are designed for the quantification and analysis of image-coded information. Among other things, they allow easy determination of fluorescence intensities, distances, areas and their changes over time. The LSM 510 META, in particular, is capable of quickly detecting and quantitatively unmixing the spectral signatures of fluorescent dyes that closely resemble each other.

Many software functions analyze important parameters such as the degree of colocalization of labeled structures, or the ion concentration in a specimen.

With their capabilities for acquiring, evaluating and presenting experimental data, LSM systems made by Carl Zeiss are tailored to the requirements of scientists of today and tomorrow.

REFERENCES

Amiri H., Schulz G., Schaefer M. (2003)
FRET-based analysis of TRPC subunit stoichiometry.
Cell calcium, 33(5-6):463-70.

Carl Zeiss (2000)
Confocal Laser Scanning Microscopy –
Principles. 45-0029 e/09.03

Carl Zeiss (2002)
Colocalization – Analysis and Visualization.
45-0012 e/11.02

Carl Zeiss (2002)
Spectral Separation of Multifluorescence Labels
with the LSM 510 META.
40-546 e/05.02

Diaspro A. (2001)
Confocal and Two-Photon Microscopy :
Foundations, Applications and Advances,
Wiley-Liss, New York

Dickinson M.E. (2001)
Multi-spectral imaging and Linear unmixing
add a whole new dimension to laser scanning
fluorescence microscopy.
Bio Techniques 31/6, 1272-1278.

Gordon G,W. (1998)
Quantitative Fluorescence Resonance
Energy Transfer Measurements Using
Fluorescence Microscopy.
Biophys J, May 1998, p. 2702-2713, Vol. 74, No. 5

Pawley J.B. (1995)
Handbook of Biological Confocal Microscopy.
Plenum Press, New York

Selvin P.R. (2000)
The renaissance of fluorescence resonance energy
transfer. Nat Struct Biol. 7(9):730-4. Review.

LINKS

Carl Zeiss website on
contrasting techniques in light microscopy
www.zeiss.de/contrasts

EAMNET website on FRAP
www.embl-heidelberg.de/eamnet/html/
teaching_modules.html

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CHAPTER 4   SYSTEM OPERATION

CONTENTS

Page

4   SYSTEM OPERATION

4.1   Purpose

This section describes the operation of the Laser Scanning Microscope with the ZEN software.

When starting up and operating the microscope system, mind the operating instruction manuals for the Axio Imager.Z1, Axio Imager.M1, Axio Observer.Z1 and Axioskop 2 FS microscopes:

– B 46-0046   Axio Imager.Z1, Operating Manual

– B 40-0111   Axio Observer.Z1, Operating Manual

– B 40-076   Axioskop 2 FS MOT, Operating Manual

– B 46-0046   Axio Imager.M1, Operating Manual (LSM 5 *EXCITER* and LSM 510 only)

4.1.1   Software

The ZEN software is used to

– control the microscope, the scanning module, the laser module, and the image acquisition process

– display, edit and analyze the images

It is a special user interface (desktop) based on the network-capable graphic 32-bit Microsoft**®** WINDOWS XP operating system.

Portions**©** Copyright 2007, Microsoft Corporation. All rights reserved.

The installation of the software for the Laser Scanning Microscope and the basic settings of the equipment components are carried out by Carl Zeiss service staff. This job includes the creation of a customized software configuration in line with the specific hardware components of the customer's microscope system.

4.1.2   Convention for the Text in this Manual

All the originally used terms of the software interface, e.g.

– names of windows

– tool groups

– panels

– input boxes

– list / selection boxes

– check boxes

– menu items

– names of buttons and sliders

– keyboard keys

are displayed in **bold letters** to allow easier identification.

4.1.3   Backup

System backup

– A complete backup of the operating system and application software is available on the enclosed system image CD-ROM.

User files backup

The following user-generated files should be included in a backup procedure controlled and carried out on a regular basis by the user (keep directory structure):

– LSM Image files: *.lsm

– Exported images: *.* (*.Tiff, *.LSM-Tiff, *.BMP, …)

– Palette files: ZEN\Palette*.lut

– Filter files: ZEN\Filter*.krn

– Pinhole setting files: ZEN\PH*.pos

– Log files: ZEN*.log

– User defined configuration .xml files: C:\Documents and Settings*Username*\Application Data\Carl Zeiss\AIMApplication\

The following files generated during the system integration should also be included in a backup procedure:

– Parameter file for pinhole setting: ZEN*.set

– Parameter file after pinhole adjustment: ZEN*.adj

– Scanner files: ZEN\bin*.bin

– Microscope stand database: ZEN\database\system_configuration_*.mdb

4.1.4   Software Operation

The ZEN software can be operated using the mouse, the PC keyboard, or both.

The operation of the mouse and the keyboard is identical to that of the Microsoft® WINDOWS operating system and is therefore not described in this manual.

If required, see the Microsoft manual or online help for relevant information.

4.2 Hardware Aspects

4.2.1 Optical Diagram of the LSM 510 and LSM 510 META (Schematic)
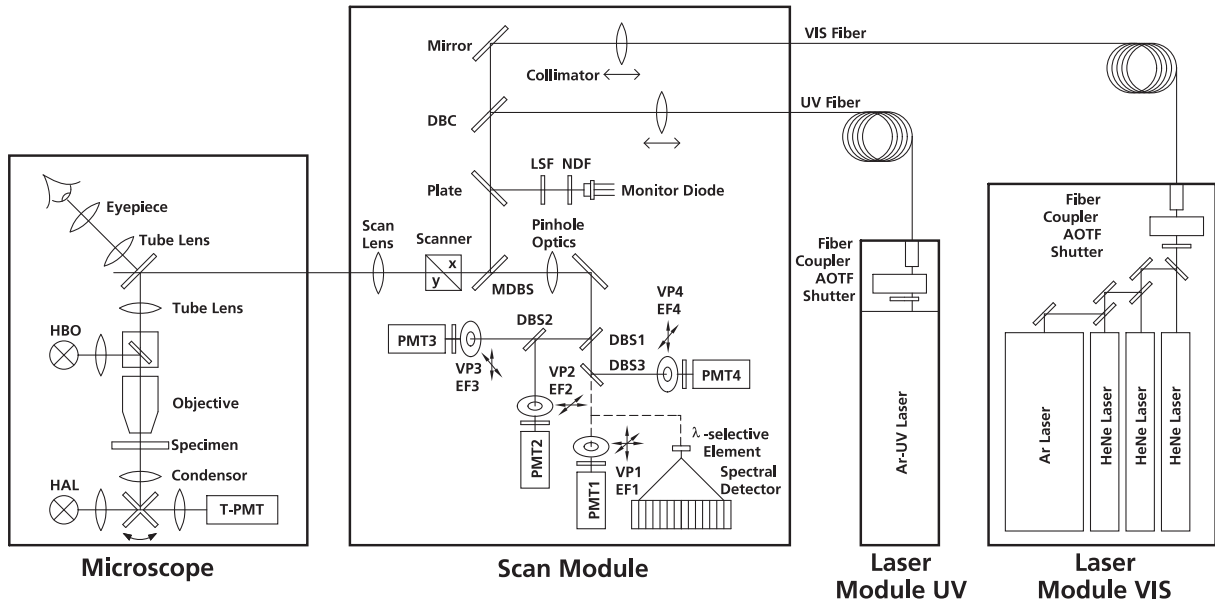
AOTF Acousto Optical Tunable Filter
DBC Dichroic Beam Combiner
DBS Dichroic Beam Splitter
EF Emission Filter
HAL Halogen Lamp
HBO Mercury Vapor Short-Arc Lamp
LSF Line Selection Filter
MDBS Main Dichroic Beam Splitter
NDF Neutral Density Filter
VP Variable Pinhole
PMT Photomultiplier
T-PMT Transmission-Photomultiplier

Fig. 4-1   Optical path, schematic (4-channel configuration)

The diagram above is a schematic representation of the LSM system.

Laser light is focused onto the specimen through an objective in a diffraction-limited mode. Light emitted at the focal plane and at planes below and above it is directed via an XY scanner onto a main dichroic beam splitter (MDBS), which separates the emissions from the excitation light. The fluorescences are separated from each other by a series of dichroic beam splitters (DBS1 ... maximally DBS4) and directed to individual photomultipliers (PMT1 ... maximally PMT4).

4.2.2 Performance and Features of the LSM 510 and LSM 510 META

Optical and Mechanical Aspects

The highly integrated system design makes for the shortest possible optical paths, top-grade optical precision and high stability. The compact scanning module can be fitted to an inverted (Axio Observer.Z1 BP or SP) or upright (Axio Imager.Z1, Axio Imager.M1 or Axioskop 2 FS MOT) microscope in less than three minutes. On the Axio Observer.Z1, the scanning module may be mounted either to the base port directly below the microscope or to the side port.

The spectral range available extends from the UV to the IR region.

For the VIS (visible-light) Laser Module, the user can select from up to six lasers with wavelengths of 633, 594, 561, 543, 514, 488, 477, 458 and 405 nm. The UV Laser Module provides wavelengths of 351 and 364 nm. A Ti:Sa Laser provides pulsed laser light from 680 to 1080 nm for Multiphoton imaging (NLO). Coupling of the laser light is through polarization-preserving single-mode optical fibers. One variable beam collimator each for the UV or NLO and visible ranges provides optimum adaptation of the respective laser wavelength to the objective used and, thus, optimum correction for Z aberrations.

Acousto-optical tunable filters (AOTF) adjust the necessary brightness for up to 8 laser lines within microseconds.

A monitor diode permanently registers the laser output; it can be used for the on-line checking of the intensity of the exciting light. This check is also possible selectively for the different wavelengths if a line selection filter is inserted.

The four internal image acquisition channels, usable for reflection or fluorescence, and an additional transmitted-light channel are ideal for the investigation of multiple fluorescence specimens. Separately in each of the four channels, the diameters of the pinholes and their XY positions can be optimized, and the desired emission filter placed into the beam path, by servo-motor control. In the case of pinhole VP1, this adjustment also includes positioning along Z. In the simultaneous registration of multiple fluorescence, identical optical sections can be obtained in each confocal channel. This is of importance, e.g., with the FISH method (fluorescence in-situ hybridization) used for genome analysis in cytogenetic studies.

The microscope's transmitted-light channel is equipped with a photomultiplier, too. It is therefore possible to superimpose a multiple fluorescence image on a brightfield, differential interference or phase image.

A fiber-optic cable connection to external special detectors, such as cooled PMTs or spectrometers, is also available. It substitutes one internal detection channel.

In addition to the emission filters for all standard and special applications, available in motor-controlled filter wheels, the user can easily install his own emission filters in two of the channels.

The high-NA C-APOCHROMAT objectives especially developed for the LSM reach the physical limit in resolving power, and can be used throughout the 380...900 nm spectral range with the same high quality, producing brilliant images.

A two-mirror scanner system, controlled by real time electronics, offers several advantages. The large deflection angle of the scanning mirrors allows a wide area to be scanned. With a 1.25× objective, the object area scanned is 10 11 × 10 mm².

The scanning field size can be freely selected between 4 × 1 and 2048 × 2048 pixels.

It is possible to rotate the XY scanning field through 360° and carry out XY scans without having to rotate the specimen itself under laser radiation load.

Selection of the specimen detail of interest for zooming is fast and convenient, and the zoomed image is automatically centered. This saves the job of placing the specimen into the center with the microscope stage.

Using a bi-directional scanning facility will double the scanning rate to approx. 5 frames/sec (at 512 × 512 pixels); if two different laser wavelengths are used for the two scanning directions (wavelength 1 for left-to-right, and wavelength 2 for right-to-left scanning), two fluorescent dyes can be viewed and documented in a quasi-simultaneous mode. This will prevent cross talk between detection channels.

The LSM 510 and LSM 510 META can be combined with a second scanner, the LSM *DuoScan*. Any of the above mentioned microscopes can be used. The *LSM DuoScan* can be equipped either with a UV Laser or a 405 nm laser both also together with a 488 and 532 nm or 561 nm lasers. The software allows to simultaneously image and bleach/manipulate a sample. The usefulness of this option is dependent on the sample.

4.2.3 Microscope Equipment of the LSM 510 and LSM 510 META System

The LSM 510 or LSM 510 META system is equipped either with the inverted Axio Observer.Z1 BP or SP microscope or with the upright Axio Imager.Z1, Axio Imager.M1 or Axioskop 2 FS MOT microscopes.

Only the differences from the delivered operating manual "Axio Observer.Z1" will be explained here.

(1) Stand

– The motorized objective nosepiece 5× H DIC is firmly fixed to the stand, where no operating elements can be found for the nosepiece. Operation will be performed via LSM 5 software control. The "Restriction of the nosepiece height to protect the objectives during motorized objective change" is inactivated. The nosepiece will be moved down automatically before each motorized objective change.

– The reflector mount is motorized and provided with the Axio Observer.Z1 reflector turret. The reflector turret has six positions: One transmitting light position, which is identical to the LSM position, and five further positions for fluorescence filter sets (reflector modules). If you want to use more than five conventional fluorescence filter sets, it is advisable to use a further reflector turret. When changing the reflector turret position you must make sure that the turret will click into position, since otherwise the image area will be cut. If the system is equipped with the LSM *DuoScan* up to four positions (depending on the laser equipment of the LSM *DuoScan*) are equipped with fixed beam combiners. Two or three positions of the reflector turret can be equipped with conventional fluorescence filter sets.

– The stand has a motorized focusing drive (fine coarse). Sensitivity of the focusing drive is adjusted to the delivered objectives by the manufacturer. If you want to use other objectives, sensitivity and parfocality can be adjusted via the Axioset program.

– The stand features an integrated power supply for the internal motors and stand electronics. The power supply can be switched at the external power supply unit for the microscope. External power supply units will be used for the mercury vapor short arc lamp or the X-Cite 120 fiber coupled lamp.

– The analyzer slider for conventional DIC methods will be operated from the right side and is located just below the nosepiece.

– When the rod is pushed in, the analyzer is located in the beam path. In the LSM-mode, the analyzer must **not** be located in the beam path, and the analyzer rod must be pulled out.

(2)   Specimen stages and fine focus drives

– Mechanical stage
  The stage with coaxial drive must be mounted on the right side of the stand.

– Scanning stage

– Piezo objective focus drive

(3)   Transmitted-light illumination

– The illuminator support contains a security circuit which activates a shutter preventing laser light from reaching the stand when the support is moved to the back. A complementary shutter built in the stand prevents laser light from reaching the eyepieces during the scanning mode.

– The illuminator support is equipped with a rotary polarizer. The Axio Observer.Z1 description contains the adjustment for the DIC mode during conventional observation.

– For scanning in the transmitted-light DIC mode, the polarizer in the transmitted light support works like an analyzer and must be adjusted in such a manner that direct laser light will be blocked.

– The conventional analyzer slider in the stand must not be located in the beam path because the laser light is already polarized.

– A fully motorized, LSM 5 software-controlled switching mirror is mounted on the illuminator support. Alternatively, the light is directed to the LSM 5 transmitted-light detector or enables conventional transmitted-light observation.

– The focusing screen for conventional transmitted-light is located in a support in front of the halogen lamp housing.

– Further information on the halogen lamp and the condensers is provided in the Axio Observer.Z1 operating manual.

(4)   Reflected light fluorescence

With the exception of the reflector slider, all the Axio Observer.Z1 fluorescence accessories can be used.

Further information is provided in the Axio Observer.Z1 operation manual.

(5)   Imaging optics

Optovar sliders cannot be used.

The analyzer for the conventional DIC mode will be operated from the right side and is located just below the nosepiece.

Use of sliders with auxiliary objects (473704/14-0000-000) is not possible.

(6)   Photo equipment

The stand does not feature an integrated SLR-port, but microscope cameras as described in the Axio Observer.Z1 and LSM 510 / LSM 510 META operation manual can be used.

(7)   Camera adaptation

The camera port at the side and the tubes can be used as described in the Axio Observer.Z1 operation manual.

The camera interface side port can be used with camera adapters 60 N or LSM adapters.

4.2.4   Computer Hardware and Software

The LSM 510 and LSM 510 META are controlled via a standard high-end PC. Linking to the electronic control system is made via Gigabit Ethernet interface. The PC comes with the WINDOWS XP or WINDOWS Vista operating system.

The instrument is fully motorized, permitting fast change-over between methods as well as automatic operation. Parameters once set or complex examination sequences once established can be saved and reproduced; therefore, complete application programs can be loaded and performed by pushbutton control.

Conversion of the light signals into a digital image is effected by means of four 12-bit A/D converters, each of which can generate 4096 brightness levels.

The software provides an enormously wide range of image processing functions, including all standard 2D/3D (stereo, projection) functions identical to sophisticated 3D reconstruction capabilities (surface and alpha rendering), digital processing of voxels and 3D measurement functions (surface areas, volumes).

4.3 Startup and Shutdown of the LSM 510 / LSM 510 NLO System

4.3.1 Startup of the System

The LSM system is equipped with a main power switch and two further switches labeled **System/PC** and **Components** that are located at the front of the System Electronic Rack. The main switch has to be set to **ON** to be able to switch the system on and off using the **System/PC** and **Components** switches. The main switch can be used to switch off the complete system with one switch only. The electronics to run the computer and the microscope are switched on with the **System/PC** switch. The laser module and the scan head are switched on with the **Components** switch. These switches are also accessible via the power remote switch (Fig. 4-2).

The "Enterprise" UV laser has to be switched on separately.

Refer to the operating manual and CHAPTER ANNEX of the printed manual for handling of any Titanium-Sapphire-Laser used for Multiphoton (NLO) Microscopy.

☞ If the UV laser shall be used, it can be switched on after the start of the WINDOWS operating system - but must always be switched on before ZEN software is started.
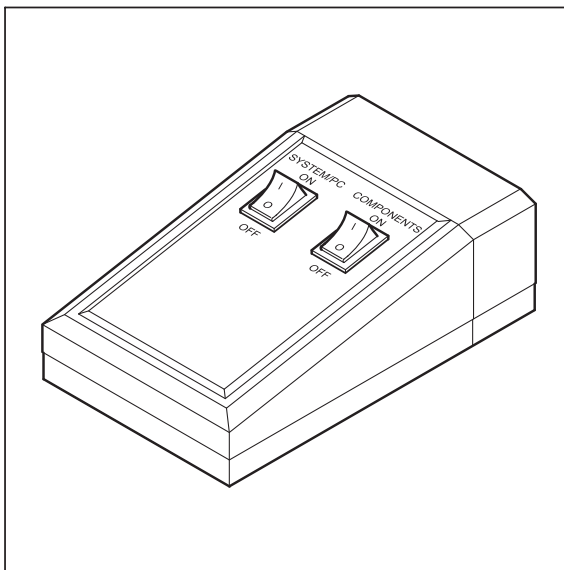
Fig. 4-2 **REMOTE CONTROL switch**

- When set to **ON** the REMOTE CONTROL switch labeled **System/PC** provides power to the computer. This allows using the computer and ZEN software offline.

☞ The drives for floppy discs and CD/DVD of the computer must not contain any data storage item.

- To switch on the system completely put the **Components** switch also to **ON**. Now the complete system is ready to be initialized with the LSM Software.

- After switching on the computer type in the user name and password to log on to the computer.

- After entries, confirm by clicking the **OK** button or **Enter**.

- The WINDOWS operating system desktop appears on the screen, showing a number of icons.

Change Filters icon

The **Change Filters** tool is used to update the filter data in the software after a change of filters in the reflector turret or scanhead. See printed manual CHAPTER 6.6.

Change Filters

Stand Select icon

The **Stand Select** tool permits a new or updated database to be assigned to the ZEN software program. This function should preferably be performed by authorized service personnel. See printed manual CHAPTER 6.3.

Stand Select

ZEN icon

Start ZEN software for operating the LSM laser scanning microscopes.

4.3.1.1 Switching on the Enterprise UV Laser

- If the UV laser is required, switch it on via the toggle switch (Fig. 4-3/**1**) of the power supply.
- It will be ready for operation after a few seconds.
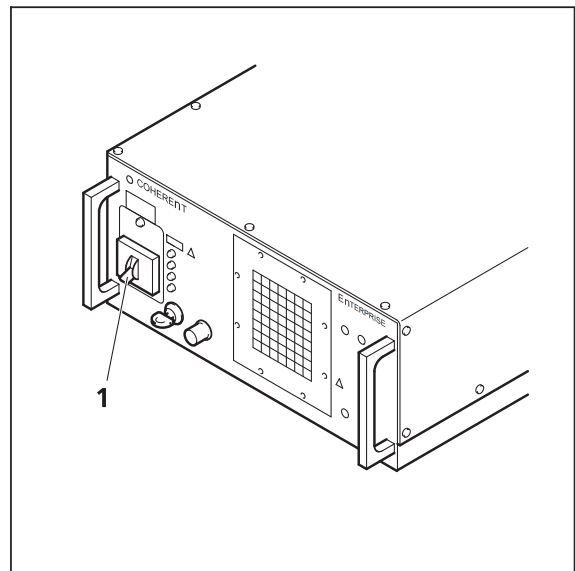
Fig. 4-3   Power supply of UV-Ar laser

a)

b)
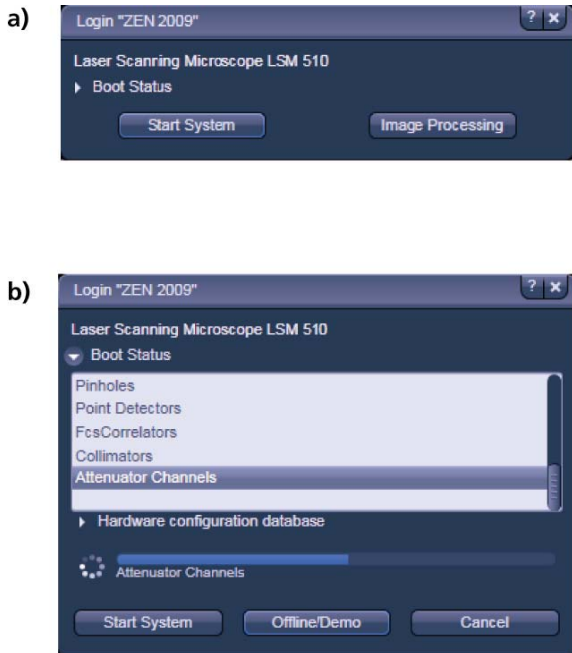
Fig. 4-4   **Starting the ZEN software**

4.3.1.2   **Starting ZEN**

The ZEN software is started by double clicking the ZEN icon. The login panel appears on top of the ZEN Main Application window (Fig. 4-4). The software can be operated in three different modes.

– Choosing Start System initializes the whole microscope system and activates the entire software package (image recording and analysis).

– The Image Processing mode ignores the hardware and activates only data handling and image processing functionality to analyze stored images.

– The Offline/Demo mode reads the active hardware database and simulates the respective hardware. This button is only available after opening the boot status display by clicking the little arrow left of the text "Boot Status" (Fig. 4-4/**b**).

4.3.2   Shutdown Procedure

⚠ Never shut down the computer by its main switch while the ZEN software is still active, or else you will lose the currently set operating parameters and the images just scanned.

☞ In the **Maintain** tab / **LSM Options** dialog window, activate **Laser off** on **Exit** in the **Shutdown** tab (section 4.5.11.3). The lasers will then automatically be switched off when you exit the LSM program.

4.3.2.1   Exiting ZEN Software

- Make sure you have saved all your image data.
- Switch of all the lasers which might still be running.
- Close the ZEN software window. This terminates the ZEN software.
- Shut down WINDOWS XP
- About 20 seconds after WINDOWS XP is shut down your computer turns off.

4.3.2.2   Switching System Power Off

☞ Please bear in mind that a cooling phase of at least 5 minutes is required between switching off of the lasers via the software and switching off of the entire system via the power remote switch or the Power Supply switch.

⚠ **5 minutes after computer shutdown**, set the power remote switches **System/PC** and **Components** or the **Main** switch on the System Electronic Rack to position **OFF**.
This puts your LSM 510 / LSM 5 MP microscope system, including the computer, off power.

4.4 Introduction to the Software Application Layout

4.4.1 Overview on the Screen Layout

A Application Bar
B Menu Bar
C Main Toolbar

D Left Tool Area

E Center Screen Area
(hosts up to 3 Image Containers)

F Right Tool Area

G Status Area

Fig. 4-5 ZEN Main Application window after Startup with empty image container

1 Main Tooltabs
2 Configurations
3 Action Buttons
4 Multidimensional tools selection panel
5 Starting multidimensional experiments
6 Toolgroup
7 Tool
8 Status Bar
9 View Tabs
10 Image Tabs
11 Displayed image
12 File Handling Area
13 View Controls
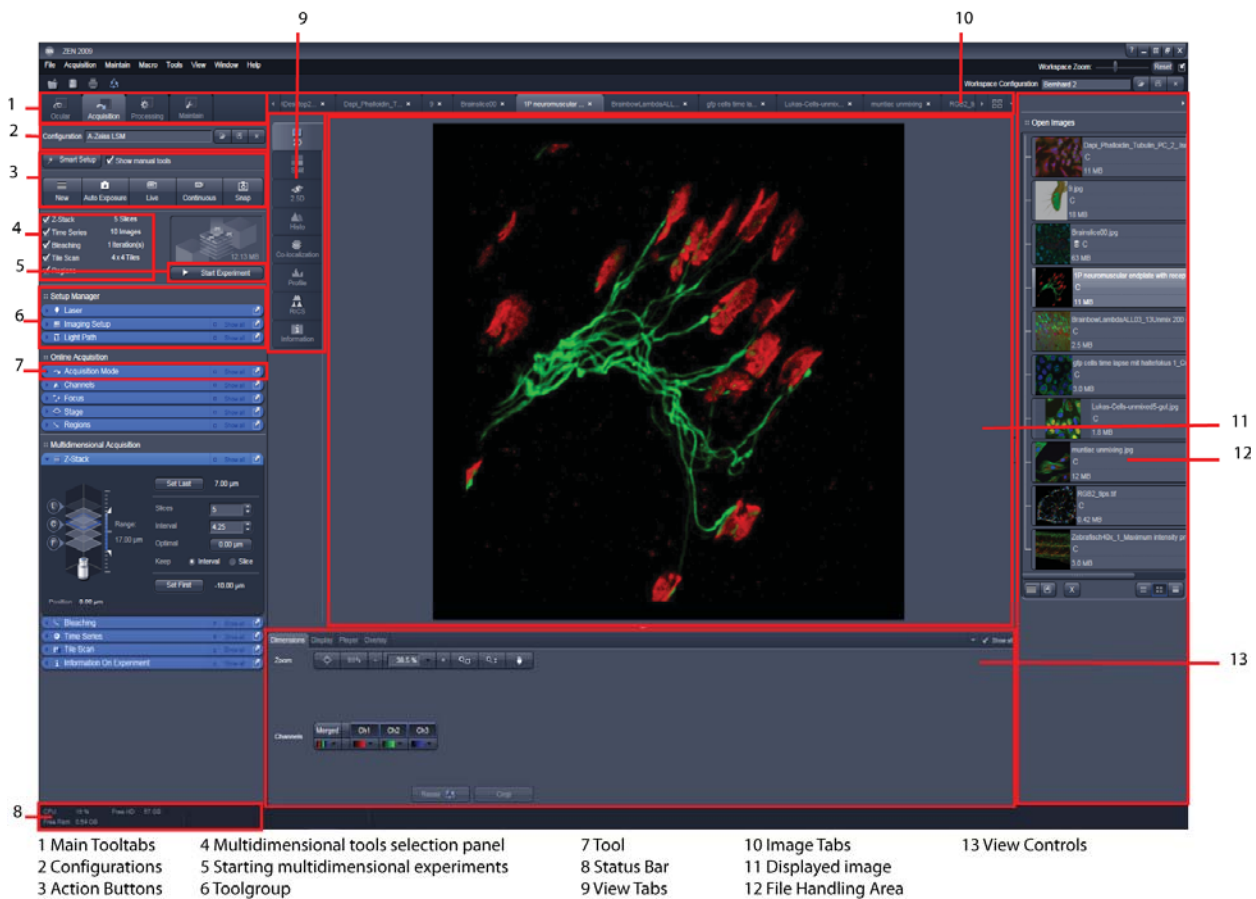
Fig. 4-6 ZEN Main Application Window after Startup with several images loaded

4.4.2   Introduction to ZEN

ZEN - Efficient Navigation - is the new software for the LSM Family from Carl Zeiss. With the launch of this software in 2007 Carl Zeiss sets new standards in application-friendly software for Laser Scanning Microscopy.

The ZEN Interface is clearly structured and follows the typical workflow of the experiments performed with confocal microscopy systems:

On the **Left Tool Area** (Fig. 4-5/**D**) the user finds the tools for image acquisition, image processing and system maintenance, easily accessible via 4 **Main** tabs (Fig. 4-6/**1**). All functions needed to control the microscope can be found on the **Ocular** tab, to acquire images use the **Acquisition** tools (Fig. 4-6/**3** and **4**). Arranged from top to bottom they follow the logic of the experimental workflow. The area for viewing and interacting with images is centered in the middle of the **Main Application** window: the **Center Screen Area**. Each displayed image can be displayed and/or analyzed with many view options available through "view tabs" which can be found on the left side of the image. According to the chosen view tab, the required view controls appear in View control blocks below each image. File management and data handling tools are found in the **Right Tool Area** (see Fig. 4-5 and Fig. 4-6).

Color and brightness of the interface have been carefully adjusted to the typical light conditions of the imaging laboratory, guaranteeing optimal display contrast and minimal stray light for high-sensitivity detection experiments. The **ZEN** software is optimized for a 30" TFT monitor but can also be used with dual-20" TFT setups.

A focus in the development of **ZEN** was to fulfill the needs of both basic users and microscopy specialists. Both types of users will appreciate the set of intuitive tools designed to make the use of a confocal microscope from Carl Zeiss easy and fast:
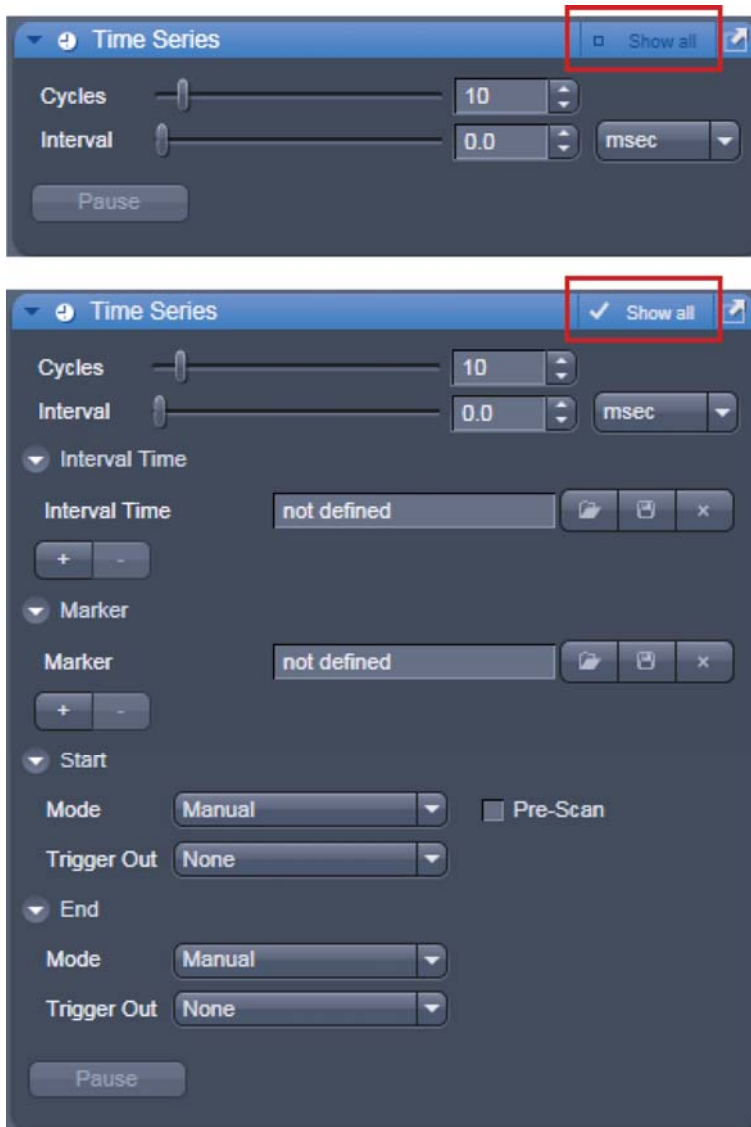
Fig. 4-7   **Show all mode**

The **Show all** concept ensures that tool panels are never more complex than needed. In the basic mode of the tools **Show all** is deactivated and the tools show only the most relevant functions, covering approximately 80 % of the users application. For each tool, the user can activate **Show all** to display and use additional functionality (Fig. 4-7).
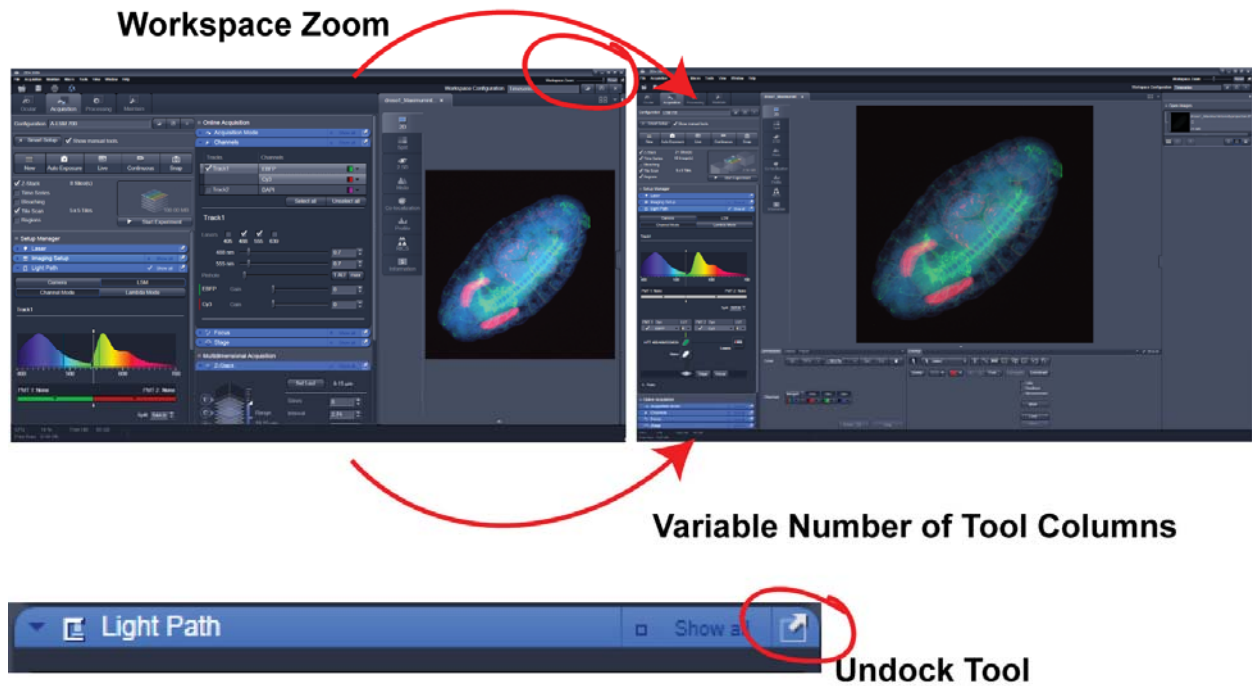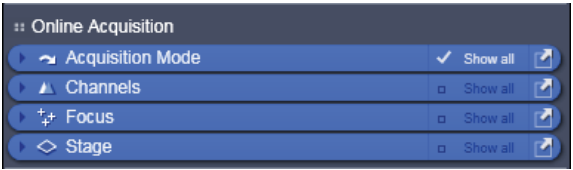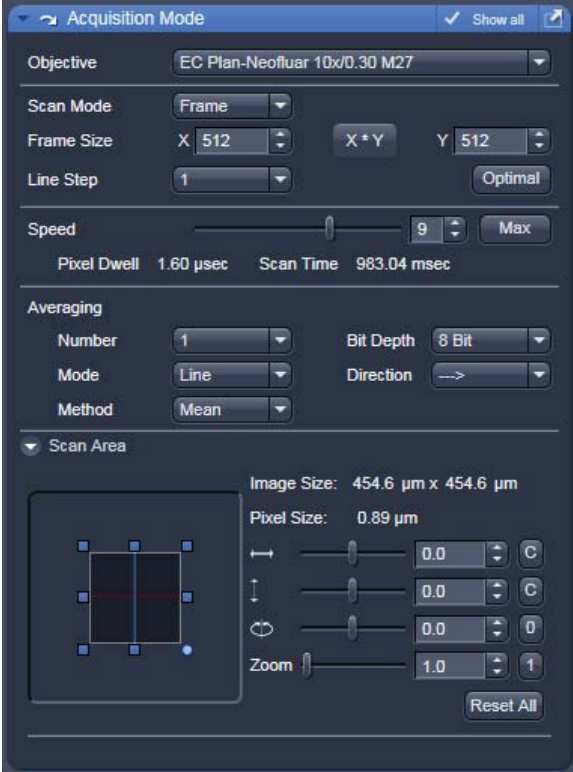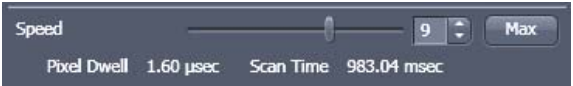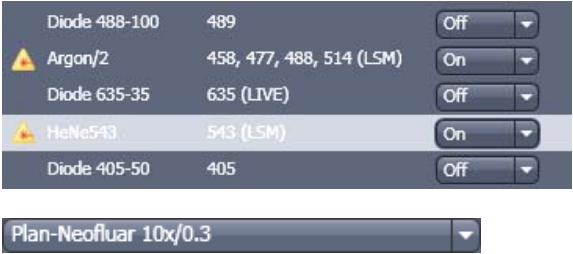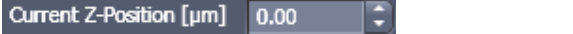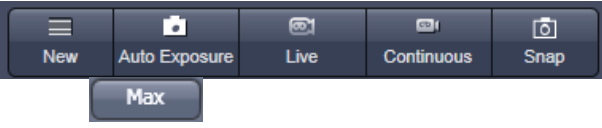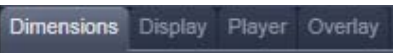
Fig. 4-8   ZEN Window Layout configuration

More features of **ZEN** include:

– The user can add more columns to the **Left Tool Area** or detach individual tools to position them anywhere on the monitor. To add a column, drag a **tool group** by the title bar (e.g., "Online Acquisition") to the right and a new tool column automatically opens. To detach a tool, click on the little icon on the very right end of the blue tool header bar or drag it by this blue header bar to where you want it (Fig. 4-8).

– Another unique feature in Imaging software is the scalable ZEN interface. This **Workspace Zoom** allows adjustment of the **ZEN** window size and fonts to the situational needs or your personal preferences (Fig. 4-8).

– Setting up conventional confocal software for a specific experiment can take a long time and is often tedious to repeat. With ZEN these adjustments have to be done only once – and may be restored with just two clicks of the mouse. For each type of experiment one can now set-up and save the suitable **Workspace Layout**. These configurations can also be shared between users.

– For most controls, buttons and sliders, a **tool tip** is available. When the mouse pointer is kept over the button, a small pop up-window will display which function is covered by this tool/button.

4.4.3 Function Elements

Function element	Description / Explanation
	Tool group
	Tool (closed) or tool window (opened)
	Panel (e.g.: **Speed** panel) – Field with a subset of tools of a tool window
	List box or selection box – Selection of one of the displayed options via a mouse click. The selected option is highlighted. – Open the box by clicking on the arrow button.
	Input box with arrows ("spin box") – Input of text or numeric values via the keyboard or using the arrows.

Pinhole [slider] 59.0	Slider with input ("spin") box and arrows – Setting of numbers in the relevant input box by moving the slider or clicking on the arrow buttons or clicking on the slider and moving via the arrow keys of the keyboard. Press the **Shift** or **Ctrl** key while clicking on the arrow button to change the numeric values in coarse or fine steps.
New — Auto Exposure — Live — Continuous — Snap — Max	Button – Selection / performance of a function via mouse click.
Dimensions Display Player Overlay	Tab – Selection of functions of a tab via mouse click (e.g.: **Track1** or **Track2** tab).
[open] [save] [×]	**Load** – **Save** - **Delete** – Buttons to load, save or delete a configuration, overlay set, track setting etc.

4.4.4 Application Bar

The application bar includes the following control elements of the ZEN software application window:

minimizes the application window

switches between one-screen mode and two-screen mode of the window

switches between maximized-window mode and compressed-window mode

closes the window

4.4.5 Menu Bar

The menu bar contains the menu items and the controls for the **Workspace Zoom**, including the **Reset** button for the Workspace Zoom and the button to re-dock all free floating tools to the **Left Tool Area**.

File Acquisition Maintain Macro Tools View Window Help
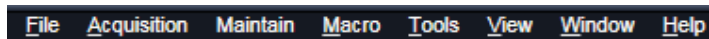
Fig. 4-9   Menu bar, left side

The menu functions are shown in the following menu overview (see next page).

Workspace Zoom

Moving the slider to left or right, changes the display size of the Left/Right Tool Area and the Center Screen Area.

Clicking the **Reset** button, sets the standard zoom factor for all areas.

Workspace Zoom: ⸺⎜⸺ Reset

Fig. 4-10   Menu bar, right side

Clicking on moves all undocked tools back to the **Left Tool Area** (see also section 4.4.8.1).

Menu overview

File	Acquisition	Maintain	Macro	Tools	View	Window
New Acquisition Document Opens a new and empty image document	**New Acquisition Document** Opens a new and empty image document	**Set Spline** Opens the dialog for setting the spline parameters	**Macro** Opens the Macro Control window for creating, loading or handling macros	**VSTA IDE**	**Text View** Displays the image tabs with text only	**Close** Closes the active window

File

New Acquisition Document — Opens a new and empty image document

Open — Opens an existing document in the active image container

Save — Saves the active document

Save As — Saves the selected document using various image formats

Export — Exports the currently active document using various file formats

New File browser — Opens a document containing a ZEN File Browser.

Login — Opens the Login ZEN dialog to change the software operation mode.

Spectra Database — Opens the spectra database

Exit — Closes the ZEN Software

Acquisition

New Acquisition Document — Opens a new and empty image document

Auto Exposure — Starts the Find procedure. This balances Gain settings.

Find Focus — Starts the Autofocus. A Line / Z Scan is performed and the focus position is determined upon contrast values.

Live — Starts the fast scanning procedure, scans with lower resolution and higher speed.

Continuous — Scans an image continuously with the currently active scanning parameters.

Snap — Scans one single image.

Start Experiment — Starts a multidimensional experiment (Z, T, ….).

Stop — Stops the scanning immediately

Maintain

Set Spline — Opens the dialog for setting the spline parameters

Camera — Opens the Camera Color Adjustment tool

Hardware Adminsitrator

Test Grid

Macro

Macro — Opens the Macro Control window for creating, loading or handling macros

Visual Macro Editor — Opens the Visual Macro Editor

Tools

VSTA IDE

VSTA Macros

View

Text View — Displays the image tabs with text only

Small Thumbnail View — Displays the image tabs with small thumbnails

Large Thumbnail View — Displays the image tabs with large thumbnails

1 Container — Sets ZEN to work only with one image container

2 Containers — Sets ZEN to work with two image containers

3 Containers — Sets ZEN to work with three image containers

Automatically layout container — The size of the containers is automatically adjusted depending on the screen space available

Shared view Controls — Shares the view controls below the images between containers

Separate view Controls — Each container is displayed with a separate view control panel

"Show all" (global) — Sets all windows, tools and control panels to "show all", all controls are always visible in all tools

Window

Close — Closes the active window

Close all — Closes all windows, a separate save dialog window will be displayed if images have not been saved

Help

Help — Opens the ZEN help

About — Opens a panel with information about the ZEN release

4.4.6   Main Toolbar

1 Open image button
2 Save image button
3 Print image button
4 Reuse button

Fig. 4-11   Main toolbar, left side

1 Workspace Configuration selection box
2 Load Workspace Configuration button
3 Save Workspace Configuration button
4 Delete Workspace Configuration button
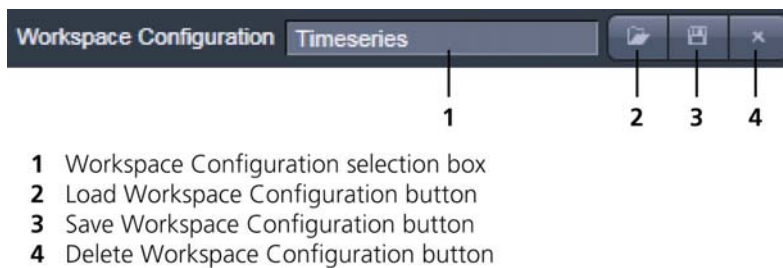
Fig. 4-12   Main toolbar, right side

Workspace Configuration

This function allows loading, saving or deleting a workspace configuration. These workspace configurations are saved settings that allow to restore a pre-defined layout of the whole ZEN application window, including status, size and position of tools and windows, workspace zoom, number of tool columns.

- To save a workspace configuration, click on the button, the following dialog will appear:
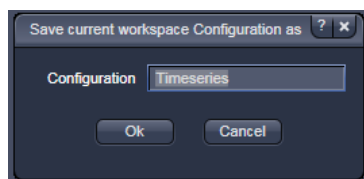
The name of the workspace configuration can be entered. Clicking **OK** saves the configuration.

- To load a workspace configuration, click on the [image] button. The following list box will appear:
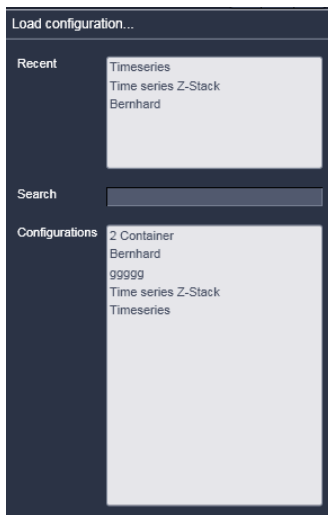
Selecting on of the list entries will load the respective configuration.

- To delete a workspace configuration, click on the [image] button. The configuration to be deleted can be selected from a list.

4.4.7　Status Bar

1　Progress bar
2　System information
3　Position and pixel intensity
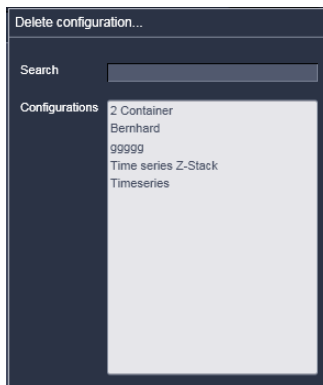
Fig. 4-13　　**Status bar**

Progress bar

Shows an overview of all running processes. If only one process is running the details of this one are shown. If more then one process is running then the single process information will be shown in the gallery.

Fig. 4-14　　**Progress bar**

System information

Shows information of the currently system tasks (state). The possible states are:

– CPU usage

– free hard disc capacity

– free RAM capacity

Position and pixel intensity

Shows the intensity values of the existing channels for the current X, Y and Z position of the mouse cursor in the image.

4.4.8 Left Tool Area

Main Tool tabs

Switches between the **Ocular**, **Acquisition**, **Processing** and **Maintain** main tools to operate the included tool

Action buttons

Action buttons are only available for the **Acquisition Main** tool to control the image acquisition process.

Tool groups

Groups special tools, e.g. **Setup Manager** or **Online Acquisition**.

Setup Manager

These tools provide access to the lasers and the hardware configuration of the light path.

Online Acquisition

Tools continuously needed for all acquisition.
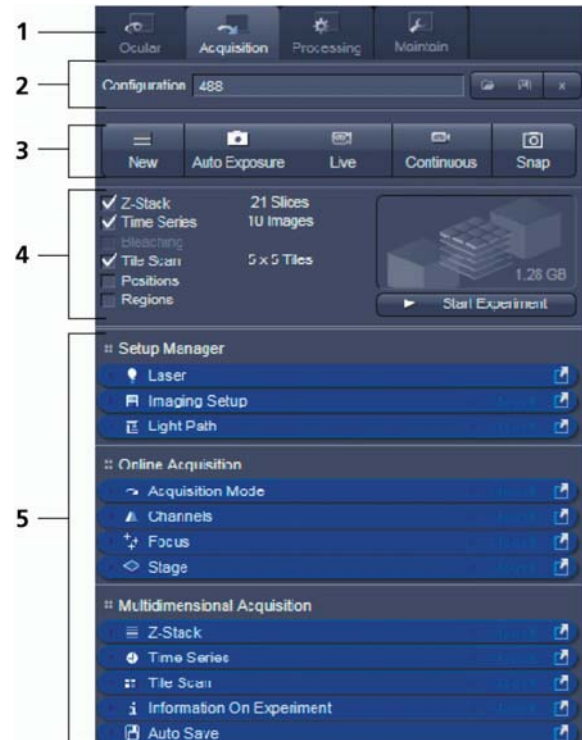
1   Main tool tabs
2   Imaging Configuration
3   Action buttons
4   Selection panel for multidimensional acquisition
5   Tool groups

Fig. 4-15   Status bar

Multidimensional Acquisition:

Tools for all multidimensional imaging

The tools of this tool group will only appear if selected in the selection panel below the start buttons. Once selected by the check box, the tool will appear in the multidimensional acquisition tool group. The tool is then active and will be used after hitting the **Start Experiment** button. The experiment type is represented above the button by a graphic.
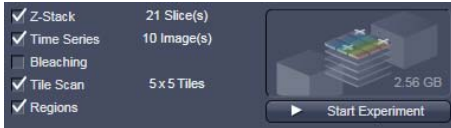
Tools / tools window

Used for setting the microscope and software functions.

Imaging Configuration
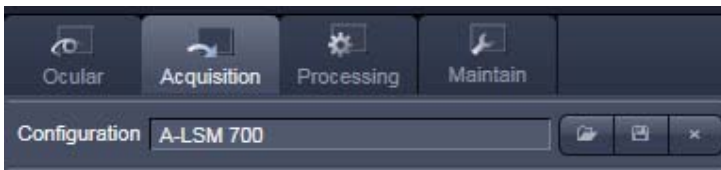
This function allows selecting an imaging configuration. The configuration can be chosen in a list box. Clicking on a configuration loads the selection.
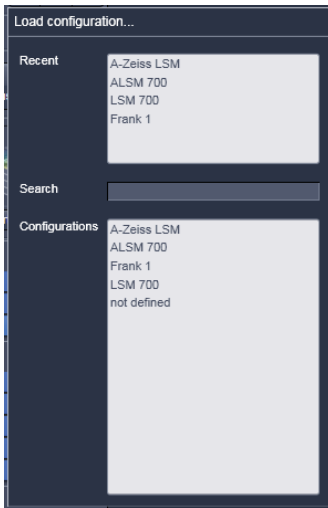
4.4.8.1   Tool Groups and Tools

Multiple Columns Layout of tool groups

This function allows moving one or several **tool groups** in a second or third column of the **Left Tool Area**. This is done by dragging and dropping the **tool groups** header. The second column opens automatically if a group is moved and closes if all groups are removed from it.

Undock function of tool windows

This function allows undocking and moving one or several tool windows from its home position in the Left Tool Area and further drags it to any position on the Monitor. This can be done by clicking the ![] button. The tools can also be undocked by simply dragging the header bar of the respective tool to the desired position. To place a tool back to its home position, click the ![] button again, drag the tool back to the **Left Tool Area** or click on the grey place holder at the home position of the tool. The last option is particularly useful if one ever gets a too crowded screen and can't find the tools any more. To float all tools back with one click use the ![] button in the top right corner of the menu bar.

To position the tool window freely on the desktop, use the drag and drop function.

Open a tool window

Clicking on the tool header opens or closes the tool pull down window. The state is indicated by a changing icon (![], ![]) left hand in the tool window header.

If a tool is undocked from the **Left Tool Area** (float function) it will be opened automatically.

Show all function of the tool window

Clicking on the ![Show all] button shows or hides additional functions of the tool.

![Show all]   Only the basic functions are shown; this functionality is adequate for most applications.
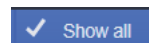
![Show all]   All the functions are activated. The full functionality of the tool is available.

Activation of tools with respect to a multidimensional image acquisition functions of the tool window

The tools for multidimensional image acquisition can be activated and selected by ticking the respective tick boxes in the selection panel:
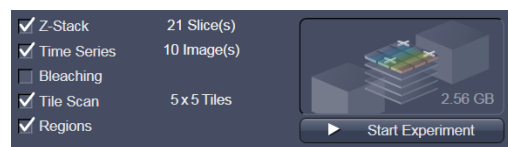
Clicking on the ![] check box of the appropriate tool selects, activates and displays the tool for multidimensional image acquisition. Once selected by the check box, the tool will appear in the multidimensional acquisition tool group. The tool is then active and will be used after hitting the **Start Experiment** button. The experiment type is represented above the button by a graphic.

4.4.8.2   Context Menu of the Left Tool Area

There are 2 different context menus in the Left Tool Area with different functionalities available.
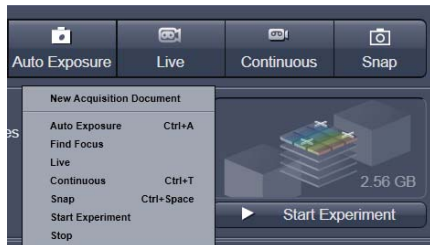
Fig. 4-16   Context menu of multidimensional acquisition area

New Acquisition Document opens an empty acquisition document in the active image container.

Auto Exposure starts the Find procedure. This balances Gain settings.

Find Focus starts the Auto focus. A Line – Z Scan is performed and the focus position is determined upon contrast values.

Live starts the fast scanning procedure – scans with lower resolution and higher speed.

Continuous scans an image continuously with the currently active scanning parameters.

Snap scans one single image.

Start Experiment starts a multidimensional experiment (Z, T, ….).

Stop stops the scanning immediately
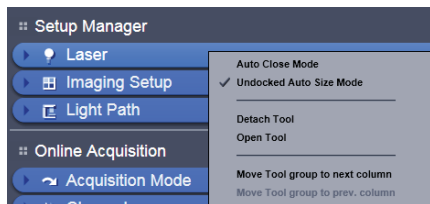
Fig. 4-17   Context menu of the tool groups area

Auto Close Mode means that the "oldest" open tool window will be closed automatically if opening a new one leads to a full tool column.

Right mouse click in the Left Tool Area opens a context menu to activate or deactivate the **Auto Close Mode**. A de-activated **Auto Close Mode** will lead to a scrollbar on the right hand side of the Left Tool Area, once a tool column is filled with open tool.

Undocked Auto Size Mode if active, detached tools are automatically re-sized. If deactivated, a scrollbar appears inside detached tools.

Detach Tool will open and detach the tool from the tool column.

Open Tool opens the tool inside the tool column.

Move tool group to next column shifts an entire tool group to the adjacent column.

4.4.9   Center Screen Area

The Center Screen Area is used for displaying scanned images or to show images in the available image views. Using the context menu of the Center Screen Area by clicking the background of a container, the view of this area can be varied individually.

The Center Screen Area can be split in a 1, 2 or 3 container view. So, it is possible to show several images in parallel.

Images can be moved from one container to another using the drag-and-drop function.

4.4.9.1   Overview of the Center Screen Area
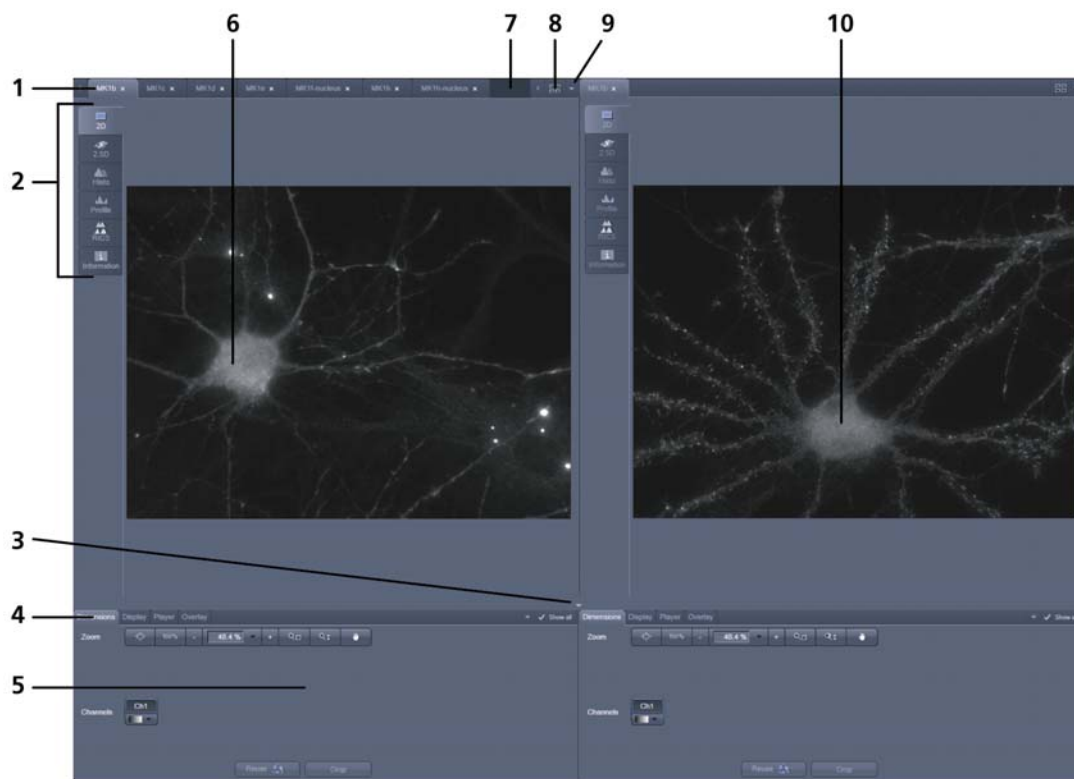
1 Image tabs with scroll arrow buttons left and right of container 1
2 Image view tabs of container 1
3 Arrow down or arrow up button to hide or show the image control area
4 Image view controls
5 Image control area
6 Image container 1 (with opened image window)
7 Background area of Container 1
8 Expose Mode button
9 Arrow down button for fast image selection
10 Image container 2

Fig. 4-18   Center Screen Area
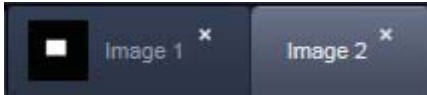
Fig. 4-19   **Image tabs**

Image tabs

For each opened image one image tab is shown in the header of the actual container. Three modes can be selected using the context menu of the **Center Screen Area** (see also section 4.4.9.2).
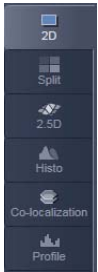
Fig. 4-20   **Image view tabs**

Image view tabs

Used to select one of the various image views (see also sections 4.6.2 to 4.6.18).

Arrow down or arrow up button to hide or show the image control area

Clicking on the arrow down button hides the image control area and increases the image view field of all containers in vertical direction for a larger image display. Clicking the arrow up button shows the image area in all containers once again.

Image view controls

Activates one or more of the various image display and analysis functions (see also section 4.4.9.2).

Image control area

Shows the tools of the selected image display or analysis functions.

Fig. 4-21   **Image control area**
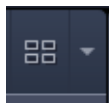
Expose Mode

Changes the one image display to the Expose display. Clicking on one of the image headers changes back to the one image display.

The main purpose of this function is to quickly find an image that might be only one of many open images which are all represented by image tabs on the top. Especially if the image tabs are configured to display only the image name

Arrow down button

Clicking on this button shows a list with all opened images for fast image selection.

Using the drag and drop function, one or several images can be moved into another opened container. If closing a container, the included images will be moved automatically to another open container.

4.4.9.2   Context Menu of the Center Screen Area

Clicking the right mouse button on the background of the container opens the context menu of the Center Screen Area.

The following functions are available:

Display of the image tabs in the container header

Three options are available:

– Text View

– Small Thumbnail View

– Large Thumbnail View
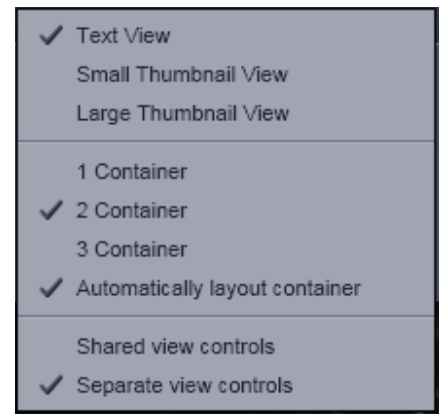
Fig. 4-22   Context menu

Number of opened containers

Up to three containers can be opened in the Center Screen Area.

Shared or separate view controls

The display of the Center Screen Area can be changed between shared view controls mode and separate view controls mode.

Separate view controls shows a set of view control tabs for each container. In shared view controls mode, only one set of view control tabs is displayed, spanning the whole bottom area under all 2 or 3 containers. They always apply to the active image/container
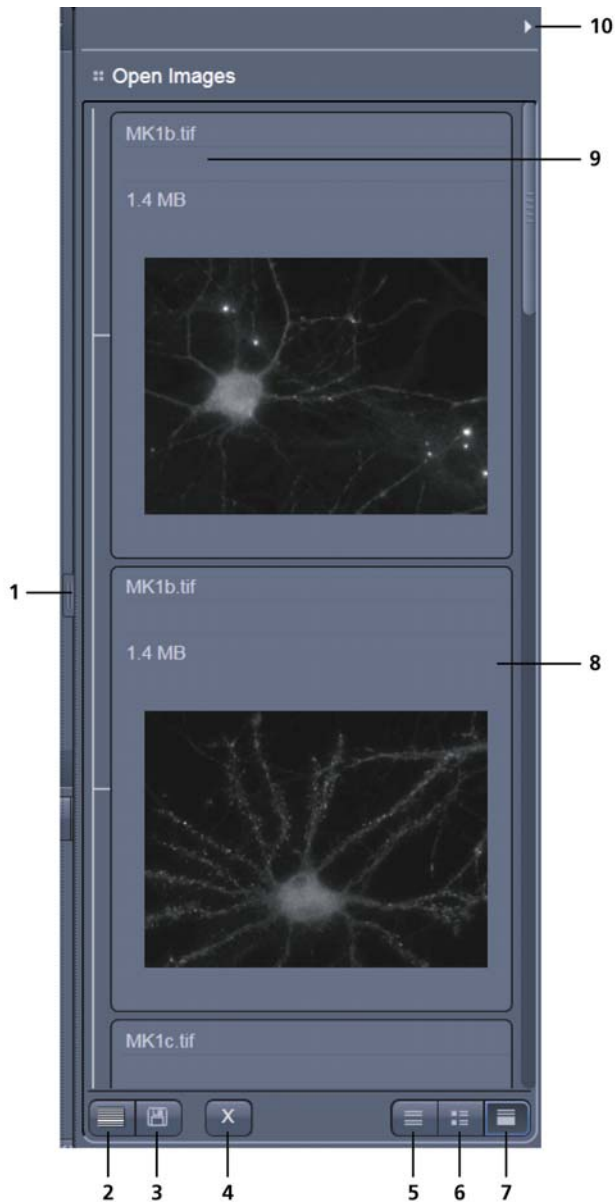
1 Expand slider
2 Save button
3 New image document button
4 Close button
5 Textual View button
6 Thumbnail View button
7 Big View button
8 Save Status icon
9 Image information
10 Hide button

Fig. 4-23   Right Tool Area

4.4.10   Right Tool Area

The Right Tool Area is used for displaying and handling the opened images, e.g. save or close. The view of this area can be varied individually, e.g. expand or hide the area, change between the view modes.

Expand slider

Dragging this slider to the left side expands the Right Tool Area by decreasing the center tool area.

Dragging the slider to the right side decreases the Right Tool Area up to the default size.

Save button

Saves the selected scanned or changed image. The **Save as** dialog appears.

New image document button
Opens an empty image document in the active container.

Close button

Closes the selected image. The **Close image** dialog appears to close the image with or without saving or to cancel the closing.

Textual View button

Shows images only with the textual characters.

Thumbnail View button

Shows images with the textual characters and an image thumbnail preview.

Big View button

Shows images with the textual characters and a big image preview.

Save Status icon

This icon appears in the image that is not saved yet.

Image information
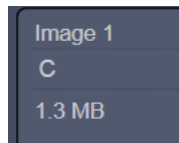
Displays the name, type and file size of the image.

Hide button

Clicking this button hides or displays the Right Tool Area.

4.5   Left Tool Area and Hardware Control Tools

The Left Tool Area contains all tools for system operation, image acquisition, image processing and maintenance. The functions are organized in the three Main tool tabs **Ocular**, **Acquisition**, **Processing** and **Maintain**. This tool area always keeps its position on the monitor.
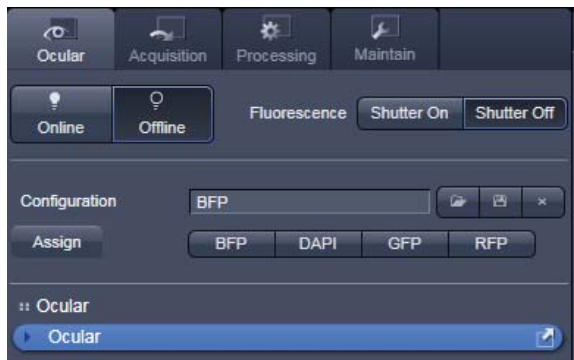
Fig. 4-24   Ocular tab

4.5.1   Ocular Tab

4.5.1.1   Basic Controls, Ocular Tool

On the **Ocular** tab, the basic switch between **manual operation** of the microscope (observation of the specimen with the eye) and **imaging** is located.

Pressing the **Online** button sets the Ocular tool active – the specimen can be observed and all changes in the ocular tool take effect immediately. When pressing the **Offline** button the system stays in the imaging mode. Changes in the ocular tool do only take effect once the Ocular tab is set in the active mode (**Online**) again. When leaving the Ocular tab the software remembers the state of the **Online** / **Offline** buttons.

Fig. 4-25   Configuration panel
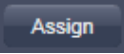
Pressing the **Shutter On** button opens a motorized fluorescence shutter if present on the system. Pressing **Shutter Off** closes this shutter.

All the settings of the tab can be stored in configurations. This includes all filters, attenuation devices, attenuation settings, shutters, and condenser settings but not the objective. Which can be stored, selected and deleted via list menus when the buttons load [ ], save [ ] and delete [ ] are pressed.

Fig. 4-26   Assign panel

These configurations can be assigned to max. 8 buttons below the configuration panel by clicking the assign [Assign] button. In the appearing dialog each button can be linked to any saved configuration. Using the delete button individual buttons can be removed from the interface.

In the **Ocular** tool, the microscope beam path is represented graphically. All the motorized components of the microscope stand can be controlled via interactive buttons and pull down lists.

4.5.1.1.1 Controls for Axio Imager

- Click on **Ocular** tab in the Left Tool Area. Click **Online** if needed.

- The currently set light path of the microscope is displayed.

- Depending on the level of motorization, the following parts can be controlled:

Reflected Light HBO Lamp	This function controls the reflected light illumination source, e. g. X-Cite 120. An additional dialogue opens which allows to open and close the lamp internal shutter and to attenuate the light intensity of the lamp via slider or input box.
Shutter Reflected Light	This turns the rear port mirror to guide the light to the specimen or to block it from the specimen.
Tube Lens	This function allows switching between available tube lenses. The appropriate lens is automatically set when switching between Ocular and LSM in the Main toolbar.
Reflector	Push and click reflector cube can be selected via graphical pop-up menu.
Objective	Objectives can be selected via graphical pop-up menu.
Condenser	Numerical aperture of the condenser is set via input box or slider. Turret position (filter) selected from graphical pop-up menu (only for motorized condensers).
Field Stop	Opening of luminous-field diaphragm (transmitted light) can be set via input box or slider.
Filter	Transmission values for attenuation filter (transmitted) are set via input box or slider for the front or rear filter wheel in accordance with the available filter steps.
Transmitted Light Lamp	Transmitted light is switched on / off via ON button in the Transmitted Light frame, setting of light intensity can be varied via input box or slider. 3200 K color temperature for photo documentation can be switched on via 3200 K button in the Transmitted Light frame.
Shutter Transmitted Light	This function opens or closes the internal shutter for transmitted light.
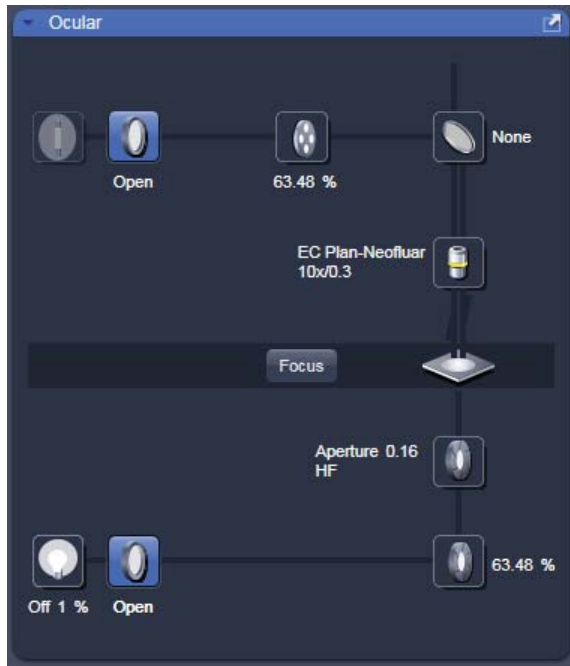
Fig. 4-27   **Axio Imager controls in the Ocular panel**

(1)   Microscope settings on Axio Imager for transmitted-light observation

- Set the reflector turret position to **None** and click the **On** button for transmitted light.

- Pull out the push rod (Fig. 4-28/**8**) to switch the beam path of the tube in LSM position.

- Control the brightness of the halogen lamp with the potentiometer (Fig. 4-28/**4**) or the **Intensity %** slider in the **Ocular** panel.

- Set the required transmission value of the gray filters in the **Filter** frame.

- Set the condenser and the luminous-field diaphragm for KÖHLER illumination.

The laser-scanning mode automatically occludes the halogen lamp even with **Transmitted Light** activated (**On**).

- Click on the **Ocular** tab in the Left Tool Area. Press **Online** if needed.

- Place specimen on microscope stage.

  – The cover slip must be facing up.

1 Switch HBO 100 power supply
2 RL button - reflected-light shutter ON/OFF
    with indicator
3 TL button - transmitted-light shutter ON/OFF
    with indicator
4 Light intensity control
5 Focusing drive
6 Control knob for X travel
    of mechanical stage
7 Control knob for Y travel
    of mechanical stage
8 Buttons for switching the beam path
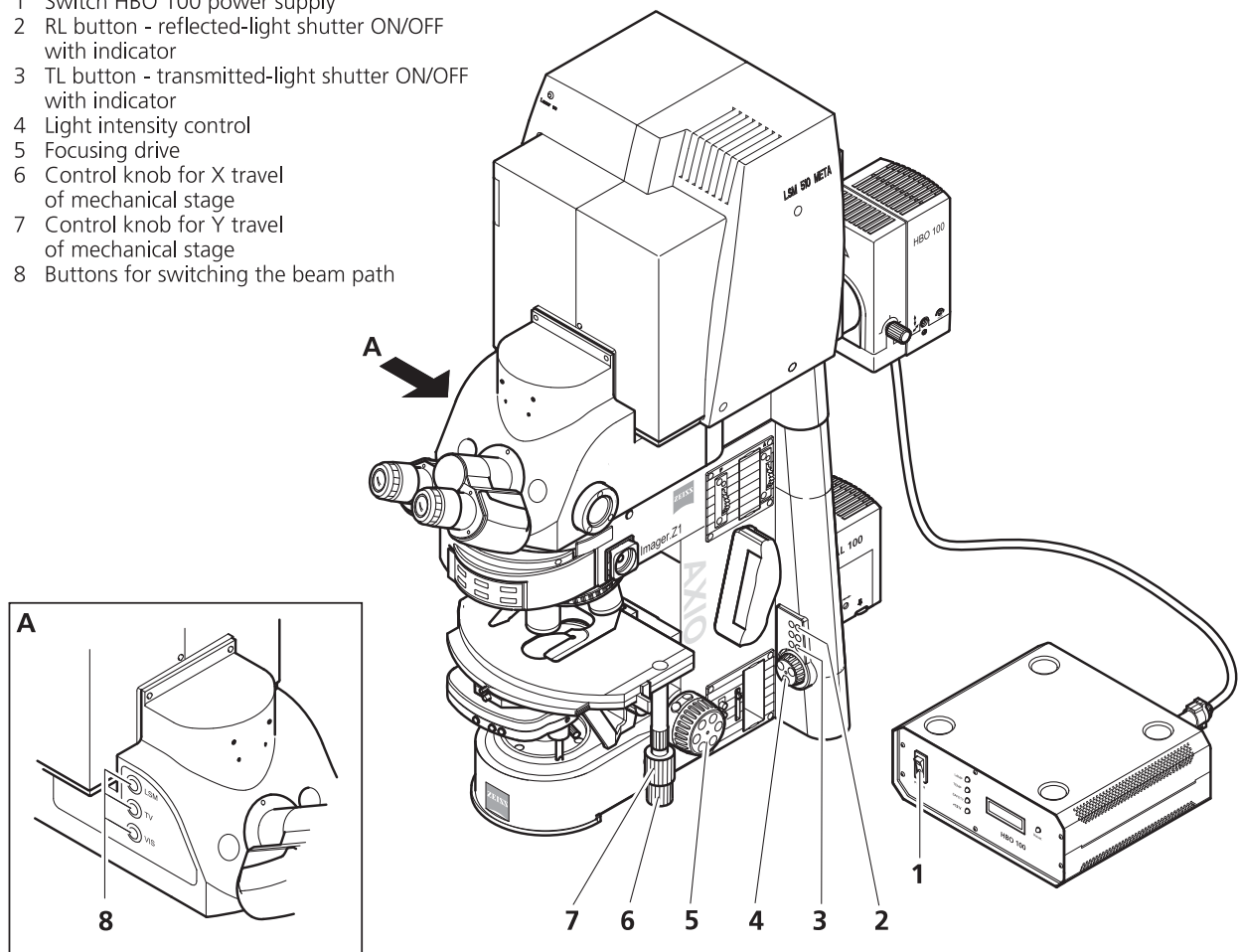
Fig. 4-28   LSM 510 with Axio Imager

- Via the **Objective** button, select the required objective as follows:

  – Open the graphical pop-up menu by clicking on the **Objective** button.

  – Click on the objective you want to select.

  – The selected objective will automatically move into the beam path.

- Use the focusing drive (Fig. 4-28/**5**) to focus the required object plane.

- Select specimen detail by moving the stage in X and Y using the XY stage fine motion control (Fig. 4-28/**6** and **7**).

(2)   Microscope settings on Axio Imager for reflected-light observation (Epi-fluorescence)

• Turn on the HBO 100 W or X-Cite power supply with switch (Fig. 4-28/**1**).

• Click on the **RL reflected light** button. The shutter opens.

To avoid excessive bleaching of biological samples, expose the specimen to the minimum possible irradiation, i.e. keep the irradiation time as short as possible. For this, use the slider in the Ocular panel to attenuate the illumination of the HBO / X-Cite.

• By clicking on the reflector turret button, select the reflector module (filter sets) to suit the type of fluorescence excitation. Proceed as follows:

• Click on the reflector turret button.

• Click on the desired reflector module.

– The reflector turret moves the selected reflector module into the beam path.

The FITC filter set consists of an excitation filter for the 450 - 490 nm spectral range, an FT color splitter for 510 nm and an LP long pass filter, which passes emission light wavelengths greater than 510 nm (FSET 09 = FITC, FSET 15 = Rhodamine, FSET 01 = DAPI).
Other filter sets:

DAPI: BP 365 FSET01
             FT 395
             LP 397

Rhodamine: BP 546 FSET15
             FT 580
             LP 590

The filter sets described in this section are examples; other sets are available on request.

The aperture setting on the condenser of the Axio Imager is performed in fixed steps.

4.5.1.1.2   Controls for Axio Observer

- Click on **Ocular** tab in the Left Tool Area.

- The currently set light path of the microscope is displayed.

- Depending on the level of motorization the following parts can be controlled:

Transmitted Light Lamp	Transmitted light is switched on / off via ON button in the Transmitted Light frame, setting of light intensity can be varied via input box or slider. 3200 K color temperature for photo documentation can be switched on via 3200 K button in the Transmitted Light frame.
Condenser	Numerical aperture of the condenser is set via input box or slider. Turret position selected from graphical pop-up menu (only for motorized condensers).
Objective	Objective can be selected via graphical pop-up menu.
Reflector	Push and click, reflector cube can be selected via graphical pop-up menu.
Tube Lens	Push and click, tube lens can be selected via graphical pop-up menu.
Shutter Reflected Light	This turns the rear port mirror to guide the light to the specimen or to block it from the specimen.
Reflected Light HBO Lamp	This function controls the reflected light illumination source X-Cite 120. An additional dialog opens which allows to open and close the lamp internal shutter and to attenuate the light intensity of the lamp via slider or input box (depending on the model of XCite connected).
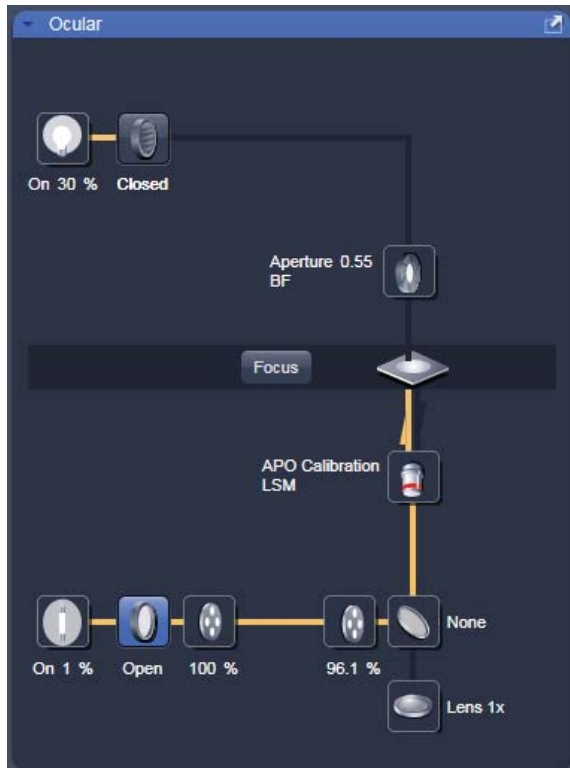
Fig. 4-29   **Axio Observer controls in the Ocular panel**

(1)   Conventional setting of the microscope Axio Observer

The Recording of microscope settings works as described for the microscope Axio Imager.

For the conventional setting of the Axio Observer, proceed as follows:

- Click the **Ocular** button in the **Main** toolbar.

- Place specimen on microscope stage.

  – The cover slip must be facing down.

- In the **Objective** list box, select the required objective.

- Use the focusing drive (Fig. 4-30/**4**) to focus the required specimen plane.

- Select specimen detail by moving the stage in X and Y via the XY stage fine motion control (Fig. 4-30/**3** and **2**).

1 Switch HBO 100 power supply
2 Stage fine motion control, X
3 Stage fine motion control, Y
4 Focusing drive
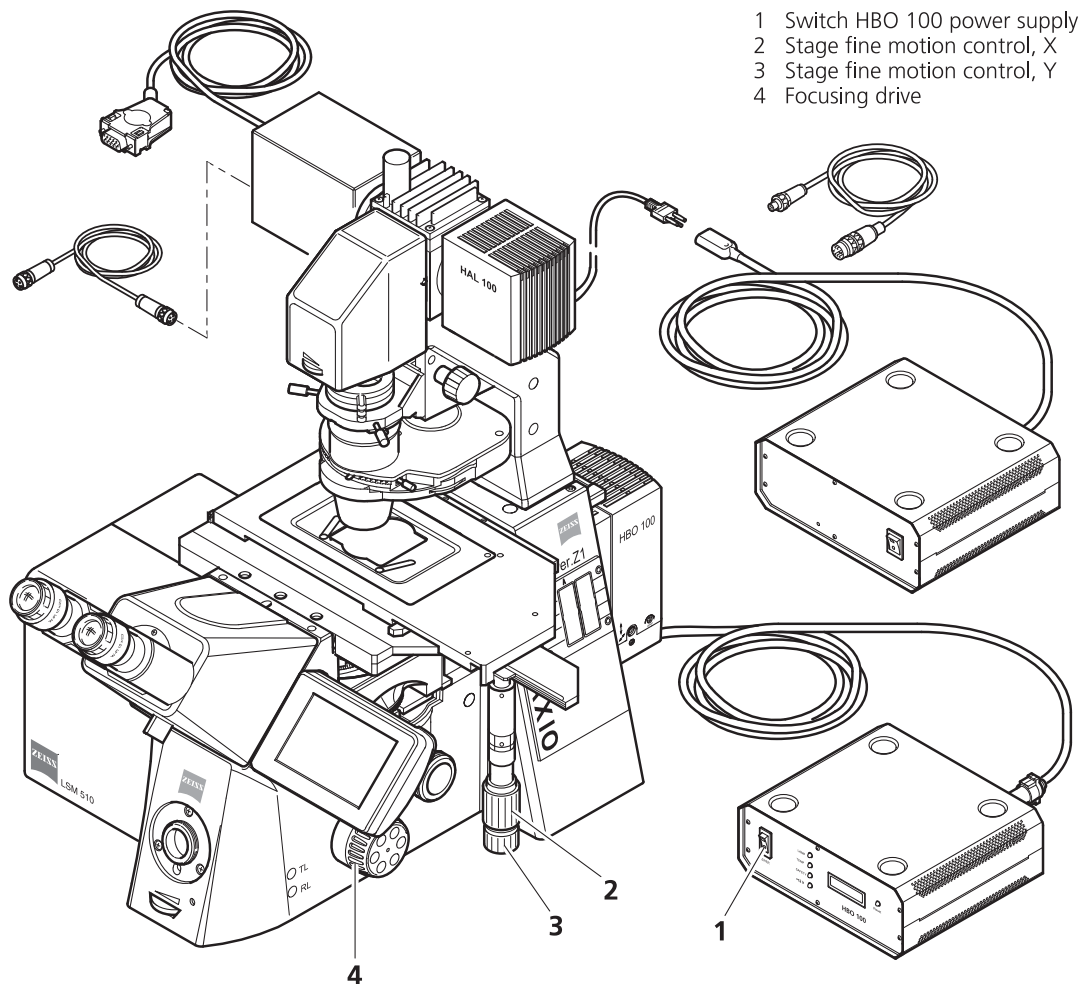
Fig. 4-30   LSM 510 with Axio Observer

(2)   Microscope settings on Axio Observer for transmitted-light observation

- Click on the **Transmitted light** button. Click the **On** button in the **Transmitted Light** panel and set the transmitted light intensity via the slider or click on **3200 K**.

- Click on the **Condenser** button and set the aperture via the slider in the **Condenser** panel. Set the filter in the **Filter** selection box.

- Click on the **Objective** button and select the objective by clicking on it.

- Click on the **Reflector** button and select **None**.

- Make sure there is no illumination from the reflected-light.

(3)   Microscope settings on Axio Observer for reflected-light observation (Epi-fluorescence)

- Turn on the HBO 100 power supply switch (Fig. 4-30/**1**).

- Click on the **Reflected Light** button and set the shutter to **Open**.

- Click on the **Reflector** button and select the desired filter set by clicking on it.

- The filter is automatically moved into the beam path to enable observation in epi-fluorescence.

- Click on the **Objective** button and select the objective.

- Make sure that the illumination from the transmitted-light is switched off.
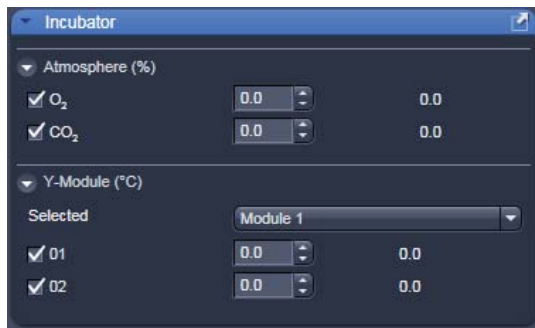
Fig. 4-31   Ocular tool, incubator control

4.5.1.2   Ocular Tool – Incubator Control

From ZEN 2009 the incubation settings can be set within the main software.

The settings for Temperature (Ch1 and Ch2) and CO_2 can be set by using the input boxes.

The measured values are displayed next to the input boxes.

(The availability of the tool depends on the type of incubator used on the system.)

4.5.2   Acquisition Tab

☞   The **Acquisition** tool tab hosts all tools for image acquisition. The content of this tab is specific for the hardware of the microscopy system.

If **ZEN** is started for image processing only (see section 4.3.1.2) this tab is not available. The same is true for the license free LE version of **ZEN**.

4.5.2.1   Action Buttons

This main tool area on the top of the **Acquisition** tab provides access to all tools for controlling image acquisition.

The prominent action buttons of this main tool tab have the following functions:

New opens a new image window. The next acquired image is displayed in this image frame.

Auto Exposure produces an image from all active channels and tracks and optimizes the settings of the Gain (Master) and offset for the given laser power and pinhole size.

Live uses the maximum speed for the given image frame and zoom setting to produce an image from all active channels and tracks in the quickest possible way.

Continuous scans images from all active channels and tracks until stopped ignoring settings for multidimensional acquisition. The last scanned frames will stay in the image frame.

Snap produces one single image using all active channels and tracks but ignoring the parameters activated in the multidimensional acquisition tool group.

Start Experiment will start the image acquisition taking into account all settings including those for time series, bleaching, Z-Stack acquisition, and tile scan.

Fig. 4-32   Acquisition tool tab

Fig. 4-33   Main Tool Acquisition tab: Action buttons

To stop an Experiment:

a) The action buttons turn into stop buttons and can be used to stop the system.

b) When performing a multidimensional experiment, the graphical representation of the experiment type (see above) turns into a stop button. Using this button will immediately stop the acquisition. Using the finish current step button will interrupt the multidimensional acquisition after the currently performed step. (i.e. when performing Z over time it will finish the current Z-Stack and then stop the experiment.)

4.5.2.2 Tool Group Setup Manager: Laser Tool

The **Laser** tool shows the types of **Lasers**, the **Laser Lines**, and **Power** status of all lasers available.

Clicking the arrow next to the indicated status of a laser opens a drop down menu which enables the user to switch between **Standby**, **On** and **Off**. Lasers which are On or in Standby are marked with a yellow triangle indicating laser radiation. Diode lasers can be set to On immediately.

The additional laser information panel (available in pro mode) shows the relevant and currently set **Maximum Power**, **Wavelength**, **Status**, **Tube Current** and **Output [%]** values of the currently highlighted laser. Click the arrow next to Laser Information to open this part of the window. Depending on the type of laser the provided information may vary.

The Argon multi-line and Argon UV laser have to be set to Standby first until they have warmed up. Once they have warmed up, they can be set to On. Use the **Output [%]** slider to set the laser power.

Fig. 4-34   Tool group Setup Manager: Laser tool

Laser operation info:

Argon Multiline laser:	Set output between 25 and 100 % of the maximum tube current. Optimum operation is at 8 A (lowest laser noise). However, the laser life is reduced if the laser is constantly operated at 8 A. Therefore, 8 A should be used only if necessary. We recommend a normal operation mode close to 4 A.
Argon UV laser:	Set output between 50 and 100 % of the maximum tube current. Optimum operation is at 20 A (Tube Current; lowest noise). However, the laser life is reduced if the laser is constantly operated at 20 A. Therefore, 20 A should be used only if this is necessary. (See also section 1 on handling this type of laser.)

To switch on the Enterprise laser, proceed as follows:

- The internal water-cooling must be running.

- Start the PC, wait until Windows XP is booted.

- Switch on the power supply of the Enterprise laser, power potentiometer turned to maximum.

- Start the ZEN software.

If the ZEN software is already running and you want to use the UV laser, do the following:

- Close ZEN software.

- Switch on the power supply of the Enterprise, power potentiometer turned to maximum.

- Start ZEN software again.

☞   All lasers are automatically set to Standby if not in use and set back to On for image acquisition.

☞   After switching on the lasers in the laser control window and their status ready the system can be used for imaging. However, for quantitative imaging it is recommended to let the system warm up for 2 to 3 hours.

☞   Please bear in mind that a cooling phase of at least 5 minutes is required between switching off of the laser via the software and switching off the entire system via the REMOTE CONTROL main switch or the Power Supply switch of the Enterprise UV laser.

4.5.2.3 Tool Group Setup Manager: Imaging Setup Tool

This tool allows the user to setup imaging **Configuration**. If no configuration matching the desired settings for imaging a specific dye or combination of dyes is available, the hardware settings first have to be chosen in the **Light Path** tool (see section 4.5.2.4) and stored as an imaging configuration.

Three different modes are available for imaging. Select the modes depending on the application.
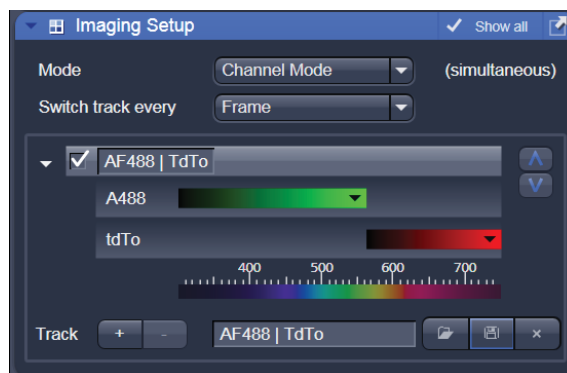
Fig. 4-35   Imaging Setup tool

Channel Mode:

This mode uses one or more detectors (including the META detector) as single channels with defined imaging parameters. All hardware parameters set to detect one ore more specific signals simultaneously are defined as a **Track** and can be stored using a name for this **Track** (available when ✓ Show all is active). The parameters include the detector(s) and detector settings like gain and offset, the pinhole size, the filters and dichroics, the laser source(s) and the laser transmission settings.

It is possible to use more than one track to detect different emission signals **sequential**ly. Up to 4 tracks with a total amount of 8 channels (incl. monitor diode and transmission channels) can be selected for sequential imaging. Ratio channels (to be selected in the Light Path tool) are displayed in addition.

Switch track every …:

The hardware settings for the different tracks can be switched every frame, every frame but fast, or every line. The latter two options only produce the wanted results if the changes in the settings between each track are restricted to activation /deactivation of channels and changes in their settings and activation/deactivation of lasers or changes in laser power. Additional hardware (filters, dichroics, and pinhole) is not moved even if the second track and all other tracks would require this. These track parameters are overwritten with the parameters of the first track upon confirmation. The confirmation to overwrite the settings is asked in any case when choosing line or frame fast as switching mode. If confirmed with No, the switching mode: **Frame** will be kept and the settings of the tracks are not changed.

The total set of parameters for one or multiple tracks can be stored as an imaging configuration.

Using multiple tracks is the preferred method to clearly separate weakly overlapping emission signals.
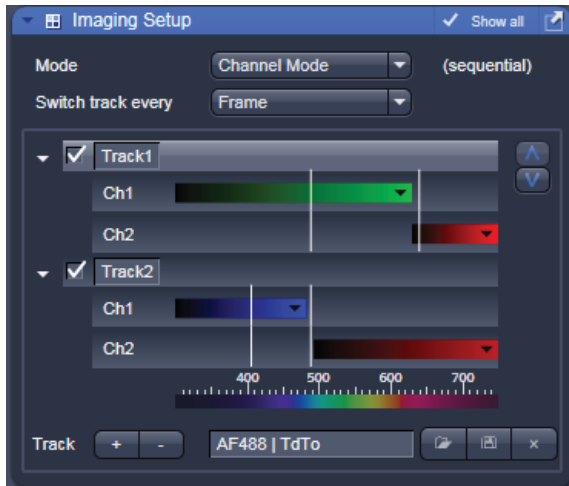
Fig. 4-36   Imaging Setup tool: Channel Mode

Fig. 4-37   Imaging Setup tool: Lambda Mode

Within the Imaging Setup tool (Fig. 4-36) information on the laser line and detection band is displayed if the arrow left of the activation/deactivation tick box of each track is clicked. The white line represents the laser line and the color bar(s) represents the detection band for the channel(s) in this track. If a track is not activated it will be ignored for image acquisition.

Tracks can be added and subtracted by clicking plus or minus. The added **Track** has the same filter settings as the first track and one active channel. In pro mode it is possible to store a track under a name. A stored track can be chosen from the drop down menu and can be loaded into the highlighted profile of a track. Thus stored tracks can easily be integrated into other imaging configurations.

Lambda Mode (only available if the LSM 510 is equipped with a META detector):

The **Lambda Mode** (Fig. 4-37) is chosen for imaging heavily overlapping emission signals. When choosing this mode in the drop down menu the display changes to show the currently active laser line(s) and the detection range of the META detector. With this imaging mode, it is possible to acquire an intensity image displaying the intensity of the fluorochrome(s) within a spectral bandwidth of 10nm. According to the detection range that is set for the META detector, a specific number of such images is acquired which is called a Lambda stack. These image data provide the intensity information within the selected detection range for each pixel. Therefore, the data allow deducing an emission spectrum for each pixel corresponding to the emission spectrum of a specific dye. This calculation is done by linear unmixing (see section 4.6.17) using the acquired Lambda stack and allow to clearly separate even heavily overlapping emission signals.

Online Fingerprinting (only available if the LSM 510 is equipped with a META detector):

The imaging mode **Online Fingerprinting** (Fig. 4-38) is based on the acquisition of a Lambda stack. It requires using already available emission spectra from the dyes which are used as markers in the imaged specimen.

The choice of the emission spectra and the setting of the detection range for the META detector are defined in the loaded configuration or using the **Light Path** tool.
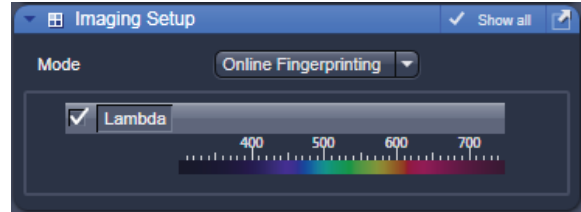
Fig. 4-38 Imaging Setup tool: Online Fingerprinting

4.5.2.4 Tool Group Setup Manager: Light Path Tool

This tool displays the hardware settings of the scan head (**LSM** tab), the beam path to image with the camera (**Camera** tab) or the Non descanned Detectors (**Non Descanned** tab). The settings can be changed in the corresponding tab and used for a new imaging configuration. The configuration can be stored in the **Imaging Setup** tool.
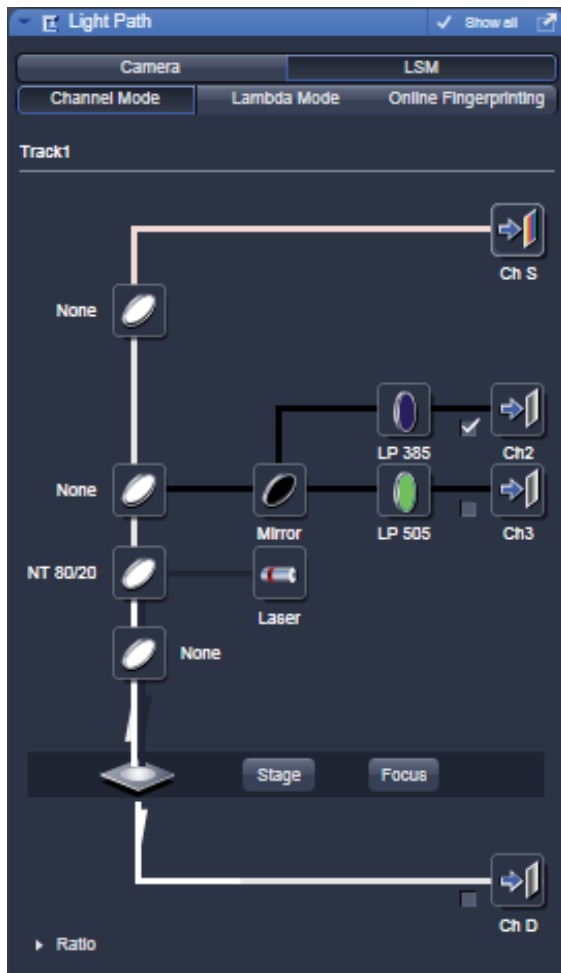
Fig. 4-39   Light Path tool: LSM tab

The **Channel Mode** tab (Fig. 4-39) displays the current hardware settings within the LSM 510 or LSM 510 META scan head for an imaging track. The display changes in accordance with the selection of a track in the **Imaging Setup** tool.

It also allows accessing the focus control, the stage position (the latter is only available if a motorized XY stage is attached to the microscope) and the laser lines. Fig. 4-39 shows the **LSM** tab when channel mode is selected in the **Imaging Setup** tool including a description of the various icons.

(1)   **Main dichroic beam splitters HT (or HFT) and secondary dichroic beam splitters NT (or NFT)**

- HT or HFT stands for main dichroic beam splitter. This beam splitter is chosen according to the selected laser line(s). It separates the excitation light from the emission signal. HT filters reflect the wavelength indicated and transmit all other wavelengths.

- NT or NFT stands for secondary dichroic beam splitter. It separates emission signals from each other and directs them to a specific detection channel. NT beam splitters are long pass filters if not otherwise stated. They transmit all wavelengths longer than the indicated one.

- To select a beam splitter, click on the icon and chose the desired one from the drop down menu.

☞   For the configuration of the beam path, please refer to the application-specific configurations depending on the used dyes and markers and the existing instrument configuration listed in the annex.

4.5.2.4.1 Activation/Deactivation of Channels / Transmission Channel and Channel Color Assignment

- To activate a channel for imaging, check the check box next to channel icon. To deactivate the channel uncheck this box.

- Use Channel D (transmission channel) to acquire an image with transmission illumination using any laser for excitation. This image will display the differential interference contrast (DIC) of the specimen if the according optical hardware for this contrast is put into the beam path in front of the detector. Check the manual of the microscope for setting DIC optics. Be aware that the laser light is already polarized and therefore a polarizer device is not necessary for transmission imaging. This imaging mode allows displaying a DIC image simultaneously to the fluorescence signal of the specimen.

- To assign a specific color to the image of a channel click onto the color icon and select a color from the drop down menu (Fig. 4-40).
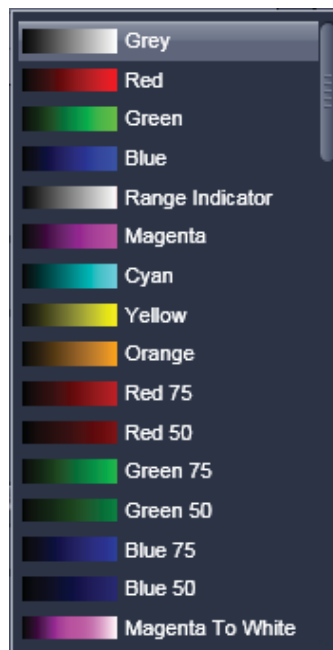
Fig. 4-40 Drop down menu for selection of the channel color

4.5.2.4.2 Reflector Turret

The filter wheel FW1 is the reflector turret of the microscope that holds push and click filter cubes and fixed beam combiner cubes. When using the LSM 510 for imaging the filter wheel is automatically set to an empty position if not otherwise defined or set by loading an imaging configuration. Standard fluorescence filter cubes are used for observation of the specimen with the eye or with the camera. Beam combiners are used for simultaneously using the excitation laser lines from both the LSM 510 META and the LSM *DuoScan* (if the system is equipped with this device). A click on the icon opens a drop down menu from which the desired filter can be chosen.

4.5.2.4.3 Stage / Focus

This icons provide access to the control for the focus (position of the specimen in Z) and the stage (position of the specimen in X and Y). But detailed description is given in section 4.5.2.8, Focus tool and section 4.5.2.9, Stage tool.

4.5.2.4.4 Setting of Laser Line and Laser Attenuation

The **Laser** icon provides access to the control of the laser lines and their attenuation (Fig. 4-41). Activate a laser line by checking the according box. This opens the attenuation slider and input box, which allows setting the desired laser power. Make sure the laser line is indicated as active with a warning triangle. If this is not the case, go back to the Laser tool and switch on the laser.
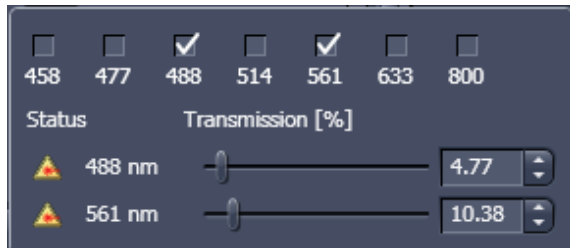
Fig. 4-41 **Laser line selection and attenuation**

- Use the **Transmission [%]** slider to set the utilizable laser intensity (recommendation: start at 5 %).
  - The transmittance of the Acousto-Optical Tunable Filter (AOTF) changes accordingly. This allows adapting the laser intensity very sensitively to the job.
  - No more than 8 excitation laser lines (wavelengths) can be active at the same time. If more than eight laser lines are connected to the system the Switch button is active and the required laser lines (if not displayed) must be allocated.

- For this purpose, click on **Switch** and select the required laser line that should be switched to the one not yet displayed.

4.5.2.4.5 Multiple Tracks

The **sequential** imaging of **tracks** permits several tracks to be defined as one configuration for the scan procedure, to be stored under any name, reloaded or deleted (see also section 4.5.2.3).

The multiple tracks can either be configured manually one after the other (identical to a single track) and then stored as recording configuration, or already existing recording configurations can be used and changed as required.

It is also possible to load already stored track configurations (single tracks) in a recording configuration.

The set up is done using both the Imaging Setup tool and Light Path tool.

In the **Imaging Setup** tool chose **Channel Mode** as the mode for image acquisition. In the **Light Path** tool define the hardware parameters like Laser line, laser attenuation, main and secondary dichroic beam splitters, and emission filters for the first track as described above. Ideally the parameters should be stored as a **Track**. Add a second **Track** within the imaging setup tool. Make sure it's selected (highlighted) before switching to the **Light Path** tool. Again set the hardware parameters in this case for the second track, and store them under a different name as a Track in the **Imaging Set up** tool. If more than two tracks are requested, repeat this procedure accordingly. Finally the total combination of tracks is stored as an imaging **Configuration** in the Imaging Setup tool.

☞ When storing a configuration all the settings of the **Light Path** plus the Detector Gain, Ampl. Offset, Ampl. Gain and Data Depth (see section 4.5.2.6 Channels tool), the scan parameters like Frame Size**,** Zoom, Rotation & Offset, and Scan Direction parameters (see section 4.5.2.5 Acquisition Mode tool) are stored.

4.5.2.4.6 Setting up a Ratio Imaging Configuration

In the **Show all** mode of the **Light Path** tool two additional ratio imaging channels **R1** and **R2** can be defined. These channels are displayed in addition to the maximum of 8 active imaging channels. They provide the possibility to display the ratio between two of the active channels.

Fig. 4-42   **Ratio channels in the Light Path tool**

A click on the arrow next to **Ratio** in the lower part of the Light Path tool window (Fig. 4-42) provides access to the ratio channel interface. A maximum of two ratio channels can be activated. These channels can display the ratio between two of the active imaging channels. The source channels for calculating the ratio have to be selected from the drop down menu next to Source 1 and Source 2. As an alternative to a second channel for the ratio imaging is also possible to choose the first images of a time series of one channel for the ratio metric calculation (e.g. to calculate F/F_0 for single wavelength dyes). The parameters for the calculation are set in the Channels tool (see section 4.5.2.6).

A suitable color is assigned to each of the two **Ratio Channels R1** and **R2**, in the same way as for the photomultiplier channels.

☞ The ratio to be formed between the selected channels can be defined more precisely using one of the four preset formulas in the **Channels** tool window after a click on the relevant ratio button (e.g.: R1) for online display of radiometric or single wavelength dyes. The Set by min/max function (in Channels tool window) allows the definition of the display scaling according to the expected minimal and maximal values.

Fig. 4-43   **Light Path tool window;
Lambda Mode activated**

4.5.2.4.7   Imaging in Lambda Mode

(to be selected in the Imaging Setup tool).

The **Lambda Mode** is only available if the LSM 510 contains the META detector. With this spectral module, the overall emission from the sample is directed onto a wavelength-dispersive element and is imaged on a 32-channel detector. All 32 photomultipliers of the detector cover a spectral width of approximately 340 nm, a single PMT covers a spectral range of 10.7 nm. In the **Lambda Mode,** images, image stacks, or time series are recorded in a wavelength selective way.

This new image format is called Lambda stack. For the acquisition of a Lambda stack, the fluorescence signal of 8 PMTs out of the 32 can be read out at once. The settings of the beam path for the **Lambda Mode** scanning procedure with regard to the main dichroic beam splitter and the META detector settings are performed in the **Light Path** tool (Fig. 4-43).

The META detector is only displayed for spectral imaging if **Lambda Mode** has been chosen as imaging configuration in the Imaging Setup tool.

The **Light Path** tool displays the laser lines, the main (dichroic) beam splitter, and the spectral range of the META detector.

You can change the settings using the following function elements:

	Activation / deactivation of the excitation wavelengths (check box) and setting of excitation intensities (slider).
	Selection of the main dichroic beam splitter (HFT) through selection from the relevant list box.
	Definition of number of PMTs out of the 32 PMT Detectors to fit the required spectral range.
	Access to the focus control (see section 4.5.2.8 Focus tool)
	Start value of required spectral range.
	End value of required spectral range.
	Step width in spectral range (10.70 recommended).
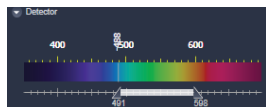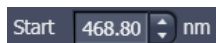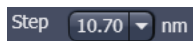
META detector settings

Use the two sliders to define the spectral detection range of the META detector. Both slider values are updated in the **Start** and **End** Input box.

The wavelength values can also be typed directly into the Start and End input boxes. The **Number of Passes** displays the number of successive scans to be performed in order to cover the required spectral range.

Step check box: Step size can be increased to 21.4 nm, 32.10 nm or 42.80 nm by binning of 2, 4 or 8 adjacent channels, respectively.

4.5.2.4.8   Online Fingerprinting Mode

(to be selected in the **Imaging Setup** tool).

The use of this function permits the selection of reference spectra together with the excitation settings, allowing an immediate display of the unmixed images while scanning. Chose **Online Fingerprinting** in the Imaging **Setup** tool to activate the according display in the **Light Path** tool.

You can change the settings using the function elements which have been described for the **Lambda Mode** plus the following:
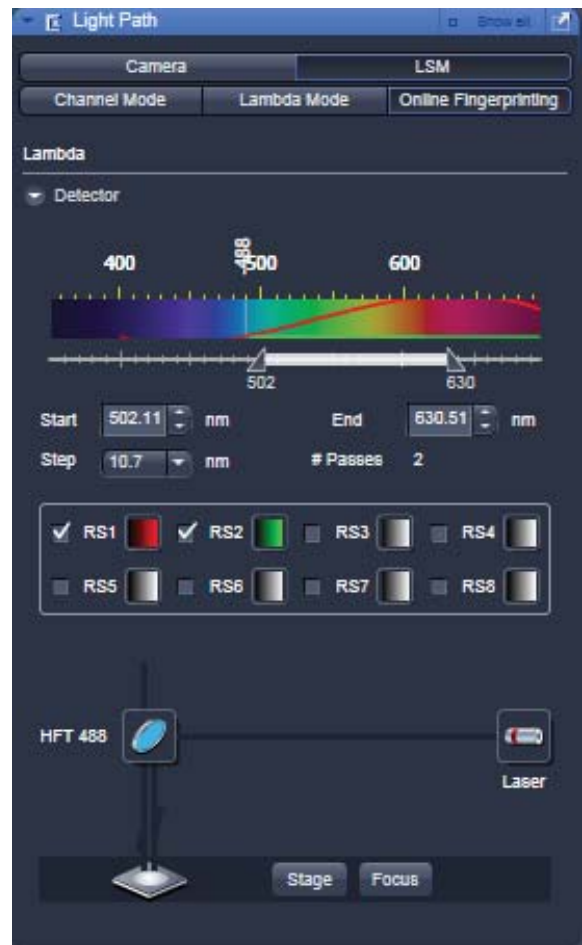
**Fig. 4-44   Light Path tool window;
Online Fingerprinting Mode**

RS1 ... 8

Menu for selecting a display color and reference spectra (reference spectra derive from earlier experiments via Mean of ROI functionality or ACE tool). The unmixed results are displayed during scanning and Lambda stack data will neither be displayed nor stored.

Fig. 4-45   Light Path tool: Camera tab

4.5.2.4.9   Camera Mode

The **Camera tab** displays the current hardware settings for an imaging track using the camera as detection channel. The display changes in accordance with the selection of a track in the Imaging Setup tool.

The Camera tab provides access to the conventional illumination sources, the halogen lamp or HBO (XCite 120). It also provides access to the condensor aperture and filters and the tube lens.

The use of this function permits the use of a Zeiss AxioCam camera (various models, see ANNEX for description) as an alternative external detector.

The camera can be used with the transmission illumination source halogen lamp, which allows acquiring a standard transmission image or, depending on the optics of the microscope, a differential interference contrast image.

When the HBO (or XCite 120) is used as an illumination source a standard fluorescence image can be acquired. In this case a specific fluorescence filter cube has to be chosen in the reflector turret filter wheel.

A corresponding tube lens (1x or 0.63x) should be used.

4.5.2.5   Tool Group Online Acquisition: Acquisition Mode Tool

The parameters for image acquisition are set using the tool **Acquisition Mode**.

The following scan modes, which can be selected from the drop down menu, are available:

Frame

–   scanning of an XY frame (Frame, Frame + Time Series)

–   scanning of XY frames with different Z-values (Frame + Z-Stack, Frame + Z-Stack + Time Series)

–   scanning of XY frames in defined ROIs (Frame + ROI + Time Series)

–   scanning of XY frames with different Z-values in defined ROIs (Frame + Z-Stack + ROI + Time Series)

Line

–   scanning of a line in the XY-plane (Line, Line + Time Series)

–   scanning of a line with different Z-values (Line + Z-Stack, Line + Z-Stack + Time Series)

Spot

–   scanning of a spot (Spot + Time Series)

The availability of the modes is also dependent on the imaging device used e.g. LSM 510 META, LSM 5 *LIVE* or camera.

Depending on the selected scan mode the additional parameters for image acquisition will change.

Fig. 4-46   Acquisition Mode - Frame with LSM tab selected in the Light Path tool

4.5.2.5.1   Frame Mode for LSM 510 META

When the scan mode **Frame** is active, a frame of variable size is scanned pixel by pixel and line by line. The laser beam is moved over the specimen line by line.

The following additional parameters and be set:

Objective, Frame Size, Line Step Factor, Speed, Pixel Depth, Scan Direction, Averaging and Zoom including rotation and offset.

A list of available objectives is displayed in the scroll down menu next to **Objective**. Choose one from the list which is appropriate for imaging a specific specimen. Pay attention to match the refractive index of the objective with the refractive index of the embedding medium of the specimen if possible.

- Select the **Frame Size** from the default sizes via the drop down menu clicking the button [X*Y] or type in the required values next to X and Y. Recommended setting to start with: 512 x 512 pixels.

    - It is also possible to enter different values for X and Y. The value for **Y** is freely selectable between 1 and 2048 pixels (integers). The value for **X** must always be an integral multiple of 4. The maximum value for **X** is also 2048 pixels.

    - The **Optimal** button sets the image resolution to an optimal value corresponding to the optical magnification (objective) the zoom and the emission range detected. This provides an image where no information is lost as optimal sampling is achieved.

- Select the **Line Step** size between 1 and 10. Only every n-th line is scanned. The lines in between are interpolated. This fast scan mode is called Step Scan.

- Select the **Scan Speed** from the 13 preset steps via slider or input box. Recommended: 7 for the first scan. A click on the **Max** button sets the maximum speed for the current zoom.

    - The **Scan Speed** determines the **Pixel Dwell** time. In the case of different image formats, the **Pixel Dwell** time is constant for the same Scan Speed, but the **Scan Time** is different.

    - **Pixel Dwell** time of the laser beam on the pixel

    - The **Scan Time** duration of the acquisition for the entire frame

Speed:	1 … 8	9	10	11	12	13
Zoom:	0.7	1.0	1.4	2.5	3.6	5.7

- Select **8 Bit** or **16 Bit** Data Depth, i.e. 256 or 65536 gray values.

- Select the **Unidirectional** or **Bi-directional** Scan Direction.

  – Unidirectional: The laser scans in one direction only, then moves back with beam blanked and scans the next line.

  – Bi-directional: The laser also scans when moving backwards, i.e. the Scan Time is halved.

  – The pixel shift between forward and backward movement (double image) resulting from bi-directional scanning must be corrected. For automatic scan correction, click on the **Auto** button. If **Auto** is off the scan **Corr X** and **Y** sliders which are indicated when selecting bidirectional scanning. Zero° rotation requires correction in the X-direction, 90° rotation must be corrected in the Y-direction. If the image was rotated, correction is required in both coordinates. Correction is performed on-line in the **Continuous** scan mode. The size of the shift depends on the Scan Speed.

- Select the **Line** or **Frame** mode for averaging.

- Select the desired scan average method **Mean** or **Sum**.

- Select the desired scan average from the available values **2**, **4**, **8** and **16** in the **Number** selection drop down menu or **Continues** (only for **Frame** average mode).

☞ The greater the number of averages selected for **Mean** average **Method**, the better the image quality will be; the scanning time will be prolonged accordingly.

Averaging can be performed in different ways, depending on whether the **Mean** or **Sum** method has been activated.

If you are using the **Mean** method, the image information is generated by adding up all scans pixel by pixel and then calculating the mean value.

In the **Sum** method, the intensity values of all images are added up, without a mean value being calculated.
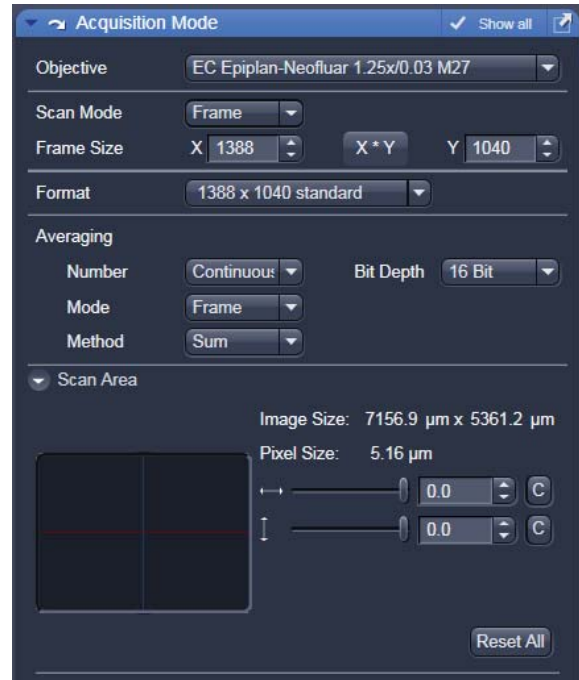
To create the image information using the **Line** average mode, each line (depending on the setting) is scanned 2, 4, 8 or 16 times during Scan Average, and then the average value per pixel is calculated. This minimizes noise interference during the scanning procedure.

If the **Frame** average mode is used to create the image information, the complete frame is scanned 2, 4, 8 or 16 times, depending on the setting. The average value is recalculated after each frame scan.

The **Frame** average mode also permits continuous averaging.

For this, select the **Continuous** option in the **Number** selection box.
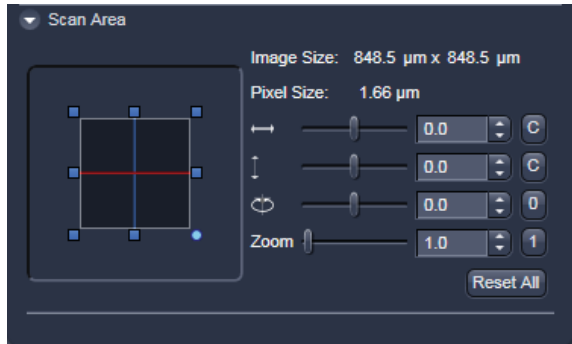
Fig. 4-47   Scan Area panel

Scan Area

In this panel, the scan field is set for zoom, rotation and offset in relation to the field of view of the microscope. The diagonals of the outer square on the left-hand side correspond to the field of view of the microscope.

The inner square contained in it (rectangle in the case of differently set frame size) represents the scan range and immediately shows the changes made to zoom, rotation and offset.

- Set the desired **Zoom** factor via the slider or by clicking on the small red squares outlining the inner square.

  – The **Zoom** factor can be set continuously in the range from 0.7 to the maximum of 40, and is displayed in the relevant input box. The value 0.7 corresponds to factor 1, and value 40 to factor 57, related to the field of view. From zoom factor 5.6 onwards, the magnification will be empty.

  – Recommended setting to start with: Zoom 1.

- To rotate the scan area, use the slider or click on the blue dot in one of the corners of the inner square.

  – Recommended setting to start with: Rotation 0°.

- Move the scan area by clicking on for offset in horizontal direction and for offset in vertical direction.

- The offset of the scan area from the center of the field of view is displayed online in µm for X and Y.

  – Clicking, holding and drawing the rectangle with the mouse permits the scan area to be moved directly within the field of view.

  – Recommended setting to start with: Offset X = 0, Y = 0

☞   During the scan procedure, the functions **Speed**, **Scan Corr**, **Zoom**, **Rotation** and **Offset** can be influenced online.

By clicking on the **Reset All** button the scan zoom is set to 1, the XY offsets are set to the zero position and the ratio angle is set to 0°.

4.5.2.5.2 Frame Mode for Camera

When images are acquired with the camera only **Frame** mode is available.

The following additional parameters and be set:

Objective, Frame Size, Format, Bit Depth, Averaging and Offset.

The exposure time is set in the **Channels** tool.

A list of available objectives is displayed in the scroll down menu next to **Objective**. Choose one from the list which is appropriate for imaging a specific specimen. Pay attention to match the refractive index of the objective with the refractive index of the embedding medium of the specimen if possible.

- Select the **Frame Size** from the default sizes via the drop down menu clicking the button ☒ or type in the required values next to **X** and **Y** all the. Recommended setting to start with: 1300 x 1300. You can select between square formats or free defined frame sizes.

Fig. 4-48   **Acquisition Mode - Frame with Camera tab selected in the Light Path tool.**

Format selects between a range of default camera resolutions. The 5x5 binning mode can be used for focusing without delay of the image display.

- Select **8 Bit** or **12 Bit** Data Depth, i.e. 256 or 4096 gray values.

- In the scan area it is possible to shift a sub region in the frame using the offset sliders if the **Frame Size** is set to a smaller value than the chosen **Format** sets it automatically.

- The horizontal and vertical **Offset** can be set to zero individually clicking **C**.

- **Reset All** Resets the **Offset** and the frame/sub region to the default value selected in **Format**.

- **Frame** mode is available for averaging.

- Select the desired scan average method **Mean** or **Sum**.

- Select the desired scan average from the available values **2**, **4**, **8** and **16** in the **Number** selection drop down menu or **Continues**.

☞   The greater the number of averages selected for **Mean** average **Method**, the better the image quality will be; the scanning time will be prolonged accordingly.

Averaging can be performed in different ways, depending on whether the **Mean** or **Sum** method has been activated.

If you are using the **Mean** method, the image information is generated by adding up all images and then calculating the mean value.

In the **Sum** method, the intensity values of all images are added up, without a mean value being calculated.

Fig. 4-49 Acquisition Mode – Line with LSM tab selected in the Light Path tool

4.5.2.5.3 Line Mode

In **Line** mode, fluorescent or reflected light along a straight or freely definable line is displayed in the form of an intensity profile. The line is scanned pixel by pixel. The laser beam is moved over the specimen along a line.

The following parameters for scanning a line can be defined:

Objective, Frame Size, Speed, **Pixel Depth, Scan Direction, Averaging** and **Zoom including rotation and offset.**

These functions operate as described in section 4.5.2.5.1. **Frame size** in this context sets the length of the line in x direction. The width of the line is one pixel.

Note: A width larger than one pixel is defined as a frame.

If **Line Mode** has been selected, the **Line Select** button appears on the bottom of the **Acquisition mode** tool. It permits positioning of the line to be scanned as required within the **Image** (Frame in XY-plane).

A frame will be scanned and the currently selected scan line and its intensity profile will be displayed. In the **View Control** the **Line–select** tools are available.

The line arrow ⬉ is active by default and allows using the mouse to draw a straight line in the image at the position where the line scan should be performed.

Alternatively the scan line ⬚ can be defined as a free shape curve (spline).

The arrow selection ⬉ is used to move the drawn line within the image.

The standard controls allow defining the color and width of the appearance of the line overlay in the image.

Once a straight line is drawn in the image, the relevant intensity profile along the drawn line will be displayed. In the **Acquisition Mode** tool Zoom, Rotation and Offset values are updated.

- When the **Line Select** button is hit again, a frame will be scanned in such a way that the selected line lies exactly in the center of the Y-axis again and is parallel to the X-axis.

☞ The position and size of the Line (rotation and offset) can also be changed directly in the **Scan Area** panel of the **Acquisition Mode** tool.
In the **Line** mode, Line Stacks can also be recorded over a defined period of time.

- When drawing a free shape curve (spline) in the **Image** using the mouse, the first click sets the starting point; each further click adds a line segment. A click with the right mouse button ends the line definition.

The scanner represented by a white line immediately begins with the on-line tracing of the defined free shape curve. The laser excitation remains inactive in this process.

If the defined free shape curve becomes too complicated or the selected **Scan Speed** is too high, the following message appears in the status bar:

Maximum scanner acceleration exceeded!

- In this case, reduce the **Scan Speed** set in the **Acquisition mode** tool.

- If the generated contour and the line traced by the scanner are not in coincidence, reduce the **Scan Speed** by a further amount.

If no sufficient coincidence of the two lines can be achieved by the reduction of the scan speed, you have to calibrate the scanner position signal.

4.5.2.5.4 Spot Mode

In the **Spot** mode fluorescent or reflected light occurring from a single voxel xyz is detected. In this mode a spot can be defined by two perpendicular lines in the **Image** using the Spot Select button which is located in the lower part of the **Acquisition Mode** tool window (Fig. 4-50).

The following parameters for scanning a spot can be defined:

Objective, Speed, Pixel Depth and Offset in X and Y (= position within the image).

These functions operate as described in section 4.5.2.5.1.

In **Spot** mode it is not possible to acquire a **Z-Stack**. After definition of the spot position the only possible scan mode is **Time Series**.
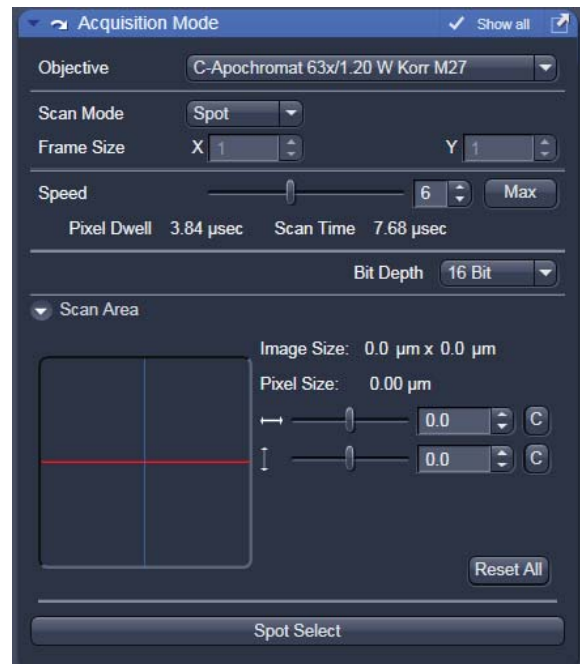
Fig. 4-50   **Acquisition Mode tool - Spot Mode**
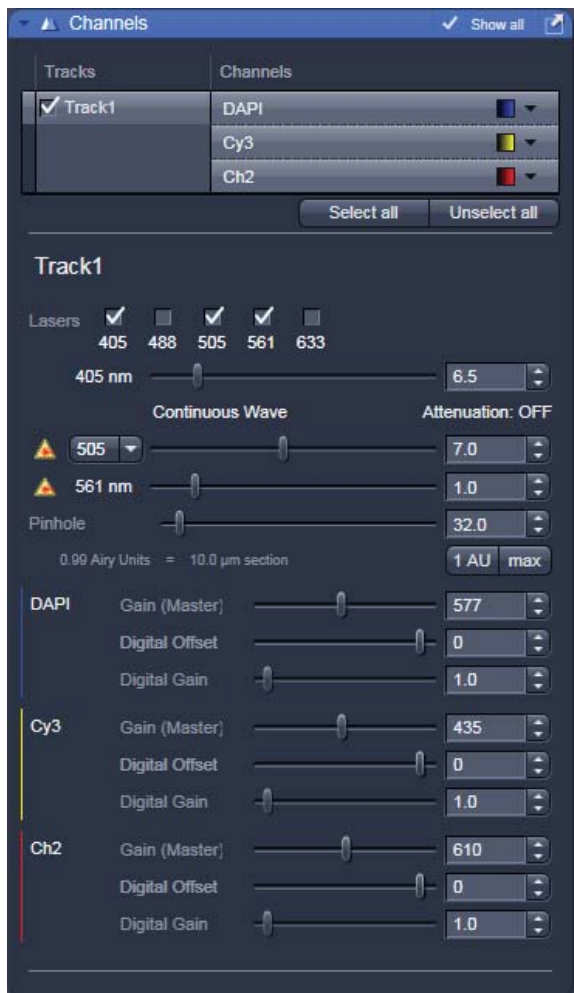
Fig. 4-51 **Channels tool using channel mode (in this case multiple tracks) for imaging**

4.5.2.6 Tool Group Online Acquisition: Channels Tool

The **Channels** tool provides the control of the parameters of the individual detection channels.

It also allows to activate and de-activate tracks by ticking and un-ticking the track check box ☑ Track1.
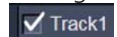
The channels and tracks defined in the **Imaging Setup, Light Path** tool or by **Smart Setup** are listed channel by channel as selectable list entries. Clicking on the list entry **Track** ☑ Track2 displays controls (laser, pinhole, gain, …) for all the channels in the respective track.

Clicking on the **Channels** [Channels EBFP] list entry displays the controls for the selected channel only.

Using shift-clicks – the controls can be displayed for any given combination of channels or tracks.
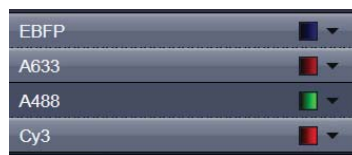
If entered in the Imaging Setup tool or the Light Path tool the names of the dyes used or the names of the tracks are displayed in the Channels tool.
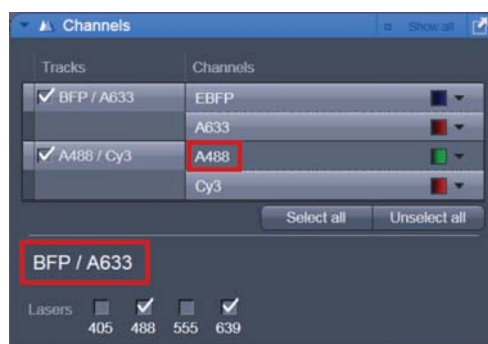
Depending on the selected **Channel** or **Track**, the currently used settings of Pinhole, Gain (Master), Digital Offset, Digital Gain, laser line and laser power are displayed. Below, all available options with **Show all** active are described.
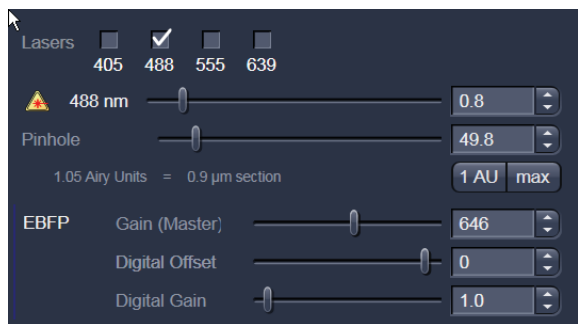
- The slider next to **Pinhole** enables you to change the pinhole diameter of the relevant channel.

  – The pinhole diameter is indicated in **µm**, **Optical Slice** and **Airy Units**. The Airy value depends on the aperture of the objective, excitations and the emission wavelength.

  – A small pinhole diameter will increase the depth of focus, but reduce the light intensity received by the detector.

  – When you vary the **Pinhole** diameter, an Optical Slice value is displayed. For optimum depth resolution, Airy values should be small (for reflection 0.3), but in fluorescence applications not below 1.0 to keep the intensity loss within a reasonable limit.

  – A click on the **1 AU** button sets the pinhole to a diameter of 1 Airy unit. A click on the **Max** button sets the pinhole diameter to the maximum.

- The sliders (and the relevant arrow buttons) next to **Gain (Master)**, **Digital Offset** and **Digital Gain** enable you to set the detector of the selected channel during continuous scanning.

  – Gain (Master): Setting of the high voltage of the PMT photomultiplier - setting of image contrast and brightness (values available between 0 and 900 or 1200 depending on the type of detector)

  – Digital Offset: Setting of the electronic offset - background of the image can be set (values available between -2 and 0.1)

  – Digital Gain: Amplification factor (values available between 0,3 and 15)

When using 12 bit grey resolution, gaps can occur in the histogram of the acquired image, in case the gain slider is moved beyond a gain level of 25.

Use the Digital Offset slider to remove underexposed pixels in the image background (no blue pixels visible with **Range Indicator** lookup table).

☞   In case the **Camera** has been chosen for imaging the exposure time is set in the **Channels** tool.
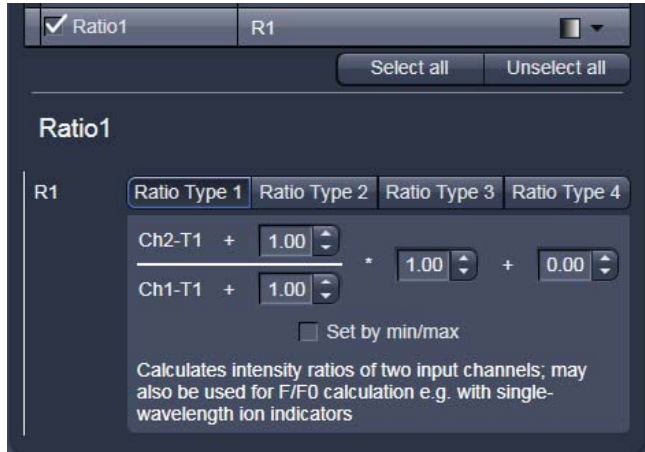
Fig. 4-52   **Channel Settings panel of a Ratio Channel**

4.5.2.7   Ratio Channels

The parameters for ratio metric imaging can be set when a ratio channel is selected.

- Click on the button of a ratio channel (e.g. **R1**).

- Clicking on the required tabs enables you to choose from four formulas (**Type 1** to **4**) for ratio calculation. The relevant decimal values can be entered in the input boxes. The entered values remain unchanged even after switchover to another formula and can be reactivated any time.

The formula type activated last is always used for ratio formation during the scan procedure. If the input box does not contain any value at all or no suitable value, the useful value last used will be activated.

The ratio channels are displayed as additional images.

Select the required formula and enter the relevant values.

Letters can be entered into the formula fields which will be valued as 1; it is also possible to make no entry, which will also be valued as 1, but will not be displayed.

Set by min/max allows the definition of the display scaling according to the expected minimal and maximal values.

- Laser lines and laser power are displayed according to each track. It is possible to vary laser intensities (in the same way as in the **Laser** tool or **Light Path** tool) and to program the AOTF for different laser lines using the Switch button.

☞   All parameters can be varied while imaging.

4.5.2.8 Tool Group Online Acquisition: Focus Tool

The Focus tool controls the position on Z of the specimen. It controls the internal Z drive of the microscope and, if available, the additional high resolution focus which can either be a Z Piezo focusing device for a single objective or a Z Piezo insert for the stage.

The **Z-Position** of the specimen stage / nosepiece is indicated as a numerical value. This value and with it the focus position can be changed by using the arrows next to it [223,40].

The **Step Size**, by which the focus moves when clicking on the arrows, can be set between 0 and 1000 µm.

The Current Z-Position can be set as **Zero Position** by clicking the [Zero Position Manually] **Manually** button.
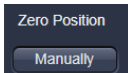
In addition a focus software joy stick is available to focus with different speeds.

Fig. 4-53   Focus tool window

The white joystick [ ] can be dragged over these areas to focus.

The stage / nosepiece can be moved to predefined positions.

Zero moves the stage / nosepiece to the position which was set as position zero before.

Work moves the specimen stage / nosepiece to the Work position. This is the position last indicated as current Z position before the **Load** button was pressed.

Load lowers the specimen stage / nosepiece to make it easier for you to change the specimen (or objective).

Use of an optional high resolution focusing device (Piezo objective focus or Z-Piezo stage insert)

The **Piezo Position** is indicated as a numerical value. This value and with it the focus position can be changed. A change of this value updates the value of the microscope focus accordingly.

The **Step Size**, by which the Piezo focus moves when clicking on the arrows, can be set between 0.005 µm and 10 µm.

As soon as the focus position of the microscope is changed (via hand wheel or software), the piezo objective focus is automatically levelled.

- A click on the **Leveling** button moves the Piezo objective focus or Z-Piezo stage insert into the center position of its travel range and the focus position of the microscope is reset to compensate for the leveling. Therefore, the same Z-level remains visible (the current position is not set to zero).

The motor focus of the stand is operated in the same way via the relevant buttons. Moving into the **Work** or **Load** position is always performed via the motor focus and not via the Piezo objective focus.

☞   Please see CHAPTER ANNEX of the printed manual for further information on the Piezo objective focus and Z-Piezo stage insert.

Fig. 4-54 **Stage tool window**

4.5.2.9 Tool Group Online Acquisition: Stage Tool

The following software description applies to systems which are equipped with a motorized scanning stage.

⚠ If an LSM 510 META scan head is attached to the side port of a inverted microscope (Axio Observer) take care not to catch your fingers when moving the motorized XY scanning stage to the maximum left position.

The **Stage** tool allows controlling the movement of the motorized stage, to move to specific locations and to mark locations taking the coordinates in X, Y and Z.

The movement of the stage is controlled via the stage software joystick or with the external control. Moving the cursor over the fields shows one or two arrows pointing into defined directions.

By clicking on these arrows the stage is moved accordingly. Alternatively the software joystick 🔵 can be used to move the stage.

The current position of the stage is updated in the numerical field of the **X-Position** and **Y-Position** in relation to the zero position. Any position can be **Set** as **Zero Position** and by clicking **Move To Zero** the stage directly moves to this position.

This movement of the stage can be halted clicking **Stop**.

The **Step Size**, by which the stage moves, can be set between1 µm and 1000 µm.

The **Marks** selection allows marking and therefore saving the coordinates of a current position in X, Y and Z (the Z position can be changed using the Focus tool) by clicking **Mark**. The new position is numbered and added to the drop down list. Single or all positions can be deleted using **Remove** or **Remove All**.

☞ If only one selected position is deleted, the position with the next number in sequence moves up one number.

☞ If a new **Zero** Position is set the coordinates of the marked positions are updated in relation to the new Zero Position.

The focus is moved to the marked position selected form the drop down menu by clicking **Move To**.

4.5.2.10   Tool Group Online Acquisition: Regions Tool

The Regions tool allows the user to define Regions of Interest (ROI) which are used for image acquisition, sample manipulation (bleaching) and image analysis (Fig. 4-55).

The control of the laser via AOTF technology enables the restriction of the illumination to the defined region. The sample is only illuminated when data are acquired.

The definition of the regions is done using an actual image of the sample and drawing the regions into this image. This makes it extremely easy to set the regions in the right position and dimension.

Fig. 4-55   Regions window

The following tools are available:

	Selects an existing region in the image. The outline of the region is marked with small rectangles to show it is active. The format and the position of the region can be changed clicking on the rectangles or the region outline and dragging it with the cursor. In addition the numerical values describing the position and size of the region are updated (see below).
	Rectangle: Drawing of a rectangle in the **Image** window; click and keep mouse button pressed, drag the rectangle in any direction, let go off the mouse button to end the procedure.
	Ellipse: Drawing of an ellipse in the **Image Display** window; first click sets the center point, displayed line permits determination of the extension, second click sets the first dimension, then the second dimension and the rotation direction can be determined, third click sets the second dimension and direction and ends the procedure.
	Circle: Drawing of a circle in the **Image** window; click and keep the mouse button pressed to set the center point, drag the diameter, let go off mouse button again to end the procedure.
	Polyline: Drawing of a polyline figure in the **Image Display** window; first click sets the starting point, each further click adds a line, double-click on the starting point closes the figure and ends the procedure.
	Closed **Bezier**: Drawing of a bezier figure in the **Image** window; first click sets the starting point, each additional click adds a line, double-click on the starting point closes the figure and ends the procedure.

A region drawn in the image will be listed in the regions tool. The regions are assigned with numbers. By checking the box next to **Hide** the regions will not be shown in the image. When highlighting a ROI in the list, the numerical parameters for **Center X**, **Center Y**, **Width** and **Height** will be shown below. In addition the ROI will be set to active in the image.

The regions can be used for different tasks. By checking the appropriate box the ROIs can be used for acquisition, bleaching, and analysis. Any combination of these tasks is possible.

It is possible to change the **Line width** and the **Color** of the outline of the region. The latter is only possible if either **Individual Color** or **Common Color** is chosen in the drop down list of the **Color Mode**. **Common Color** assigns the same color to all regions. If **Automatic Assignment** is chosen in the Color Mode a color will be automatically assigned to each region as it comes. The choice of Color is inactive in this mode.

The **Creation Mode** offers three different settings for defining ROIs. **Switch to selection mode** activates the arrow tool once the region is drawn in the image. **Multiple elements** allow drawing multiple regions of the same type in a sequence. **One element only** restricts the number of ROIs to be drawn in the image to one. Any new region deletes the previous one.

The laser usually scans the entire line length, but is limited in the Y-direction by the ROIs outline. The Scan Time is therefore reduced. It is possible to reduce the movement of the scanner also in the X direction along the line by checking the box next to **Fit frame size to bounding rectangle of regions**. This then corresponds to zooming in onto the region. The frame size will be updated accordingly in addition to the scan time. This applies for image acquisition using a ROI.

If a region is used to define a position for bleaching within the sample, the bleach process can be accelerated by checking the box next to **Zoom Bleach (fast, less accurate)**. In this case the scanner movement will be restricted to the bleach region zooming in onto this region. This may result in a less accurate positioning of the region as the definition of the region has been made in a different zoom in the image. The bleach process will be faster than without this option. The gain in speed is dependent on the speed that is used for bleaching. If a high speed has already been chosen for bleaching the gain in speed will be minimal.

The numbers of the different regions can be shown in the image if the check box next to **Show numbers** is checked.

The regions can be saved to be loaded later on. This process stores all regions currently displayed in the Regions tool in one file.

4.5.3 Tool Group Multidimensional Acquisition: Z-Stack

To activate the Multidimensional Acquisition tools the check boxes below the action buttons have to be checked. Once checked the tools are active and will be used for acquisition. If the Z-Stack tick box below the action buttons is checked the Z-Stack tool is visible. When **Start Experiment** is pressed the system will perform a Z-Stack.
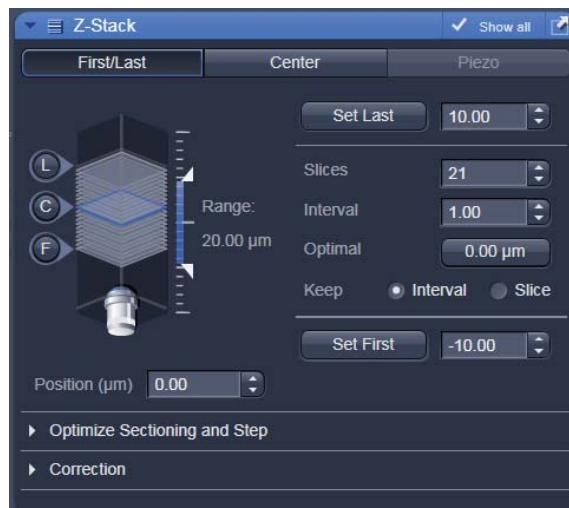
Fig. 4-56   Z-Stack tool First/Last mode with Z-Stack Navigation displayed

The Z-Stack function permits scanning a series of XY-images in different focus positions resulting in a Z-Stack, thus producing 3 dimensional data from your specimen.

For the definition of the first and last image of the stack it is either possible to use the **Mode First/Last** (Fig. 4-56) or to use **Center** (Fig. 4-57). In case a **Piezo** focusing device is attached to the system, the Piezo device can be chosen for the acquisition of the stack.

4.5.3.1.1 How to Proceed Setting a Z-Stack Using First/Last Mode

Focus onto the specimen and start a **Continuous** scan. While scanning change the focus using the **Focus** tool (see section 4.5.2.8), or the **Z-Stack Navigation** (see below), or use the focus drive of the microscope (see manual of the microscope). When you reach the position within the specimen, where you would like to start the stack, hit **Set First** and this position will be taken as the first position in the stack. Move the focus into the other direction until the end position within the stack is reached, then hit **Set Last**. These focus positions are then indicated next to Set First and Set Last. In addition, **Range** shows the total height of the stack in micrometers. The Focus **Position** indicates the current position of the Focus with the Range.

The **Number of Slices** and the value for the **Interval** between the slices are adjusted accordingly. With **Keep Interval** checked the number of slices is adjusted, with **Keep Slice** being checked, the value for the Interval between the slices is adjusted.

Changing the value for **Number of Slices** using the slider or the editing box, also changes the **Interval** but keeping the total **Range** for the stack.

Changing the value for the **Interval** changes the **Number of Slices** while keeping the total **Range** for the stack within the possibilities of fitting the calculated number of slices into the Range. The total Range may therefore vary to higher and lower numbers around the initial value of the Range.

Optimal Interval sets the **Interval** to match the Nyquist criteria (see below) and accordingly adjusts the **Number of Slices** to keep the **Range** set with First/Last.

Fast Z Line performs a fast XZ scan for overviews using a continuous movement of the microscope focus (only in Line scan mode and not with the Piezo). The stack size is retained; the interval is adapted depending on the scan speed. Not available in **Frame** mode.

The **Z-Stack Navigation** displays in a graphical manner the navigation in the Rang of the Z-Stack. The blue slice is the current focus position. This interface displays the actual position of the current focus also while scanning. By dragging the blue slice with the cursor, the current focus position changes accordingly. This tool might also be used for defining the first and last position for the Z-Stack.

☞   It is not relevant that the first and last position in the software match the absolute first and last position. The microscope will always move against gravity when acquiring the stack ignoring the assignment first and last.

☞   When using an Axio Imager the precision of the Z-Stack position can be optimized for very critical applications by taking care of the following steps: The approach to the first and the last slice when marking the positions should be made from the same direction as the Z-Stack is finally acquired. As the Z-Stack acquisition is always performed against gravity it is recommended to lower the stage, then approach the first slice by moving the stage up towards the objective, mark it, then go on focusing to the last slice and mark it. During this process be sure to move the stage in this one direction only, do not refocus.
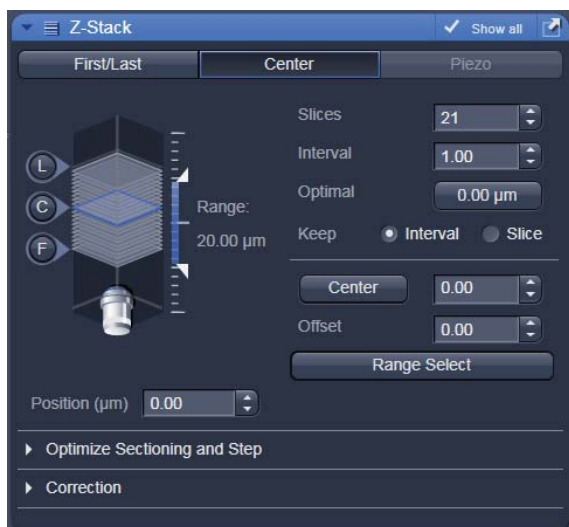
Fig. 4-57   **Z-Stack tool Center mode with Z-Stack Navigation displayed**

4.5.3.1.2   How to Proceed Setting a Z-Stack Using Center Mode

The **Center** mode (Fig. 4-57) allows setting the Range of the Z-Stack in relation to the current **Focus Position** which is indicated as **Offset**. In addition it is possible to use the image of a XZ scan (line scan in Z) for positioning the Z-Stack range. This tool then also allows setting the first and last slice independently from each other and adjusting the center position of the range according to these settings. You should have an idea about the size of the stack when using this mode. It is useful when several Z-Stacks of the same range should be acquired at different positions within the specimen, as the whole range of the stack can be set to a different position at once using the Offset function.

The values for **Number of Slices** and **Interval** are taken over from any settings done in First/Last mode. The **Offset** defines the **Center** position of the stack in relation to the **Focus Position**.

When defining a specific **Offset** the range of the Z–Stack is shifted to a different position in Z.

Pressing the [icon] button moves the actual **Focus Position** to the **Center** position and acquires an image at that position. A Z-Stack is then acquired at the new position when starting the experiment. Hitting **Center** moves the **Center** position back to the actual **Focus Position** setting the **Offset** again to zero.

Use ![C], ![L] and ![F] to get images from these three specific positions. The **Focus Position** updates accordingly. The **Offset** value then might be set to half of the Interval depending on the Interval value and the number of slices, as with a lower number of slices or a large Interval the real center position of the stack might be just between two slices.

Range Select produces a XZ-scan within the **Range** of the Z-Stack. This helps setting the Range of the Z-Stack without continuously scanning complete frames. The position of the first and last slice is indicated with a red line, the **Focus** position, which initially corresponds to the Ceter position, is indicated as a green line.

Changing the **Offset** value moves the red lines in parallel to the new offset position, the green line, the **Focus Position** is kept constant. ![C] sets the **Focus Position** to the indicated **Center** position setting the **Offset** back to zero and producing an image at the newly defined Focus Position. Alternatively hitting Center sets the indicated Center position back to the actual Focus Position moving the red lines back to the original position and setting the Offset back to Zero. The original values for Number of Slices, Interval and Range are always kept constant.

The red lines can be moved independently from each other allowing an optimal adjustment of the Z-Stack range to the specimen. **Center** sets the green line to the center position between the red lines and adjusts the Focus Position accordingly. The original values for **Number of Slices** and **Range** are adjusted. The **Interval** is kept constant.

☞   The size and position of the Line where the XY scan should be performed can first be defined using the line scan function in the Acquisition Mode tool.

4.5.3.1.3 How to Proceed Setting a Z-Stack Using Piezo Mode

The Piezo objective focusing device allows stacks to be produced considerably quicker than via the focus of the microscope stand. Clicking on the **Leveling** button in the **Focus** tool moves the Piezo objective focus to the zero position, while the microscope focus moves into the opposite direction at the same time, i.e. the position of the object in relation to the objective remains unchanged. This function is used to set defined initial conditions.

* Define the first and last slice using **Start/End** mode. Activate **Piezo** mode (Fig. 4-58). The total Range of the Z-Stack is limited to 250 µm when using the Piezo device. Adjusting the value for **Number of Slices** or **Interval** is independent form each other and affects the Range of the stack. The Focus Position is not changed.

Fig. 4-58   Z-Stack tool Piezo mode with Z-Stack Navigation displayed

When changes are made to **Interval** or **Number of slices** in **Start/End mode**, the values are taken over for the **Piezo** mode provided they are within the Piezo objective focus work range. This also works in the opposite direction.

Range Select produces a XZ scan as described above. Dragging the green line moves the whole stack updating the **Focus Position** accordingly. Dragging a red line changes the **Number of Slices** and therefore the **Range** of the stack keeping the **Focus Position** unchanged. The second red line is set to keep the green line in the center of the stack.

The scanning **Direction** for acquiring the stack using the Piezo can be performed unidirectional or bidirectional. This means the data acquisition is done also when the focus is moving backwards to the initial start position of the stack. This only applies if multiple stacks are acquired in a time series. For one stack only the direction settings have no effect. A correction slider **Corr Z** is available to correct for possible shifts of the stacks in Z.

Fig. 4-59   **Optimize Sectioning and Step: Optimal Interval is set starting with one Airy unit for all channels**

Fig. 4-60   **Optimize Sectioning and Step: Match Pinhole to Step resulting in equal optical sections for all channels**

4.5.3.1.4   Optimize Sectioning and Stack

The **Optimize Sectioning and Step** function helps to set the **Optimal** for a given excitation wavelength, detection range and used objective. If more than one channel is used, the optimal interval is determined for each channel independently. For given pinhole diameters, half the value of the currently smallest optical section (typically of the channel which detects the shortest wavelength range) is used to determine the optimum interval. For example if the optical section for the channel detecting DAPI is calculated as 1.5 µm, the optimal interval is 0.75 µm (Nyquist criteria). It is recommended to start with setting one airy unit (1 AU) for all channels in the **Channels** tool before starting to optimize.

☞   Note that the actual values might slightly vary around these theoretical values.

This now produces stronger overlapping optical sections for the channels detecting longer wavelength ranges. The optical sections are not identical and the image data acquired are redundant. Especially for analyzing co-localization of signals from different channels the optical sections have to match.

The function **Match Pinhole** sets all pinholes to match this optimal interval. The pinhole diameter is set such that the values of the resulting optical sections from the different channels are identical and have double the value of the optimal interval.

☞   Note that the settings of the pinhole might not result in matching optical sections. This is then due to the chosen objective which would require a smaller setting of the pinhole that is mechanically not possible. In this case chose a higher magnifying objective or start with a larger pinhole setting for the channel detecting the shortest wavelength range.

Undo resets the just before altered value.

X:Y:Z = 1 matches the settings in Z to the settings in X and Y. This produces a cubical voxel.

The **Refractive Index Correction** considers the different refractive indices between the immersion medium of the objective (n') and the embedding medium of the specimen (n), which can be set between 0.5 and 3.

4.5.3.1.5 Correction

The **Refractive Index Correction** considers the different refractive indices between the immersion medium of the objective (n') and the embedding medium of the specimen (n), which can be set between 0.5 and 3.
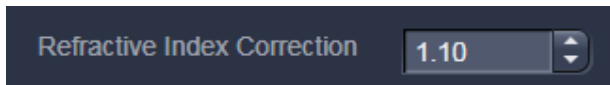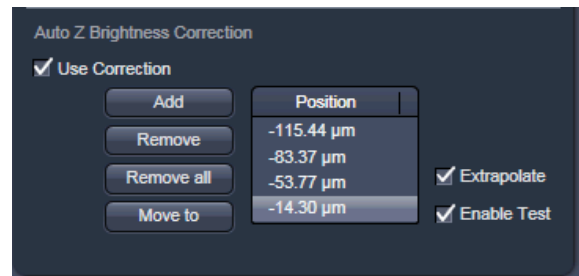
Fig. 4-61   **Auto Z Brightness Correction**

$$Ratio = \frac{n}{n'}$$

Auto Z Brightness Correction (Fig. 4-61) allows setting of different values for the scan parameters **Detector Gain**, **Ampl. Offset**, **Ampl. Gain** and **Laser Attenuation** of up to 10 freely selectable slices within a Z-Stack. During the scan procedure, the values of these parameters are automatically linearly interpolated between the neighboring positions within the Z-Stack. This function can be activated and deactivated with the check box **Use Correction**. **Add** takes the above mentioned parameters and correlates them with the current Focus Position which is then added as a **Position** in the list (for example the first slice of a stack). Adding a new position with a defined set of channel parameters cannot be done while scanning. The positions don't need to be listed in a specific order, the program will sort the values automatically in the background. **Move to** moves the actual focus to the highlighted **Position** in the list without scanning. Starting a **Continuous** scan then allows setting or correcting the parameters and storing them again hitting **Add**. Highlighted positions can be removed (**Remove**) or the whole list can be removed (**Remove all**). The interpolation between the **Positions** in the list can be extrapolated (check the box next to **Extrapolate**) to the actual first and last slice of a Z-Stack if those are not part of the range of the **Positions** in the list.

When **Enable Test** is active, scanning at single positions of the stack or while focusing through the stack is done using the settings of the **Position** list or the interpolated values calculated for the respective focus position. This allows controlling the settings before starting the actual acquisition of the stack.
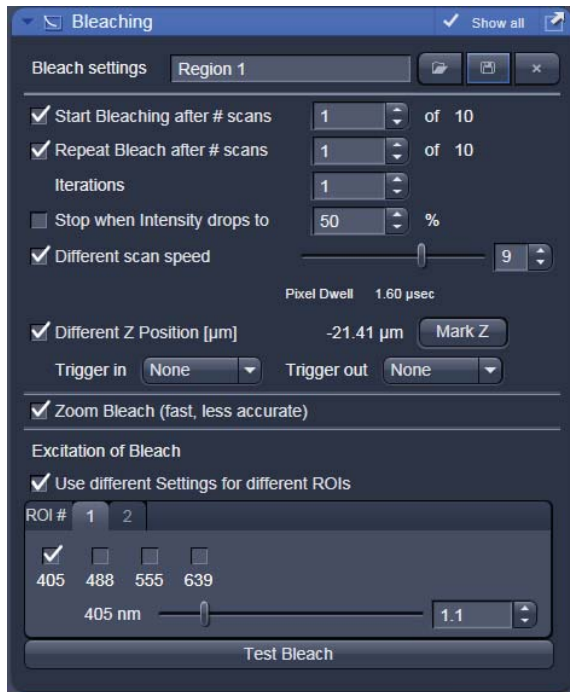
Fig. 4-62   Bleach Control window

4.5.4   Tool Group Multidimensional Acquisition: Bleaching

The use of this function permits setting the bleach parameters using the LSM 510 for bleaching in spot, line or frame mode.

The **Bleach Settings** can be saved under a name and reloaded for later use. Existing settings can also be deleted from the list.

Bleaching works in conjunction with the acquisition of a series of images over time. During the sequential imaging of frames, lines or a spot, bleaching of the specimen can be performed according to the bleach settings defined in the Bleaching tool.

Start Bleaching after # scans expects a number, which indicates the amount of scanned frames, lines, or spots after which then the bleaching of the sample happens.

Repeat Bleach after # scans expects a number, which indicates the amount of scanned frames, lines, or spots after which then the bleaching of the sample is repeated.

The repeated bleaching also allows bleaching the sample to an intensity value calculated as % of the initial intensity within the region to bleach. To achieve this indicate the percentage value in the box next to: **Stop when Intensity drops to**.

The number of **Iterations** indicates the total amount of scans which are performed for bleaching the selected region (see also section 4.5.2.10) during each bleach process.

A **Different scan Speed** for the bleaching process can be set independently from the scan speed which is used for imaging. A lower speed results in a longer pixel dwell time, which increases the efficiency of bleaching.

Different Z Position: If this option is active you can set the current stage position as the one in which the bleaching will be done by clicking **Mark Z**. This function is only available using the **Line** or **Frame** scanning mode.

When the imaging is performed in **Spot Mode** (chosen as acquisition mode in the Acquisition Mode tool), a **Different XY Spot Bleach Position** can be selected. Click **Spot Select** in the **Acquisition Mode** tool. A new image appears with a green **crosshair**. When clicking and dragging the crosshair a red crosshair becomes visible. The red crosshair marks the spot that is imaged (This is also indicated in the **Scan Area** of the **Acquisition Mode** tool. The green crosshair marks the spot that is bleached. Move the center of the crosshairs to the desired positions.

Use one out of four triggers with **Trigger In** to trigger the bleaching process. In addition, one out of four triggers can be used as **Trigger Out** signal to control an external device. (For a detailed description on the trigger use and interface see Chapter 7 ANNEX).

If a region is used to define a position for bleaching within the sample, the bleach process can be accelerated by checking the box next to **Zoom Bleach (fast, less accurate)**. In this case the scanner movement will be restricted to the bleach region zooming in onto this region. This may result in a less accurate positioning of the region as the definition of the region has been made in a different zoom in the image. The bleach process will be faster than without this option. The gain in speed is dependent on the speed that is used for bleaching. If a high speed has already been chosen for bleaching the gain in speed will be minimal.

Define the region(s) for bleaching in the Regions tool (see section 4.5.2.10). Only a region activated under Bleach is considered for bleaching. In the **Excitation of Bleach** area (Fig. 4-63) chose the laser line and laser intensity which is then used for bleaching.

It is possible to choose different laser lines and/or laser intensities for the bleach process of each region when activating the option: **Use different Settings for different ROIs**. A maximum number of six regions is possible. The numbers of the regions correspond to the numbers indicated next to **ROI#**. For each region, a tab is active which allows setting individual laser parameters for bleaching.
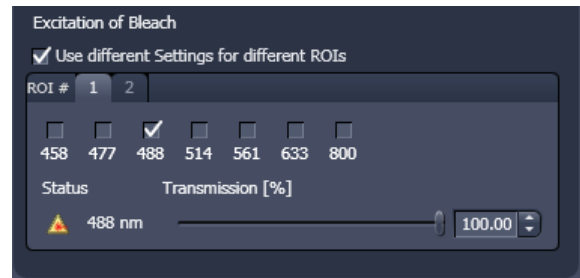
Fig. 4-63   **Excitation of Bleach area for setting laser line and intensity for bleaching**
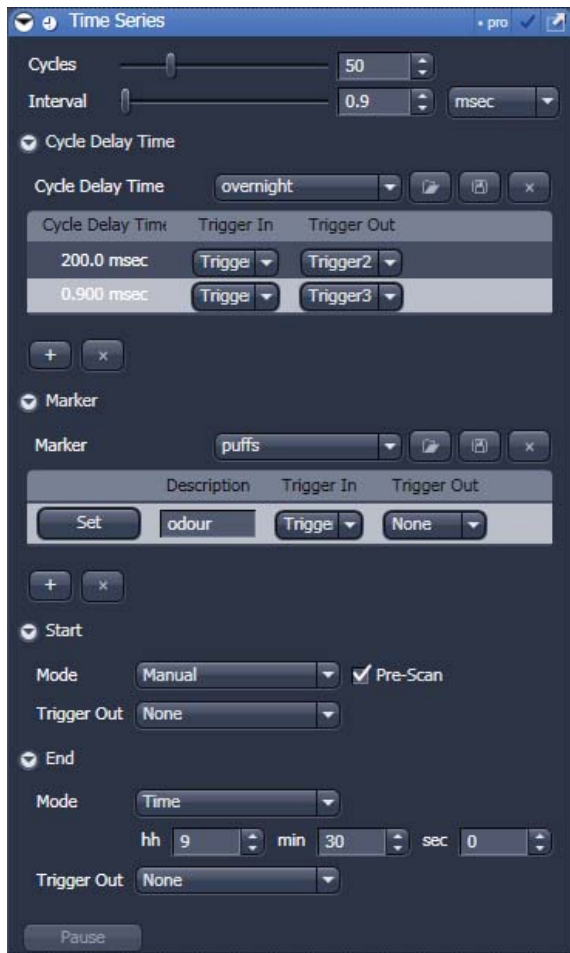
Fig. 4-64   **Time Series tool**

4.5.5   Tool Group Multidimensional Acquisition: Time Series

In the **Time Series** tool the parameters for the sequential acquisition of image frames and lines as time series are defined. Imaging in spot mode is only possible in combination with the settings of a time series acquisition. The options in this mode within the Time Series tool are limited and explained below.

The **Time Series** function for frame and line scan offers multiple functions for creating an image series.

To set up a simple time series acquisition it is enough to define the number of **Cycles**, which corresponds to the number of frames or lines, imaged. A maximum value of 100,000 can be set. The amount of data that can be handled by the system is limited. If the maximum amount is exceeded a warning will appear. In this case, it is necessary to either change the destination for data storage and/or reduce the amount of cycles. The limit also depends on the imaging setup and the acquisition mode. Note that it is an upper limit of handling data files but this is beyond the value of 100 Gigabits.

Interval defines a potential time interval, set in milliseconds (ms), seconds (sec) or minutes (min), to separate the beginning of two cycles. A time shorter than the scan time for a frame or line is not effective. The interval time can be changed while scanning.

A **Interval Time** is a potential time interval between the end of one cycle and the beginning of the next cycle. The time and time unit for the delay time is set using the slider and input box of time interval. A cycle interval time is not effective if a cycle delay time is defined (interval is always included in the delay). A total of six different delay times can be defined by adding further lines clicking on the + button. The delete function deletes the currently highlighted delay time. Each delay time can be controlled using the **Trigger** function. If no trigger is set the highlighted delay time will be effective for the time series acquisition. Additionally it is possible to send a trigger signal out at the start of each delay time. Settings for cycle delay times can be stored and reloaded. The delay time can be changed while scanning.

The **Marker** functionality provides the possibility to indicate the start or end of an external process in the data of the image series by connecting this external process to the selected **Trigger In** for that Marker. The **Description** of the Marker is then visible in the image data using the Mean ROI functionality or the image gallery display mode (see section 4.7.3.1). A **Trigger Out** signal can be set with each defined marker. By clicking **Set** a marker is set manually at any time during scanning. A maximum of seven different markers can be defined using the + button. The delete function deletes the currently highlighted marker. Settings for markers can be stored and reloaded. When the image series is stored, all the markers, including the time indication and the comments, are stored along with the image contents.

The markers visible in the image series, have different colors with the following meaning:

- **red**:   manually set marker with time indication and comments
- **blue**:   automatically set marker with change of delay
- **green**:   automatically set marker at the beginning and at the end of a bleaching procedure

The acquisition of a time series is started using the **Start** action button.

Pre-Scan   If this box is checked, a continuous scan is performed when clicking **Start**, but no images are acquired until you hit the button ▮ Go ▮. This button becomes visible next to **Pause** in the lower part of the control window.

Alternatively, you can start the acquisition using a **Trigger In** or using the system **Time** of the computer. In any case it is possible to send a **Trigger Out** signal with the start of the times series.

When starting a Time Series via Trigger or Time the message **Waiting for Trigger** or **Waiting for Start Time** will then be displayed in the status line when hitting **Start**.

Pause   This function interrupts the Time Series. The labelling changes to **Resume**. The current scan finishes with the end of the frame or line. When Resume is clicked, the Time Series is immediately continued.

The same options as for starting a time series apply for ending a time series.

Focus Stabilizer   This check box activates the Definite Focus unit.

If the time interval between two time points is below 11s – the focus stabilizer will not be activated between the two time points.

☞   If the entered number of cycles is processed without a set trigger impulse been given to end the procedure, the Time Series is finished.
If a trigger signal to end the scan arrives before the cycles have been processed, the Time Series will only be interrupted. **Waiting for Trigger** will be displayed in the status line. The Time Series can now be continued via a new trigger signal or ended via **Stop**.

☞   A Time Series is finished when the end **Time** is reached.
If the entered number of **Cycles** has been processed, the Time Series is finished before the end Time. If the number of cycles has not yet been processed until the end Time, the Time Series is also finished. The end time for the Time Series can be changed online. Bleach times will be added to the total time for image acquisition.

☞   If a time series is interrupted before its programmed end, the programmed number of images will be taken over in the database. However, only those images are stored which were created before interruption of the time series. This is due to the fact that the original image parameters are to be taken over via the **Reuse** function.
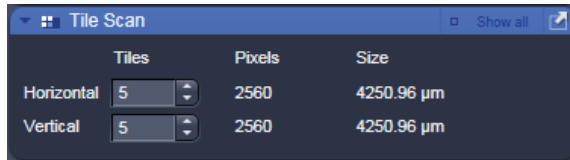
Fig. 4-65   Tile Scan tool

4.5.6   Tool Group Multidimensional Acquisition: Tile Scan

This function permits a frame to be created as an overview image of the specimen. The size of the final tiled image depends on the settings for the image format set in the **Acquisition Mode** tool and the number of tiles.

The application of the **Tile Scan** function requires an objective with a minimum magnification factor of 2.5x.

Indicate the number of **Tiles** in **Horizontal** and **Vertical** direction. A maximum of 100 tiles can be set. According to image format the number of **Pixels** changes. According to the objective used for imaging the **Size** of the Tile Scan changes.

In case the stage does not move exactly horizontal to the scan field a **Rotation** value can be set to correct for this (available in **Show all** mode). This can either be calibrated beforehand using a grid slide or any other specimen which displays clearly visible structures in horizontal direction. Acquire smaller tile scans to see the effect of a specific value for Rotation until you find the right one. This value should be taken for the Rotation. Alternatively use the Tile Scan Rotation Macro which provides an automatic Rotation calibration function. Take over this value to the main software before acquiring the tile scan.

When **Bi-directional** is active (available in **Show all** mode) the image acquisition is performed also on the horizontal backwards movement of the stage.

The Tile scan is created around the absolute starting position of the stage.
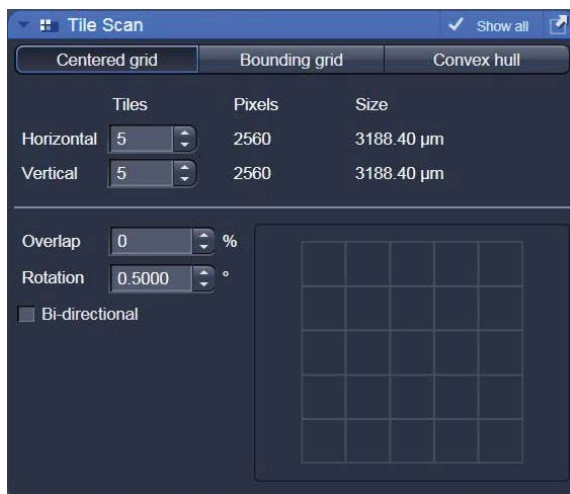
Fig. 4-66   Tile Scan tool with StitchArt*plus*

Tile Scan with StitchArt*plus*

The StitchArt*plus* license allows creating tile scans with overlap. The overlap can be used to stitch singe 2D images or Z-Stacks three-dimensional.

Centered grid: The current position will be the center of the tile scan.

Bounding grid: Positions that should be part of the tile scan have to be marked using **Add**. Using these positions a bounding grid is created, which finally defines the dimensions of the tile scan.
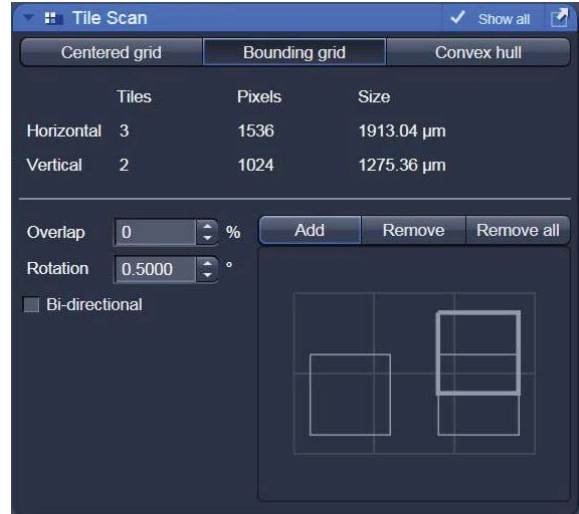
Fig. 4-67   Tile Scan: Bounding grid

Convex hull: Positions that should be part of the tile scan have to be marked using **Add**. Using these positions a convex hull is created, which finally defines the dimensions of the tile scan.
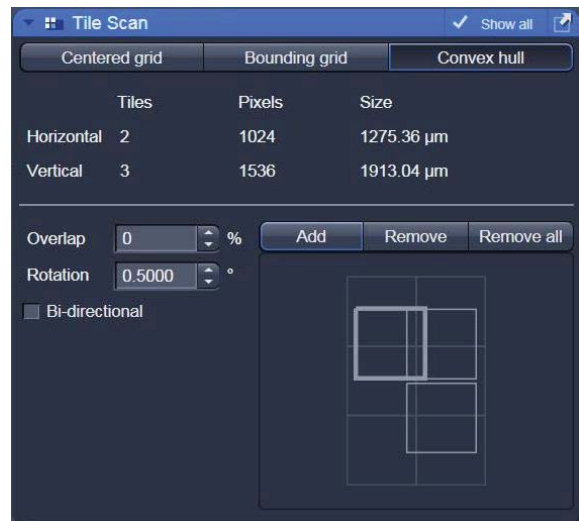
Fig. 4-68   Tile Scan: Convex hull
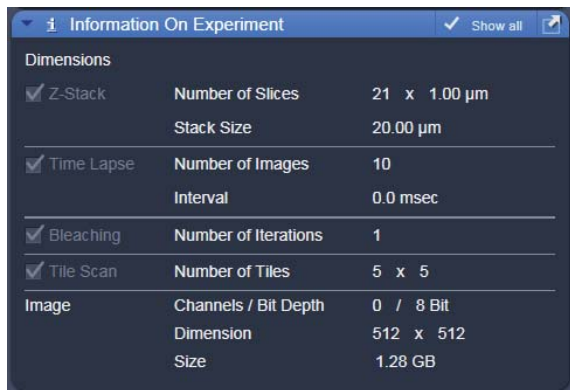
Fig. 4-69   **Information on Experiment**

4.5.7   Tool Group Multidimensional Acquisition: Information On Experiment

This tool displays the information of the current experiments in terms of the acquisition mode and type of data produced. In addition this tool allows controlling the use of the multidimensional acquisition modes. When an acquisition mode is selected with the check box, the acquisition mode will be considered. If not, it will be ignored. The check boxes correspond to the check boxes on the individual tool bars. Thus the control over the use of multidimensional acquisition functions is presented in an easy to use overview. Additional information on the potentially acquired image or image series is displayed.

4.5.8   Tool Group Multidimensional Acquisition: Positions

This function comprises two main applications. First, it is used to take images of a specimen at freely defined positions. Second, it is used to image specimens, typically cells, which are grown in sample carriers like plates with a set number of wells.

Fig. 4-70   **Positions tool, Position List tab; sub-tab Scan Overview Image**

Using the first tab, **Position List** (Fig. 4-70), provides the following functions:

Clicking **Add,** adds the current position of the scanning stage to the list of positions. Highlighted positions can be removed (**Remove**) or the whole list can be deleted (**Remove All**). It is also possible to move the stage to the highlighted position clicking the button **Move To**.

The list of positions is consistent with the positions defined in the stage tool (see section 4.5.2.9).

Note: When the list of positions is deleted in the **Position list**, it is also deleted in the Stage tool. This is not the case the other way round.

For the image acquisition a method for **Auto-Focus** can be chosen from the drop down list which is either based on the reflection from the cover slip or the fluorescence of the sample. If **Reflection** is selected the system determines the imaging configuration for the auto-focus. If **Fluorescence** is selected, the auto focus looks for the brightest fluorescence signal using the present imaging configuration.

Scan overview image opens a new interactive window (Fig. 4-70) to define the number of **Tiles**, the **Objective** (chose from the drop down menu) and a potential **Zoom** factor with which to scan an overview image. This overview image can then be used to navigate in the sample (i.e. define positions for imaging). **Scan** starts the tile scan, **Cancel** closes the window without further action.

The **Offset** defines the distance between the actual position of the sample in Z and the position of the cover glass, which is determined with **Auto Focus** method **Reflection**. This offset can also be determined automatically. For this, focus onto the sample to be imaged then click **Find current position offset**. The value for the determined offset will then be written into the editing field. To test the performance of the Auto-Focus method (if Reflection is chosen, also the value for the Offset will be taken into consideration), defocus the sample and click **Test**. The auto-focus procedure will be performed at the actual position and an image of the sample will be on the screen when completed.
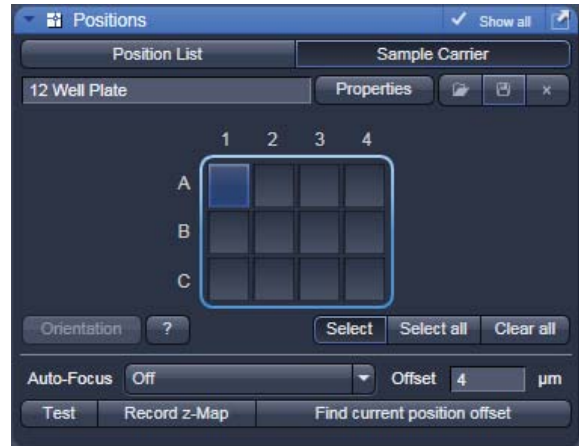
Fig. 4-71   **Positions tool, Sample Carrier tab**

Using the second tab **Sample Carrier** (Fig. 4-71) provides the following functions:

Define the sample carrier by clicking on **Properties**. This opens the interface to determine the number of **Columns** and **Rows** of the sample carrier and the **Distance** in mm between the individual wells which is assumed to be equidistant within rows or columns (Fig. 4-72).

To assign the actual position of the stage with a position in the sample carrier, move to the center of a well in the actual plate under the microscope then click **Orientation** followed by clicking the field in the sample carrier representing this well of the plate. This assignment will be kept until changed repeating this procedure.

The positions to be scanned in the sample carrier can be selected. Click **Select**, and then click onto the wells where images should be acquired. With **Select all**, the whole sample carrier will be imaged. **Clear all** deselects all positions.
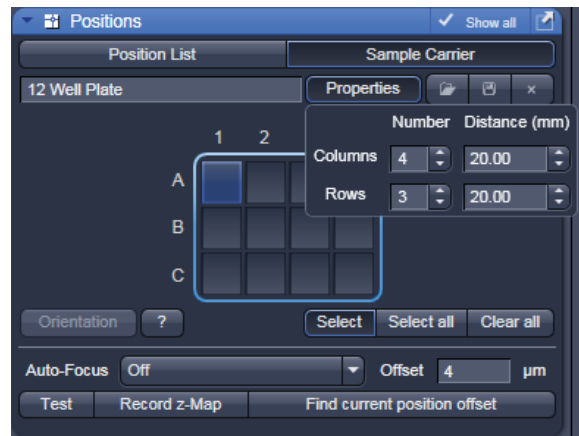
Fig. 4-72   **Positions tool, Sample Carrier tab, Properties interface**
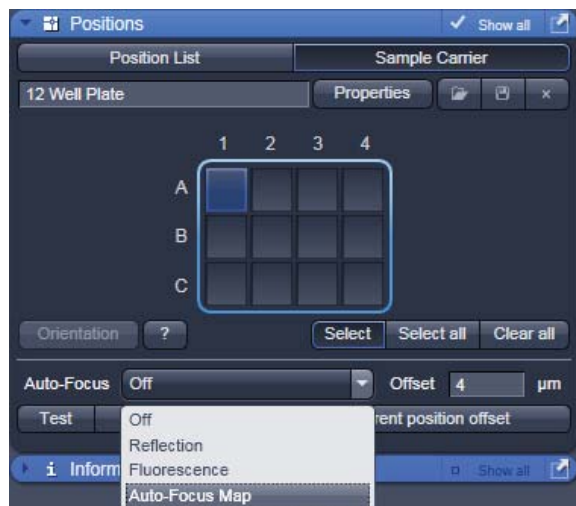
Fig. 4-73   Positions tool, Sample Carrier tab, choice of methods for Auto-Focus

For this application and additional method for Auto-Focus is available: Auto-Focus Map (Fig. 4-73).

When this method is chosen, click **Record z-Map** for the system to define the auto-focus position for each of the positions in the sample carrier. This creates a **Z-Map** of the bottom layer of the sample carrier. This map is used to keep the sample in focus until another sample carrier is chosen or the carrier's orientation is changed.

4.5.9   Image Optimization

This section describes an example how to acquire an image, using an excitation wavelength of 561 nm and a fluorescence emission range above 570 nm. Use the MBS 488/561 as the main dichroic beam splitter. We presume that no predefined configuration is available for this imaging task.

The description applies to the use of any microscope.

4.5.9.1.1   Requirements:

- Position the specimen under the objective and focus onto the desired imaging plane.

- The first steps define the Imaging parameters.

- Activate the main tool tab **Acquisition** and open the Imaging Setup tool. Chose Channel Mode for the image acquisition and check the box next to Track1 (Fig. 4-64).

- Switch on the HeNe Laser 543 nm.

- Open the **Light Path** tool.

- Click on the laser icon, select the 543 line, and set the transmission slider to about 10 % (this laser has an output power of only 1 mW, other lasers have up to 25 mW depending on the type of laser and the imaging device used; be careful when setting the power for imaging with other lasers for imaging other dyes). Make sure other lasers are not checked (Fig. 4-75).

☞   Optimize the laser intensity subsequently via the **Transmission** slider when imaging.
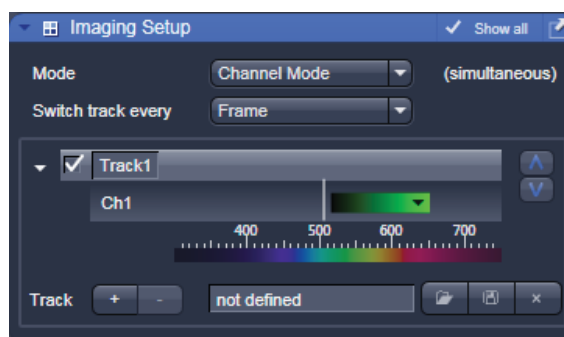
Fig. 4-74   Imaging Setup tool – imaging mode selection and track activation
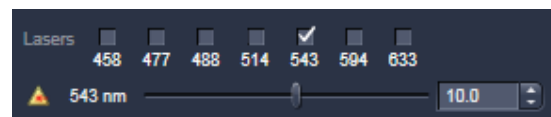
Fig. 4-75   Laser line and laser power selection

Fig. 4-76   Channel activation and color selection

- Activate channel 1 (or ChS) via the check box. You may want to assign a different color to the channel which will be taken over for the initial display of the image. Click the channels icon to open the color table and select the desired color (Fig. 4-76).

- When using ChS move and adjust the slider to set a detection window.

- If required, deactivate all the other channels (Ch2, 3, monitor diode, transmission, R1-2) via check box.

- Click on the icon of the main dichroic beam splitter and select **MBS 488/543** or any other HFT indicating the 543 line.

Fig. 4-77   Light Path tool window

The beam path in the Light Path tool now displays the way the potential emission signal takes. Channel S receives the red part of the spectrum. The settings are suitable for imaging dyes like Rhodamin, TRITC or Cy3.

For overlaying fluorescence and transmitted-light images, click on the **T-PMT** (Transmission) button in the **Beam Path and Channel Assignment** panel.

All transmitted light applications like

- phase contrast

- differential interference contrast (DIC)

- polarization contrast (Pol)

- darkfield

can be performed if the hardware of the microscope is equipped with the necessary optics. We recommend DIC as standard contrast because the suitable objectives have a higher transmission than the ones for phase contrast. This enhances detection sensitivity.

- The **Imaging Setup** tool now shows the active channel and the detection range for the signal. You may want to save these settings as a specific imaging configuration (Fig. 4-78).
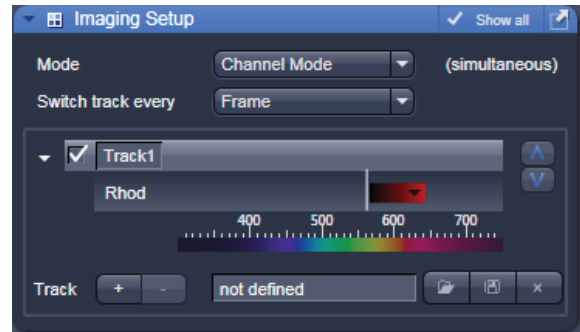
Fig. 4-78   Imaging Setup tool

4.5.9.1.2   Define the Scan Parameters:

- Open the Acquisition Mode tool (Fig. 4-79).

- Select **Frame** mode and a **Frame Size** of 512 x 512 to start with.

- The **Optimal** button automatically adjusts the frame size to match the magnification and resolution of the objective. Thus, images are acquired without collecting redundant image information.

- The Scan Speed is set to 9 per default. A lower speed produces better images as the light collection per pixel is higher. Use a speed from 7 to 9 to begin with.

Fig. 4-79   Acquisition Mode tool

- Start with the following settings on the **Averaging** panel:
  Bit depth: 8 bits
  Average: Number: 1 (no averaging)
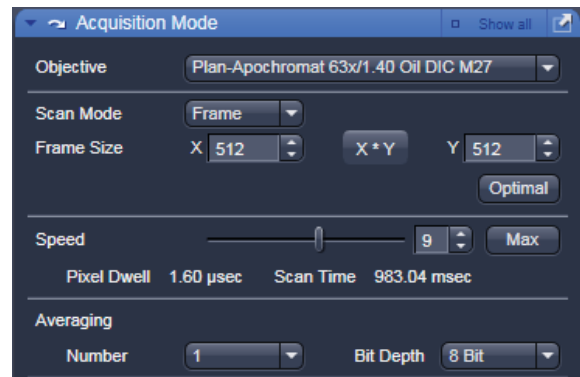
☞   Additional parameters like scan direction are displayed in **Show all** mode. Scan direction is per default set to unidirectional.

☞   The default settings for Acquisition Mode are suitable for good image quality and do not need to be changed for initial scanning.

☞   Using the **Live** button is a convenient way of creating an overview scan.
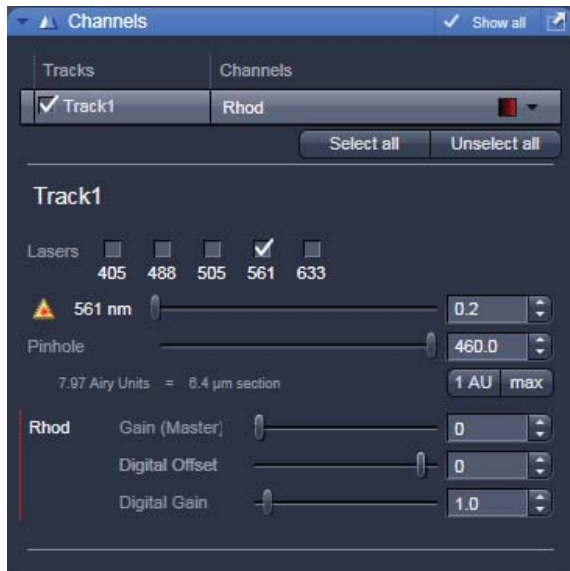
Fig. 4-80   **Channels tool**

4.5.9.1.3   Define the Detection Parameters:

- Activate the **Channels** tool (Fig. 4-80).

- Set the **Pinhole** to one Airy Unit clicking **1 AU**.

  – The pinhole diameter should be so small that there is still enough variation for the setting of the Gain (Master) and that sufficient image information is still available. 1 Airy is a good value to enable a confocal fluorescence XY-image to be obtained.

  – A small pinhole diameter will increase the depth of focus, but reduce the light intensity received by the PMT photomultiplier (for reflection mode confocal images start with a pinhole value of 0.5 Airy Units).

- Use the **Auto Exposure** button.

  – The system determines the settings for the Gain (Master) and digital offset to match the scan speed, pinhole size and laser power.

  – Finally, the system builds up the image.
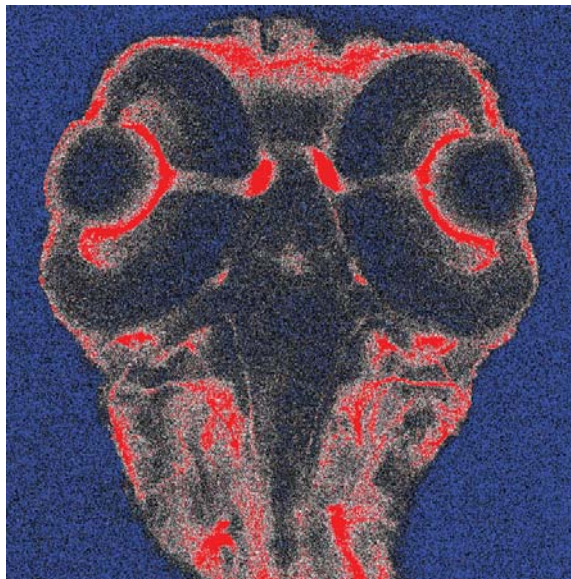
Fig. 4-81   **Image displaying bad adjustment of gain and offset (blue regions are under-exposed, red regions are overexposed)**

4.5.9.1.4   How to Optimize Image Quality:

As a rule, the first scanned image is not ideal, since the settings of the laser and therefore also the photomultiplier might not match the light output. More often than not, the screen image is dull and needs subsequent optimization.

- Use the lookup table **Range Indicator** (see section 4.6.2.1). This display mode shows overexposed regions in red and underexposed regions in blue. Some red and some blue pixels should be visible to ensure the signal is spread over the dynamic range of the detector and no image information is lost.

- Start a **Continuous** scan and quickly adjust the laser power, Gain (Master) and offset to set the image into the dynamic range.

- Try to lower the impact of laser light onto the sample. Reduce the laser power and adjust the gain accordingly.

- To adjust the black level (background), use the **Digital Offset** slider so that areas without picture content just show a trace of blue.

- If necessary, re-amplify brightness with the **Ampl. Gain** slider.

- Stop scanning.

- Save the imaging configuration again as this time it will also save the correct settings for the detector, the laser, and the pinhole.

The signal-to-noise ratio can be substantially improved by reducing the scan speed to an acceptable level and averaging over several scans (i.e. with an average **Number** greater than 1 for the **Mean** average **Method** in the **Scan Control** window).

- Use the **Speed** slider in the **Acquisition Mode** tool set the slowest acceptable scan speed.

- In the **Number** text box of the **Averaging** panel enter the number of images to be averaged.

Fig. 4-82   **Image displaying good adjustment of gain and offset**

☞   Image optimization can be achieved faster if you select a smaller frame, since less data have to be processed.
The greater the number of averages selected using **Mean** as average **Method**, the better the image quality will be; the scan time is prolonged accordingly.

A similar procedure applies for image optimization if more than one channel is used and simultaneous image acquisition is performed.

Make sure the suitable lasers are switched on and the channels are activated.

Pay attention to address the right channel when pinhole size, detector gain, and amplifier offset are controlled as these are set individually for each channel.

Use the split view (see section 4.6.3) to see the effect of the controls onto each channel separately.

If the acquisition is performed sequentially in with multiple tracks it is faster to activate only one track at a time for image optimization.

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4.5.10   Processing Tab

4.5.10.1   General Structure of the Processing Tab

The functions of the **Processing** tab are designed to cover a large range of Image Processing methods to process and analyze already stored as well as just scanned images including mathematical operators and algorithms.
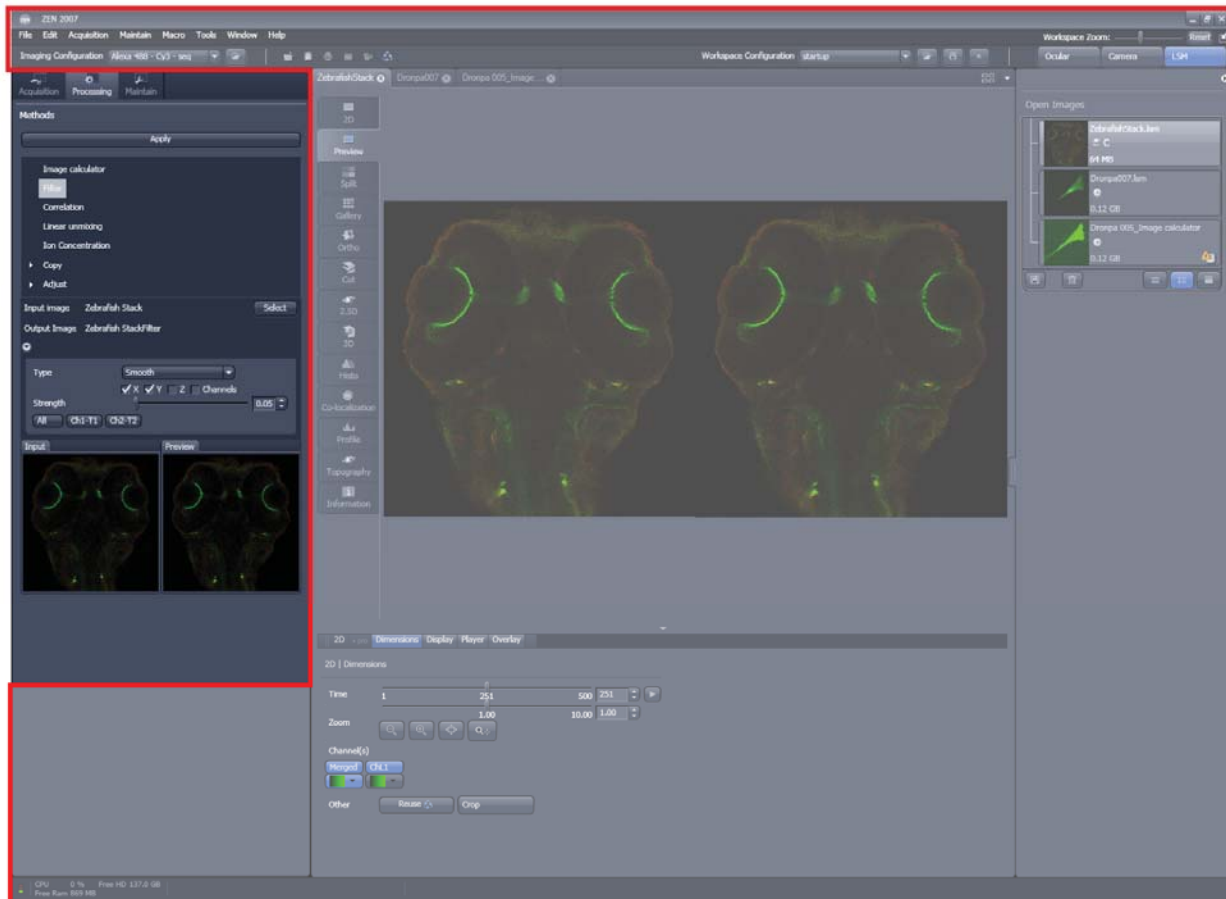
Fig. 4-83   The Main Application window with the Processing main tab activated.

By clicking on the third of the four Main tool tabs in the top left corner of the **Main Application** window (see Fig. 4-83), the list of available processing tools becomes available.

The **Processing** tool tab is structured as follows (see Fig. 4-84): On the top, under the **Main** tool tab, is the **Processing** tab header with the [Apply] button. To process a loaded image, first select the required methods from the List of available processing methods (Fig. 4-84); select the active image as the input image with the [Select] button(s). The thumbnail panel at the bottom of the **Processing** tab now shows thumbnails of the selected input images and a preview of the processing result. In the method-specific settings, all the parameters for the active methods can be set, edited or viewed.

When an input image in a processing tool is selected, in addition to the **Preview** panel at the bottom of the **Processing** tab, a new **View** tab – the **Preview** tab - in the Centre Screen Area is opened. The **Preview** View tab shows the preview in more detail, while the preview thumbnail is always displayed in the **Processing** main tool tab, even when the view in the Image Display is changed. If in doubt which Image is selected as processing input, always refer to the displayed thumbnails.

The Preview – in the thumbnail as well as in the automatically generated Preview View tab in the centre screen area – is immediately updated.

When everything is set, click [Apply] on the top of the **Processing** tab and the set processing action will be performed.

All actions in the **Processing** tab automatically generate a new image document if the Processing Output is not explicitly directed to an already existing channel or image.
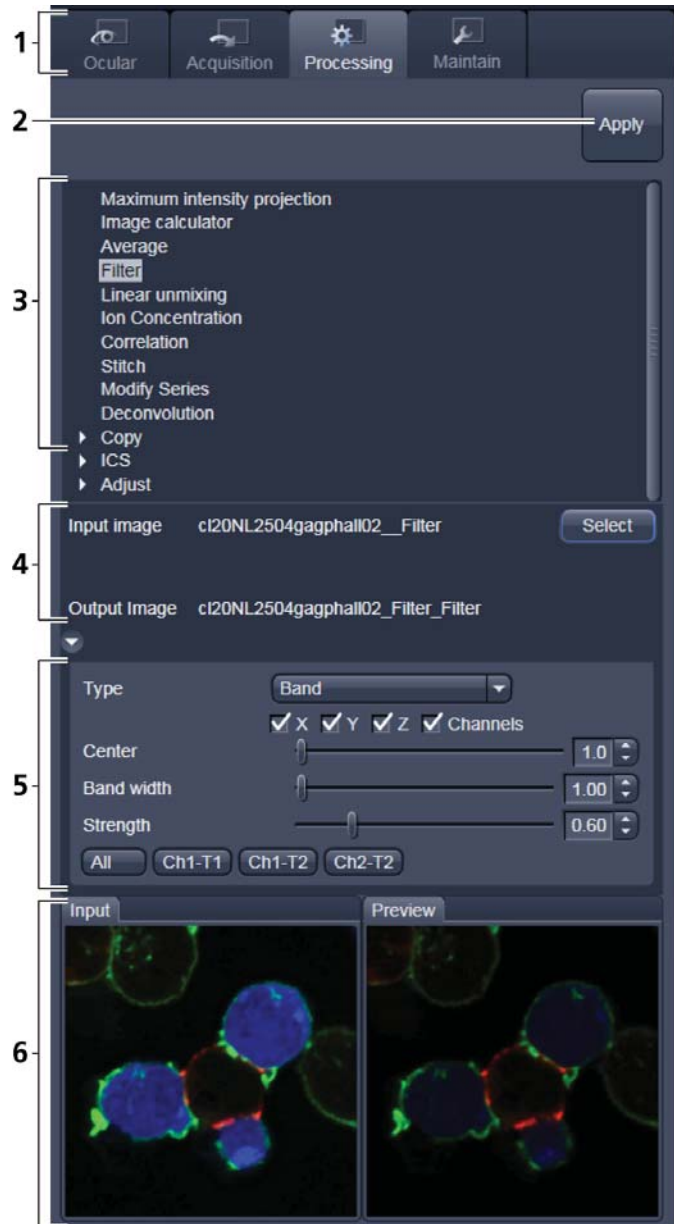
1 Main tool tabs
2 Apply button
3 Available processing methods
4 Input image selection panel
5 Method-specific settings
6 Input and preview thumbnails

Fig. 4-84   The Process tab structure

Fig. 4-85   Maximum Intensity Projection tool

4.5.10.2   Maximum Intensity Projection

The **Maximum Intensity Projection** function generates a maximum intensity projection image along the z-, time- or channel dimension of a multidimensional image data set (Fig. 4-85).

To open the **Maximum Intensity Projection** Processing tool, select it from the list in the **Processing** main tab.

Select the **Input Image** with the [Select] button and choose the dimension along which you want to generate a maximum intensity projection from the **Coordinate** pull down menu (x, time, channel). Clicking the [Apply] button creates the maximum intensity projection in a new image document.

The maximum intensity projection creates an output image each of whose pixels contains the maximum value over all images in the stack at the particular pixel location.

4.5.10.3   Image Calculator

The **Image Calculator** tool provides a calculator-style interface to apply arithmetic operators to the selected image.

- To open the **Image Calculator** tool (Fig. 4-86), click on the **Image Calculator** in the list of methods in the **Process** main tool tab.

  – This opens the **Add** window.

  – Click on the **Close** button to quit the **Add** window.

The two ⬚ **Select** buttons in the selection panel select the active image in the **Image Display** as Input Image and Input Image 2. The Output Image is automatically fed into a new image document. A specific input channel from a multi-channel image or series can be selected with the pull down menus next to the **Select** button.

The ⬚ **First images 3** option reduces the Input 2 Image series to the selected first frames of a time series. This can be used to normalize a time series to the values of its first image(s).

After loading 2 input (source) images and specifying the input channels, the calculator keyboard allows the definition of an operator or a combination of operators which is represented by a formula in the formula display. The operators will be inserted into the formula at the cursor position. The formula can also be typed in directly. For an overview of the available more advanced operators, please refer to the formula panel that is shown in a pop-up window after clicking the ⬚ **Operators ...** button (Fig. 4-86).

By highlighting the selected operator a description of the function of this operator is displayed in the lower part of the operators list (Fig. 4-87).

All image calculations in this tool work pixel by pixel and start with the upper left pixel regardless of the image size of the two images or image series.

Fig. 4-86   **The Image Calculator window**

☞   When choosing images of different data depth the check box next to **Intensity range 1...0** should be marked. This normalizes the image intensity for all images to values between 1 and 0.
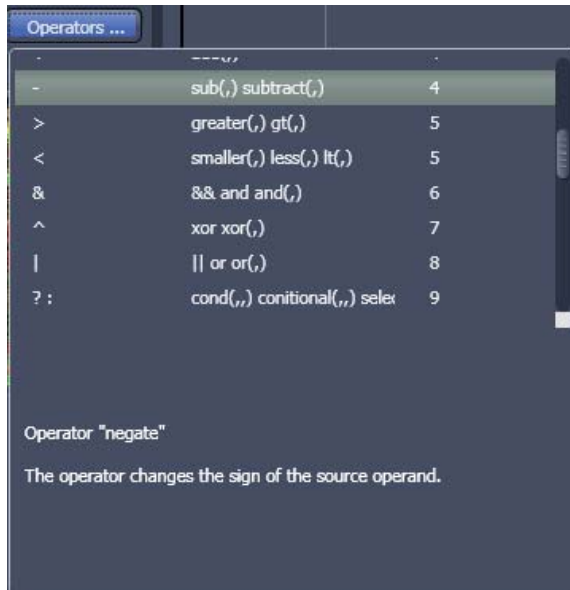
Fig. 4-87   Operators window

All actions performed in the Image Calculator have immediate effect on the previews. After pressing the **Apply** button on the top of the **Processing** main tab**,** a new image document with the resulting image is crated.

A good example for an application of the Image Calculator is offsetting/normalizing two images or an image series with a reference image to enhance contrast for small changes in intensity:

- Select a time series as **Input 1** and the first frames of this series (or another offset image) as **Input 2**.

- Then type/edit an operator as shown in figure Fig. 4-88 that enhances the relative changes in the image with respect to the relatively bright signal level, for example:

S1-S2/2 = source 1 – ½ * source 2.
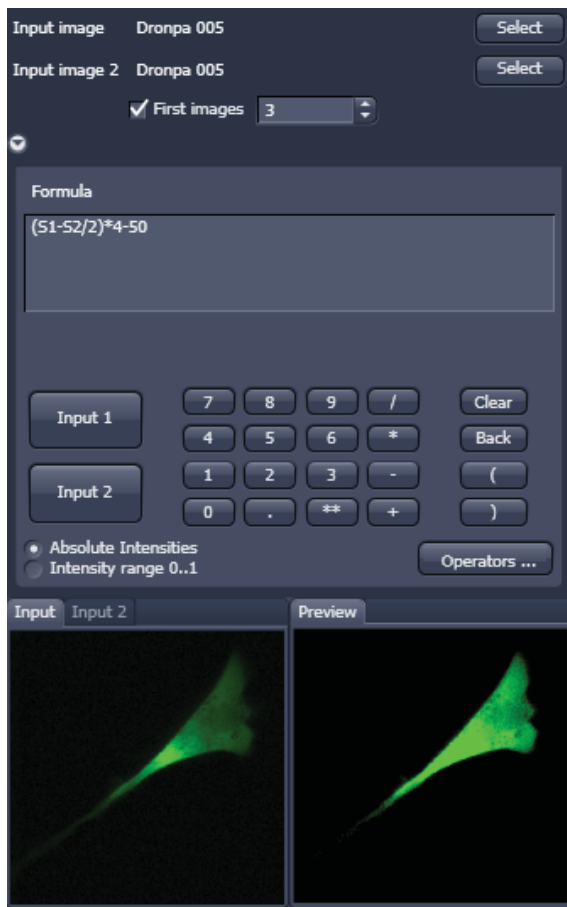
Fig. 4-88   Image calculation window

4.5.10.4   Average

The **Average** processing function will calculate an image that can be averaged in pixel and time.

- Activate **Average** in the **Processing** tool.

The **Average** menu will appear (Fig. 4-89). The **Average** processing function will calculate an average image.

- To select an image, press the **Select** button and click into the image in the container. This will be your **Input** image.

- Select how many pixels you want to bin in x-direction, y-direction and time points by setting the respective values in the **X Average Pixels**, **Y Average Pixels**, **Z Average Pixels** and **Time Average Pixels** input boxes, which can be opened by clicking on the arrows.

☞   Note that **Z-Average Pixels** and **Time Average Pixels** are only available for Z-Stacks and time series. You can either enter a value in, or use the sliders or arrows of the input boxes. If a moving average should be performed, the respective **Moving Average** box must be checked.

In this case consecutive averages comprising the specified number of frames will be computed.

An average output image will be calculated according to the settings and the result presented in the **Preview** window.

The size of the resulting image in **X**, **Y**, **Z** and **T** can be viewed in the **Size of result image** display.

If the **Apply** button is pressed, the averaged image will be opened as a new image in the container.

Fig. 4-89   Average window

Fig. 4-90   **Filter window**

Fig. 4-91   **Median filter settings**

4.5.10.5   Filter

The filter function permits the processing of scanned images with a **Median**, **Smooth**, **Sharpen** or **Bandpass** filter.

- To open the **Filter** processing tool, select it from the list in the **Processing** main tab (Fig. 4-90).

- Select the **Input Image** with the [Select] button and choose the filter of your choice from the **Type** pull down (Median, Smooth, Sharpen, Band).

- Select the channels you want to process with the [All] [Ch1-T1] [Ch2-T2] buttons. In the filter panel, the filter-specific parameters can be set as described in the following sections.

Median Filter

With the median filter, the gray value of each center pixel is replaced with the median value of the neighboring pixels. The viewed neighbor pixels are defined by a square of a certain size called the kernel. The modified pixel now is the center pixel of this pixel matrix.

The median value is defined as the middle value (not average) of all the gray values sorted in ascending order within a matrix.

Image noise will be reduced by the application of the median filter. The cut-off of regions will slightly blur. Local maxima will be flattened. The dynamic range will be reduced considerably.

The effect of the filter increases with the matrix size. This matrix / kernel size can be set for each dimension with the three sliders in the filter panel or by setting the size in the spin-box on the right hand side of each slider (see Fig. 4-91). A larger kernel, however, also increases the processing time. Other settings of this filter can not be modified.

Smooth Filter

The smooth filter is a lowpass filter with Gaussian characteristic. The gray value of each center pixel is replaced with the weighted average value of the surrounding neighbor pixels. The "neighborhood" is limited by the cut-off in pixels, set by the **Strength** slider. It has an analogous effect as the kernel size (see Fig. 4-92). If a dimension is processed by this tool is set with the tick boxes. The modified pixel now is the center pixel of the filter matrix.

Image noise will be reduced by the application of the lowpass filter. Sharp edges of regions will blur. Local maxima will be flattened. The dynamic range will be reduced considerably.

Fig. 4-92   **Smooth filter settings**

Sharpen Filter

With the sharpen filter, the original image is filtered with a lowpass filter first. The result of this filtering is then subtracted from the original image. This will improve image sharpness.

The tick boxes to choose the dimensions as well as the **Strength** slider (see Fig. 4-93). They are the same as in the **Smooth Filter**.

Fig. 4-93   **Sharpen filter settings**

Band Filter

With the band filter, the original image is filtered with a "band blocker" filter. It filters out a certain frequency band with position **Center**, width **Band Width** and a steepness of the edges set by **Strength**.

This is particularly useful to remove periodic perturbances in the image.

Fig. 4-94   **Band filter settings**

Fig. 4-95   **Correlation window**

4.5.10.6   **Correlation**

- Activate **Correlation** in the **Processing** tool. The **Correlation** menu will appear (Fig. 4-95).

The **Correlation** processing function will display the spatial correlation of an image or image stack.

- To select an image press the **Select** button and click into the image in the container. This will be your **Input** image.

As an output image the correlation image is computed and the result presented in the **Preview** window.

- You can select which kind of correlation you want to perform by activating the **X**, **Y**, **Z** and **Time** check boxes to perform spatial correlations in x, y and z-direction or a temporal correlation.

Note **X** and **Time** check boxes are only available for Z-Stacks or time series stacks.

- If the **Apply** button is pressed, the correlation image will be opened as a new image in the container.

4.5.10.7   Linear Unmixing

The **Linear Unmixing** processing tool permits to extract the emission of single fluorescence dyes (e.g. GFP only, YFP only etc.) from strongly overlapping multi-fluorescence data acquired in so-called "Lambda stacks" (see section 4.5.2.4.7).

Linear Unmixing of spectral information from multi-spectral imaging is an established technology known from remote sensing and satellite imaging. The basics of this increasingly popular image analysis technique in life science microscopy are described in the following resources (selection from a large literature, without claiming completeness):

–   Landsford, R., Bearman, G. and Fraser, S.E. / Resolution of multiple green fluorescent protein color variants and dyes using two photon microscopy." / Journal of Biomedical Optics (2001); 6, 311-318.

–   Dickinson, M.E., Bearman, G., Tille, S., Landsford and Fraser, S.E. / "Multi-spectral imaging and Linear Unmixing add a whole new dimension to Laser scanning fluorescence microscopy" / Bio Techniques (2001); 31/6, 1272-1278.

–   Zimmermann T, Rietdorf J, Pepperkok R. / "Spectral imaging and its applications in live cell microscopy." / FEBS Lett (2003);546:87-92.

–   Timo Zimmermann / "Spectral Imaging and Linear Unmixing in Light Microscopy" / Adv Biochem Engin/Biotechnol (2005) 95: 245-265

–   or have a look at *http://zeiss-campus.magnet.fsu.edu/* and the respective brochures from Carl Zeiss MicroImaging GmbH

In brief, with the knowledge of the spectral characteristic of individual components of a multi-component sample, even heavily overlapping individual spectral characteristics can be mathematically extracted from experimental multi-channel data. This method is a strictly pixel by pixel image analysis procedure.

Experimentally, fluorescence spectra of mono-labeled samples are acquired and stored in the Spectra Database (see section 4.6.17) as an external reference. Then a Lambda stack of the multi-labeled sample is acquired. Finally, the individual components are mathematically extracted using the information from the reference spectra. Up to ten different reference signals can be fed into the least-square-fit based algorithm to produce a 10-channel multi-fluorescence stack without any partial overlap between the channels.

If no mono-labeled samples are available, the references can be obtained by the following methods:

a) Interactively by user-selection of regions in the image where only one fluorescence dye is present (only available in the **Unmixing View** tab (see section 4.6.17)

b) Automatically by software analyses of what the individual spectral signatures are. This processing function is called "Automatic Component Extraction" (ACE). Note: in some cases, spectrally acquired images are not appropriate for ACE and linear unmixing can lead to wrong results.
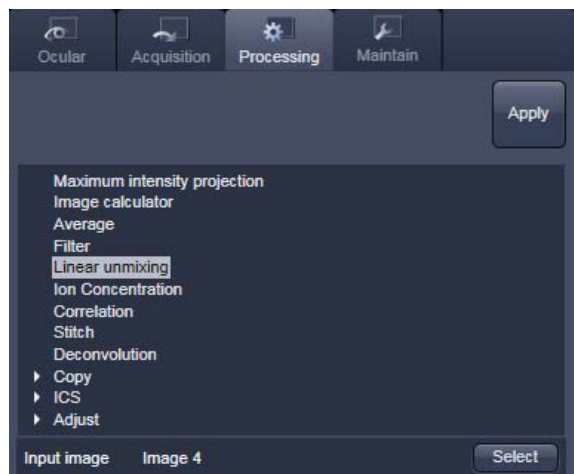
Fig. 4-96   Linear Unmixing in the Processing tab

Fig. 4-97   Linear Unmixing panel

- To open the **Linear Unmixing** tool, click on **Linear Unmixing** in the **Processing** tool list in the **Processing** tab (Fig. 4-96).

- Pressing the Select button loads the active image from the **Image Display** and displays it as Input Image.
  Only Lambda stacks can be loaded.

- In the **Linear Unmixing** panel (Fig. 4-97), the number of spectrally distinguishable fluorescent components of the imaged sample can be selected from the Components 3 selection box. The number of extractable components can not be higher than the number of acquired channels. The ZEN software is limited to a maximum of 10 components.

- Select the sample-specific fluorescence dye reference spectra from the **Spectra Database** (Fig. 4-98).

- Assign appropriate colors to the fluorescence channels by clicking on the color flag and choose from the available lookup tables (LUTs) (Fig. 4-98).

- If no predefined reference spectra exist:

  – Prepare reference samples and create spectra via the **Save to Spectra DB** button in the **Unmixing View** (see section 4.6.17).

  – or Use the "Multi Channel Unmixing" functionality described below. (Note: in some cases, spectrally acquired images are not appropriate for "Multi Channel Unmixing" and linear unmixing can lead to wrong results – see discussion below).

- After definition of the required reference spectra click **Apply**.

A new image with the resulting (unmixed) channels will be created (Fig. 4-99 and Fig. 4-100).

The following additional settings and functions are available for linear unmixing (see Fig. 4-97):

Auto scale balances the intensity of the unmixed channels to equal levels.

Display Channel with Residuals generates an additional channel in which the intensity values represent the difference between the acquired spectral data and the fitted linear combination of the reference spectra. In essence, the residual value is the biggest remaining "residual" from the least square fit routine. The residuals are a general measure for how good the fit of the algorithm has performed. The higher the intensity in this additional channel, the worse is the fit of the spectra to the dataset. This shows that either the acquired data is not representing the sample (e.g., when information is lost by pixel saturation) or wrong/insufficient reference spectra have been chosen. In this case, new image acquisition with no overexposed/saturated pixels is strongly recommended. In the case of wrong reference spectra, choosing/acquisition of different/new reference spectra can be necessary to improve the results.

Background: Select a background spectrum from the list of selected components. This spectrum is then subtracted from the images prior to unmixing. For example: three components selected and one of them marked as background will subtract the selected background from the acquired images and generate a 2-channel unmixed image. For the generation of background spectra refer to the Unmixing View type/Lambda stack – specific view (see section 4.6.17). Even small background contributions to the image should be indicated to the unmixing algorithm to ensure best unmixing results.

Multichannel Unmixing: When this option is chosen the unmixing algorithm is applied to a multi-channel image (up to 10 channels) without the use of reference spectra. See also section 4.6.17.1.

Fig. 4-98   Linear Unmixing: Spectra and LUT assignment

The calculation of residuals and the subtraction of background based on a background spectrum are not available. For heavily overlapping signals this method will yield poor results.

☞   Avoid saturation of fluorescence signal in the data set to be unmixed. Saturation will generate a high signal in the residual channel. To get the best unmixing results, define an extra background channel, if possible.

☞ **Reference Spectra** used for **Linear Unmixing** of Lambda stacks processed in the Unmixing View are stored with the resulting image and can be re-loaded in the **Processing** → **Linear Unmixing** tool with the **Read parameters from image** button. This facilitates processing of a series of images or Lambda stacks all with the same parameters / reference spectra.
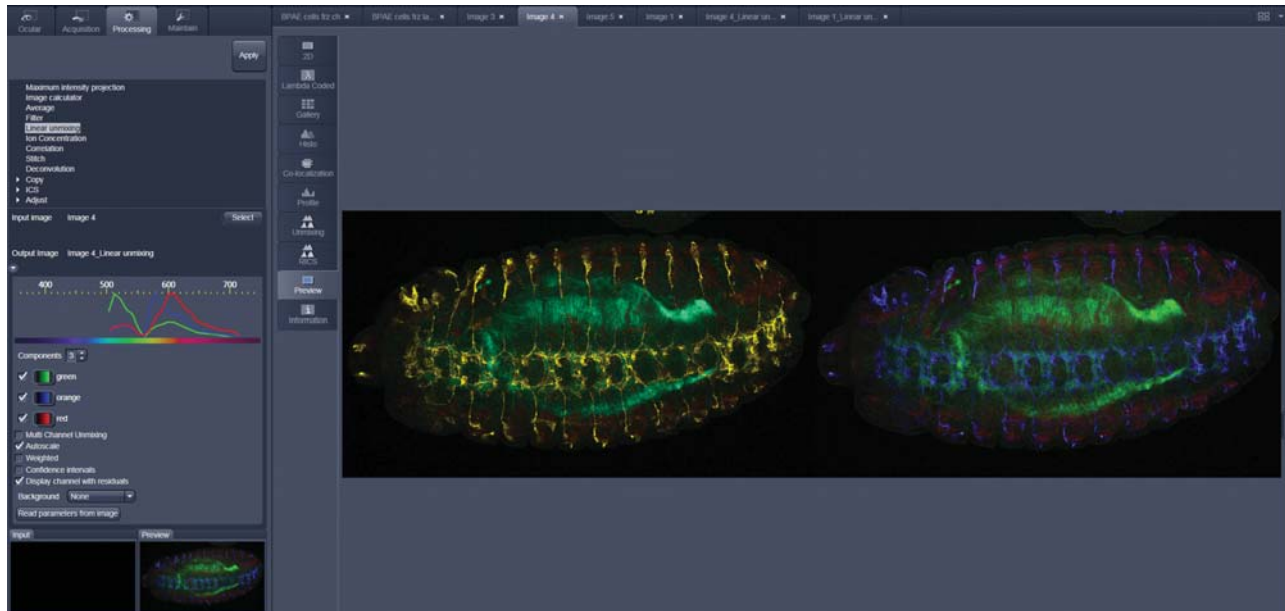
Fig. 4-99   **Image Display window before unmixing in Preview**
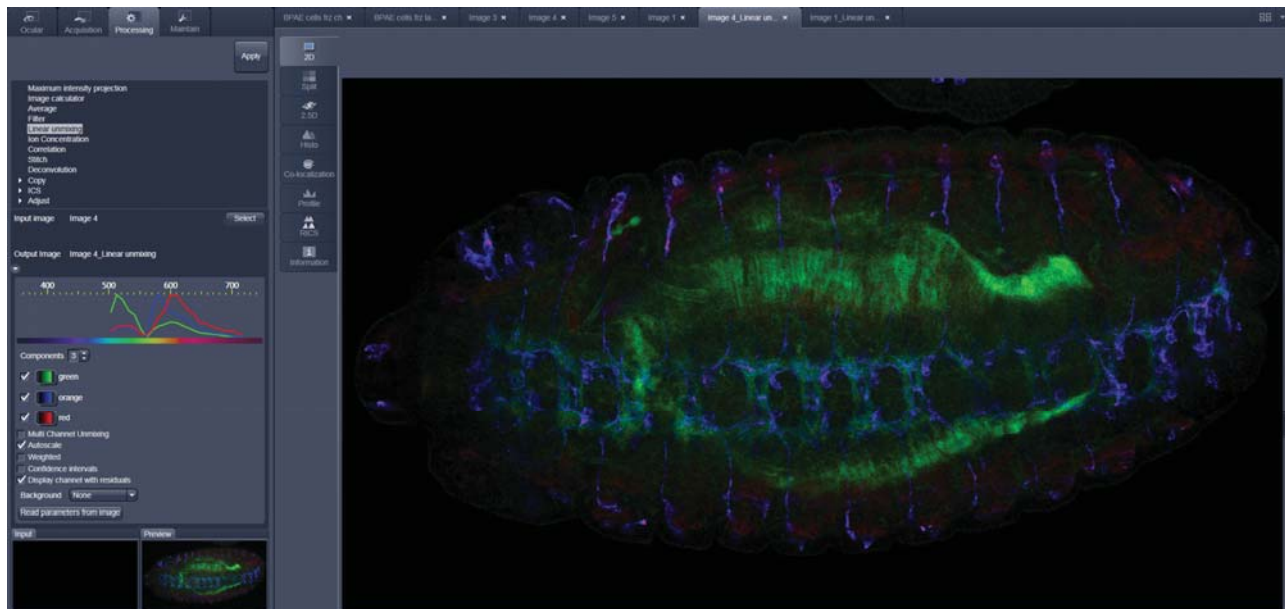
Fig. 4-100   **Image Display window after unmixing**

4.5.10.8   Ion Concentration

This Processing function permits the calibration of ion concentrations in physiological experiments.

To open the **Ion Concentration** tools click **Ion concentration** (Fig. 4-101).

The two [Select] buttons select the active image in the **Image Display** as Input Image and Input Image 2 (for background subtraction). The name of the automatically generated Output Image is shown under the name of the Input Images.

At the bottom of the **Ion concentration** tool window thumbnails of the input images as well as a preview of the output image are displayed.

After opening the full window by clicking the little arrow under the Output Image description (Fig. 4-102), the full tool window opens and the following functions are available (Fig. 4-103).
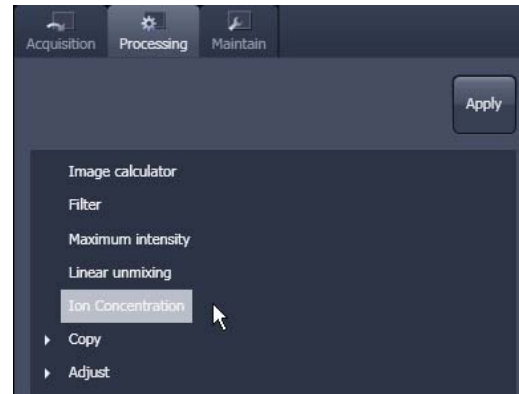
Fig. 4-101   Ion Concentration in the Processing tab
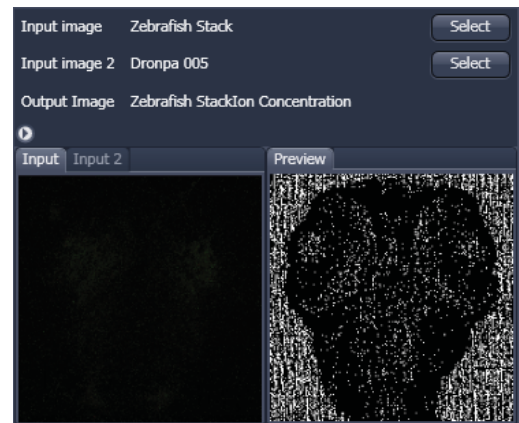
Fig. 4-102   Ion Concentration tool window

[Calibration graph]	Opens the display for the resulting calibration curve.
[Load ...] [Save ...] buttons	To load & save calibration curves from / to files.
[✔ Background (Input 2)] tick box	To select Input Image 2 as background image.
Dispay minimum concentration 0.10 / Dispay maximum concentration 1.00 — Image scaling	Spin boxes to set the Min and Max in the Output Image
Pull down menus for Dye / Channel 1 / Channel 2 / Method / Calibration	To set three different calibration options, according to the dyes (single wavelength, vs ratiometric), the calibration method and if the calibration is carried out in vitro/situ. Ch 1 and 2 select the channels from Input Image 1.
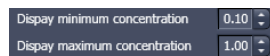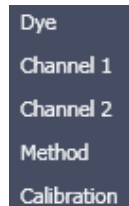
From these pull downs, the following combinations of parameters can be chosen:

Dye: **single wavelength** vs. **ratiometric**

Available methods for **single wavelength**: **Titration** or **equation** (both, **in vitro** and **in situ calibration**).

Available methods for **ratiometric** dyes: **Ratio**, **titration**, **equation** and **unmixing**.
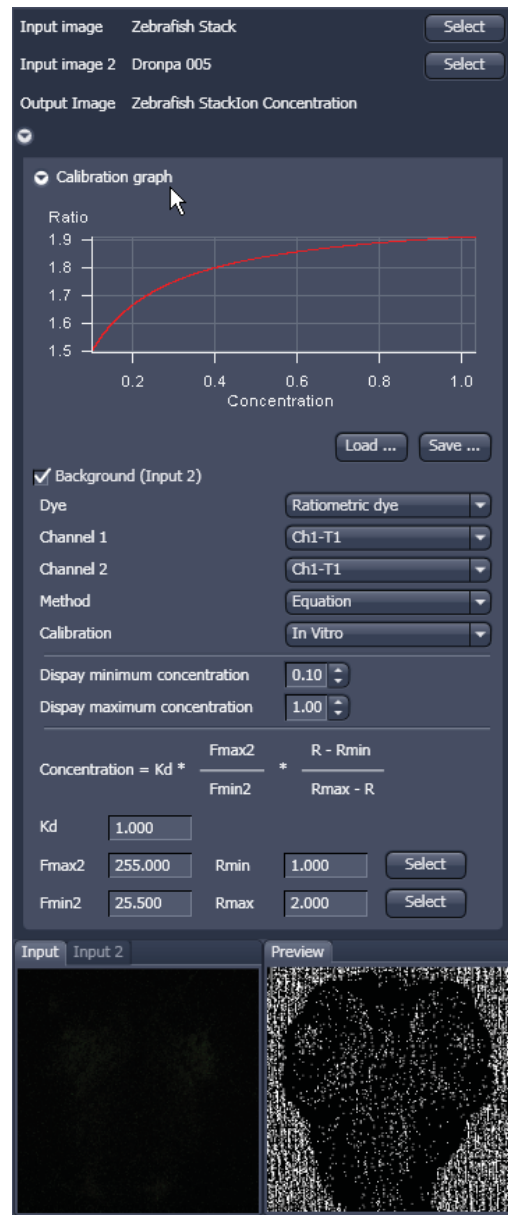
Fig. 4-103   Fully opened Ion Concentration tool window - Ratiometric dye in equation mode

Single Wavelength Dyes – Offline Calibration (Fig. 4-104)

- Subtract background/autofluorescence image from raw images to obtain better raw data to start with.

- Perform equation- or titration calibration (compare F with a calibration curve → titration calibration or put F values in calibration formula).
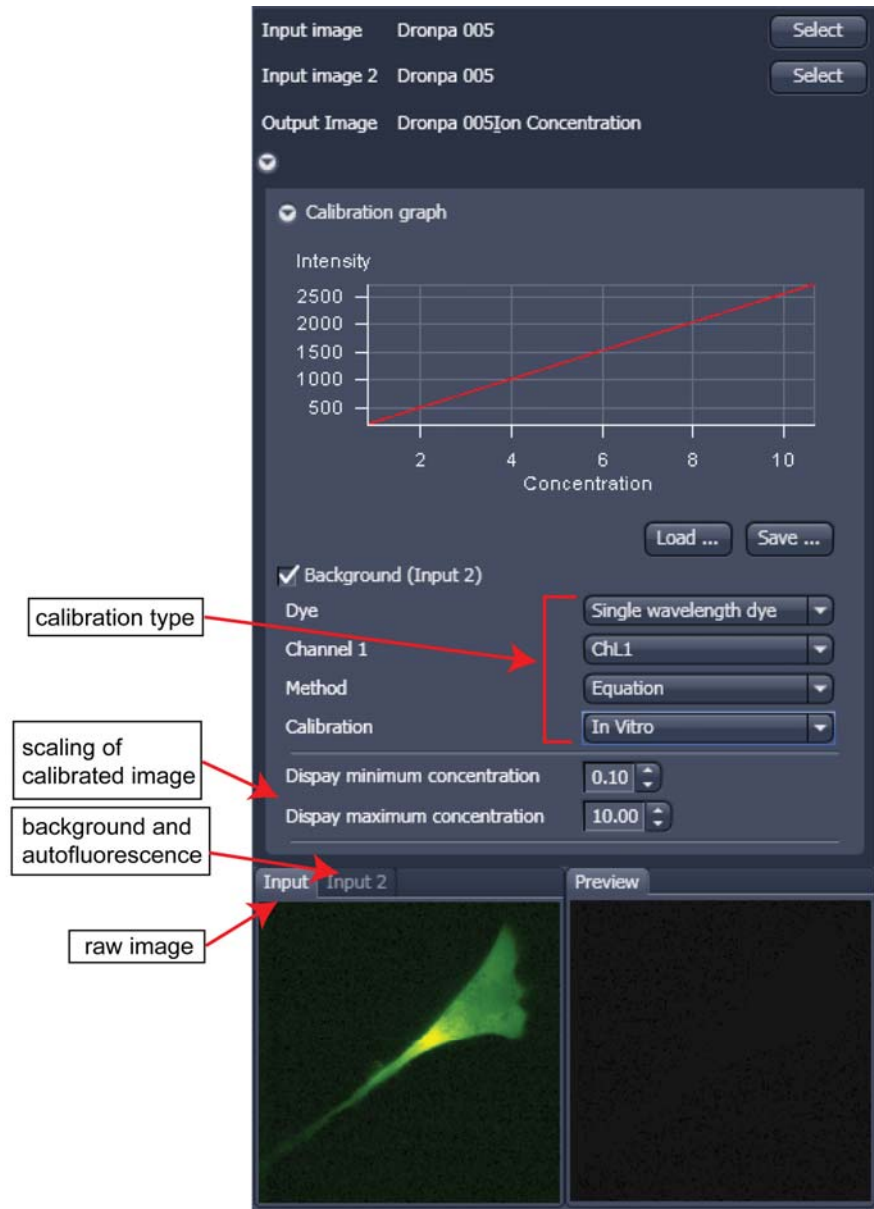
Fig. 4-104   Ion Concentration panel

Ratiometric Dyes

- Fura-2, Indo-, SNARF, Cameleon, Ratiometric Pericam, Phluorin.

- Display fluorescence ratio R over time

- Display fluorescence ratio R corrected for background/autofluorescence over time

- Calculate absolute ion concentrations (pixel by pixel) via titration calibration (known ion concentrations applied to the cells – in situ – or in solutions – in vitro or equation calibration where possible [Fura-2, Indo-, SNARF]

- Calculation of R eliminates artifacts and uncertainties caused by

  - inhomogeneous dye distribution

  - photobleaching

  - may be applied with moving cells.

Ratiometric Dyes - Online Ratio (Fig. 4-105)

$R(t1) = F1(t1) / F2(t1), R(t2) = F1(t2) / F2(t2) \ldots$
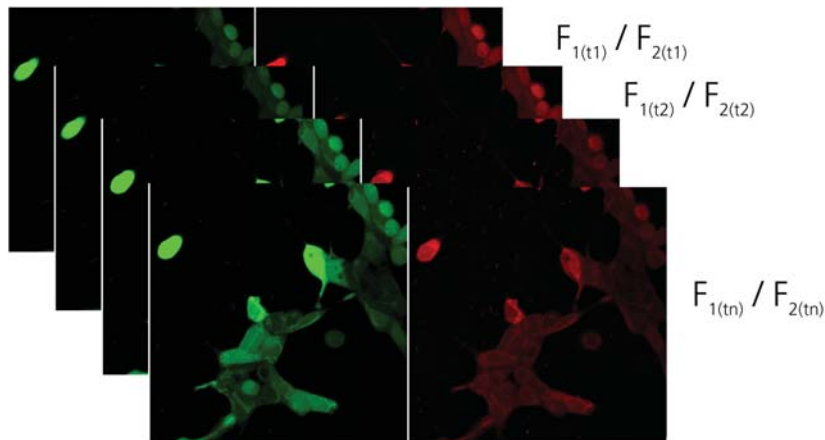
Fig. 4-105   Ratiometric Dyes – Online ratio

Ratiometric Dyes – Calibration (Fig. 4-106)

- Subtract background/autofluorescence images from raw images to obtain

  $R_{korr} [=F_1-F_{1Background})/(F_2-F_{2Background})]$

  when calibration reference is not obtained with the experimental sample (in situ).

- Calculate ratio R.

- Perform equation- or titration calibration (compare R with a calibration curve → titration calibration or put R values in calibration formula).

Fig. 4-106   Ratiometric Dyes - Calibration

Fig. 4-107   Ion Concentration - Ratiometric dye, Equation Calibration

Ratiometric Dyes - Equation Calibration (Grynkiewicz) (Fig. 4-107)

Fura-2, Indo-1,..

K_D (dissociation constant) taken from literature

R_{min}: derived from ion-free state of the dye (e.g. 0 Ca^{2+})

R_{max}: derived from ion-bound state of the dye (e.g. saturated with Ca^{2+})

F_{min2} and F_{max2} are the minimum and maximum fluorescence intensities at wavelength 2

R_{min}, R_{max}, F_{min2} and F_{max2} may be determined in the cells under investigation (in situ) or in solutions (in vitro)

Calibration parameters may be saved and reloaded (*.cal)

Options for Calibration Image Selection (Equation- or Titration Calibration)

- Click into image window.
- Select source channel(s).
- Optional background subtraction
- Optional calculation of parameters from overlay region(s)

4.5.10.9 Stitch

With this function tiled images acquired with Versions of ZEN 2009 and the tiling function can be stitched together in 2D and 3D.

Select a tiled image (or stack) with the select button.

The **correlation threshold** is defining the accuracy with which the algorithm detects similarities in the adjacent image planes.

If a 3D tile scan image has been acquired (Tile Scan combined with Z-Stack) then additional functions for the stitching procedure are available.

Ignore Z Correction will in some cases reveal better results for the 3D stitch. Depending on the image data the last step of the stitch algorithm, which makes a correction of the Z-Stacks in Z looking at the overall image, can lead to unwanted shifts of the stacks in Z. This can be avoided when this option is checked.

Calculate Topography provides a topographic image of the 3D stitched image data. This calculation can be done in several modes (see also section 4.6.12).

Fig. 4-108   Stitch function

First / Last maximum

- Select **First maximum** to calculate the topography surface by using the first maximum coming from the top. This mode provides better result for surfaces of semitransparent materials with inclusions of higher reflectivity or transparent multi-layers with subsurface layers of higher signal intensity.

- Select **Last maximum** to calculate the topography surface by using the last maximum coming from the top.

First / Last threshold

- Select **First threshold** to calculate the topography surface by using the first slice coming from the top, where the intensity reaches the value defined by the lower intensity threshold.

- Set the **Threshold** to define the lower and upper intensity thresholds used for calculation of the topography surface. Use of this function is recommended to find the real surface in the case of images with pronounced noise. All image pixels with intensity less or higher than the thresholds set are ignored for the surface calculation.**Correct bleaching** applies a correction factor to the double-exposed pixels. Crossfading is the only method available here and does an interpolation of the brightness values over a number of pixels in X or Y direction. The **Correction Width** in pixels can be adjusted.

4.5.10.10   Deconvolution

The 3D Deconvolution option is used for the resolution enhancement of fluorescence image stacks.

When a three-dimensional object is reproduced by an optical system the resulting image of the object does not correspond exactly to the object's actual form. The image of the object is "distorted" as it passes through the optical system. In physical terms the actual object is convolved by the optical system's **Point Spread Function** (PSF).
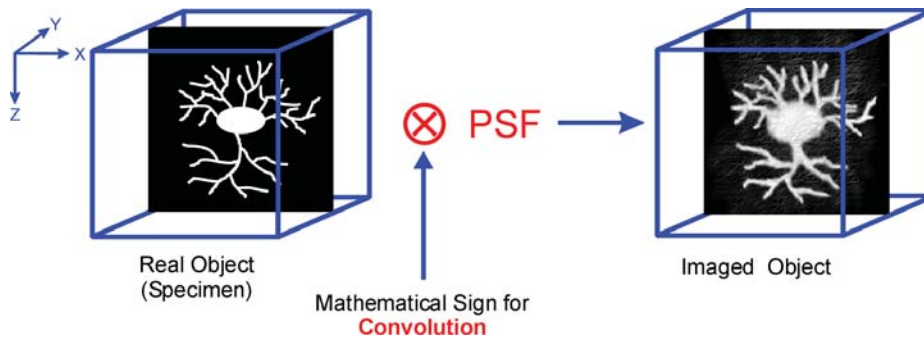
Fig. 4-109   Point Spread Function (PSF)

The **Point Spread Function** describes how the light of a point object is distorted by the optical system. This "convolution" makes the image appear grainy and structures in the image seem blurred. This effect is most prominent in the axial (Z-)direction as each lens is optimized for the two-dimensional image of the object.

If the PSF is known it is possible to use mathematical algorithms to undo this distortion. The image of the object is deconvolved using the PSF and the actual form is reconstructed:
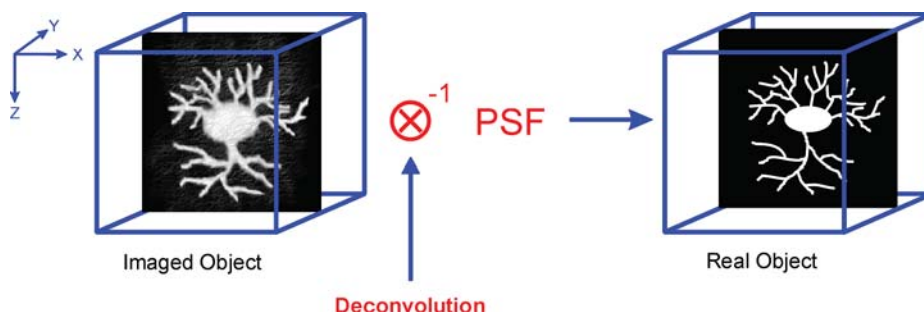
Fig. 4-110   Point Spread Function (PSF)

The effect of 3D deconvolution can be demonstrated impressively on objects with a known form. As a rule fluorescent beads are used for this purpose. The following figure shows the 3D deconvolution of an image stack with a fluorescent bead with a diameter of 1 µm.

As the resolution of an optical system is significantly lower in the axial direction than in the lateral (X/Y-)direction, the greatest improvement in resolution can be achieved in the Z-direction.

The Z-Stack must meet the following requirements:

– At least two-fold oversampling in xyz (z: half of optimal interval button)

– High signal-to-noise ratio

– Detector gain < 500 V

Calculation is either made for one channel of the opened image which must first be selected accordingly, or for all channels of a stack.

Calculation is started via **Apply** and can be stopped using the **ESC** key, if required.
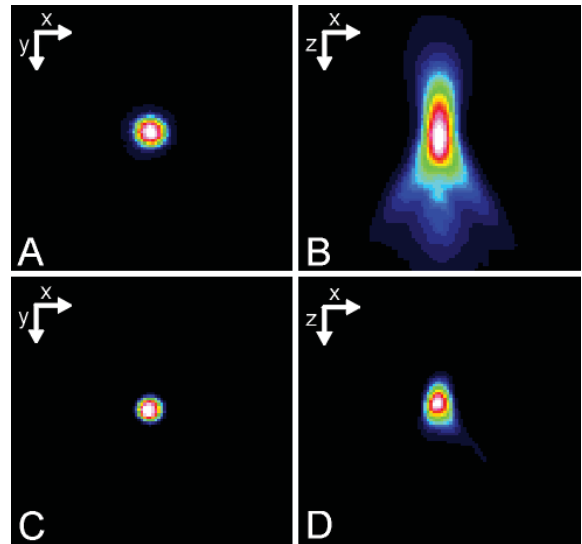
Fig. 4-111 **Image of a fluorescent bead with a diameter of 1µm before deconvolution (A,B) and after deconvolution (C,D)**
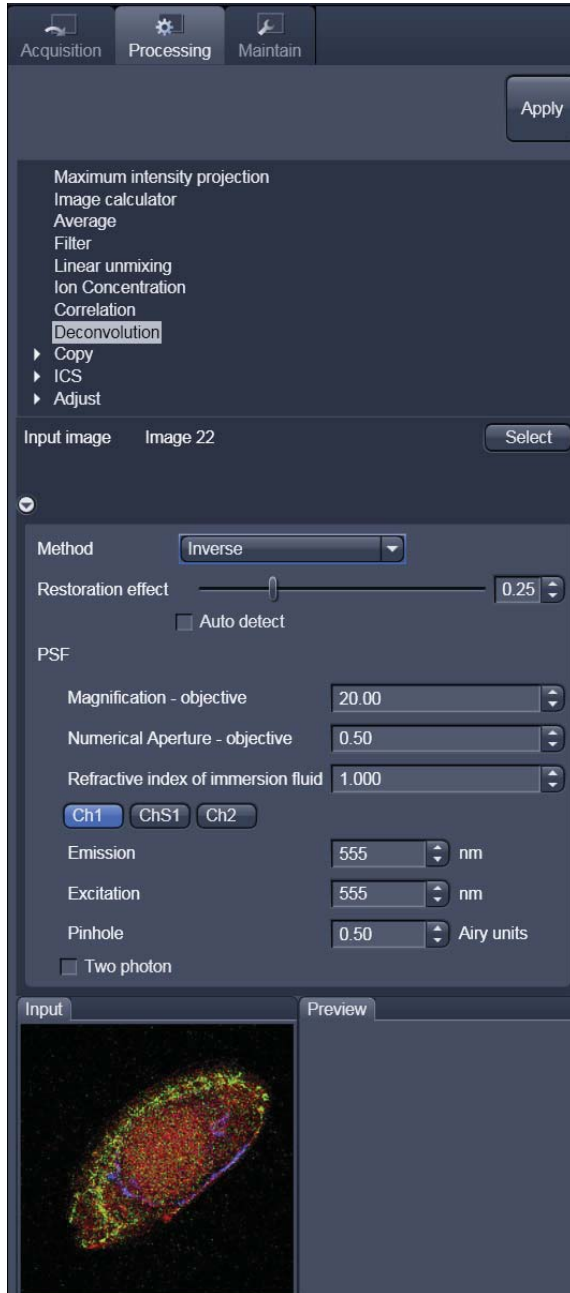
Fig. 4-112   Deconvolution window

To open the **Deconvolution** tools click **Deconvolution** (Fig. 4-112).

4.5.10.10.1 Methods

The **Method** pull down menu (Fig. 4-112) permits the selection between the calculation methods **Nearest Neighbour**, **Inverse** and **Iterative**.

(1)   Inverse Filter

The regularized inverse filter generally achieves better results than the Nearest Neighbor algorithm. It is well suited to process several image stacks for a pre-selection of images for the use of the iterative high-end methods.

(2)   Iterative

The best image quality is achieved using the Constrained Iterative Maximum Likelihood Algorithm. Increasing the resolution in the image, especially in the Z-direction, is only possible with this method. Due to the complex mathematical method, depending on the image size and the PC being used the calculation can take up to several hours.

In the **Inverse** method, the **Restoration Effect** slider permits the noise-to-signal ratio to be selected between the settings **Weak** (low noise) and **Strong** (pronounced noise).

(3)   Nearest Neighbor

The Nearest Neighbor method is the simplest and fastest algorithm which in principle corresponds to a 3D sharpness filter.

Activation of the **Auto detect** check box will start a routine for the automatic determination of the noise level in the entire image part of the Z-Stack (not available in the **Nearest Neighbour** method). If **Auto detect** is enabled, the **Restoration Effect** slider is disabled.

The **Iterative** method permits (in addition to the parameters of the **Inverse** method) the maximum number of iterations to be entered between 1 and 200 under **Maximum Iterations** and the **Auto Stop** function to be activated / deactivated. The **Auto Stop** function interrupts the calculation depending on the set image improvement (delta between last but one and last cycle in %), no matter whether the value under **Maximum Iterations** has been achieved or not.

The **Nearest Neighbour** method permits entry of the **Number of Neighbours** and the **Sharpness in Focus** value in addition to the **Restoration Effect**.

4.5.10.10.2 PSF Tab

In the **PSF** settings section of the **Deconvolution** tool, a theoretical point spread function (PSF) is calculated from the systems settings (objective data, wavelengths, pinhole diameter).

For wavelengths above 700 nm, the **Two photon** button is automatically activated.

The displayed values are always taken from the hardware parameters / images meta data, but can be edited subsequently for simulation purposes.

Fig. 4-113   PSF data input fields
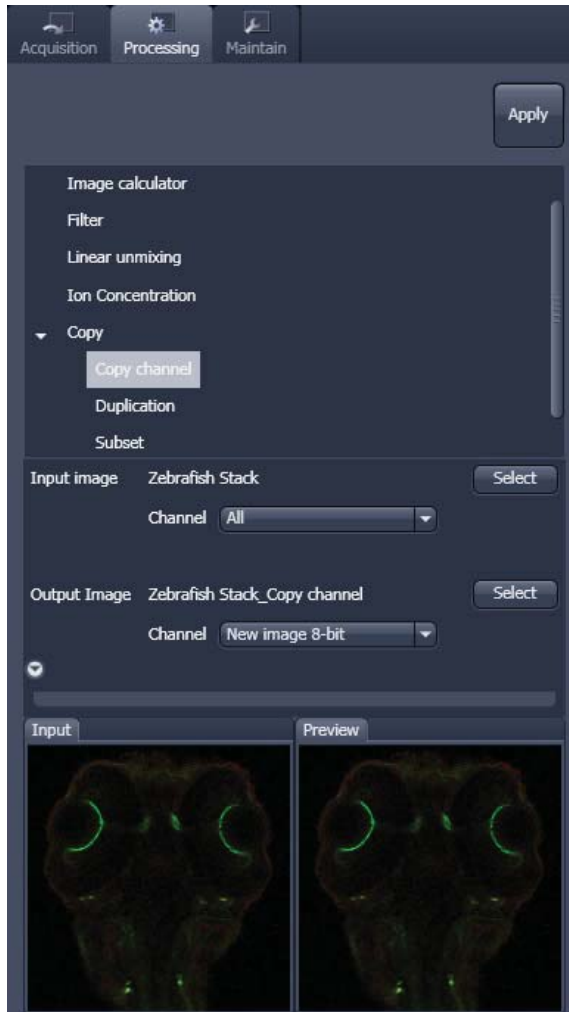
Fig. 4-114   Copy window

4.5.10.11   Copy

The **Copy** function provides means to copy or duplicate images.

Copy Channel (Fig. 4-114), produces a copy of the selected image.

The first [Select] button in the selection panel selects the active image in the **Image Display** as Input Image. The [Channel All ▼] selection pull down specifies which channel to copy.

The second [Select] button is used to choose a destination image if the copied channel is to be added to an existing image document. In the second [Channel New image 8-bit ▼] pull down menu, the destination channel is specified – or a new image document can be chosen as destination.

[Apply] starts the copy process.

☞   For Z-Stacks or Time Series, the entire series of the selected channel is copied.

Duplication

Duplication creates a new image document which is a duplicate of the selected input image.

Delete Image

Single images or series of images can be deleted from the selected image series (input image) acquired as time series or Z-Stacks or combinations of those.

4.5.10.12   Subset

The **Subset function** is a set of tools to truncate a multidimensional data set in all available coordinates to the desired size (Fig. 4-115).

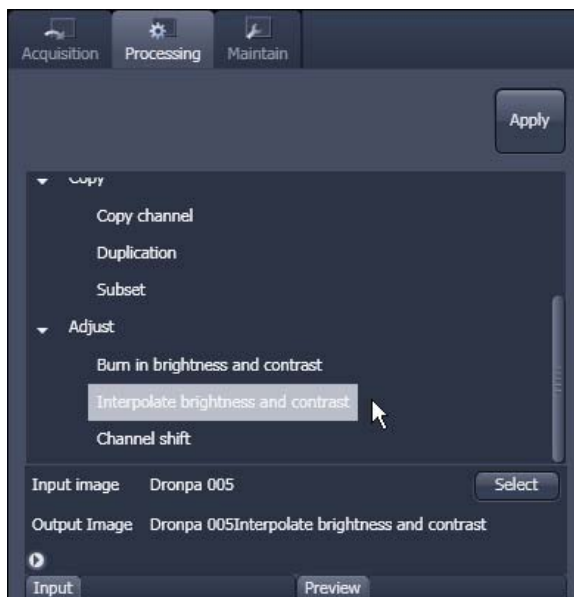The [Select] button selects the active image in the **Image Display** as the Input Image to be processed.

In the tool-specific settings panel, the parameters for the interpolation procedure are set.

Opening for example the **x**-subset panel (Fig. 4-117) brings up three sliders to set the **Start** and **End** coordinates of the new data set.
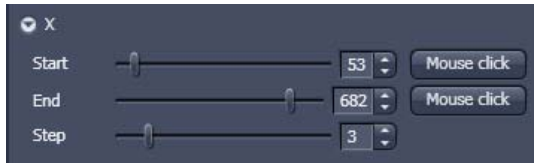
Fig. 4-117   Subset panel for the x coordinate

[Mouse click] Selects the respective coordinate by clicking in the original image in the **Preview View** in the image display area (centre screen area). The **Step** coordinate determines how many data points in the respective dimension are dropped out.

For example: setting **Step** to 3 takes 1 and drops 2 pixels along the respective dimension, reducing the data to 1/3. The scaling is calculated to stay correct in the resulting image.

This subset-panels are available for all coordinates of the selected image (x, y, z, t, channel, position and tile). The panel **Selection for all visible coordinates** is shown in Fig. 4-116. It always applies only to those coordinates with the subset-panel (above) open.

The **Select by mouse click in image** option allows to click in the Image Display and the respective coordinates are set (if displayed in the image: x, y, tile in the 2D view, z and position in the gallery view, channel in the split view, etc).

The assign from viewer selects the coordinate from the slider in the **Dimensions View Options** control block.
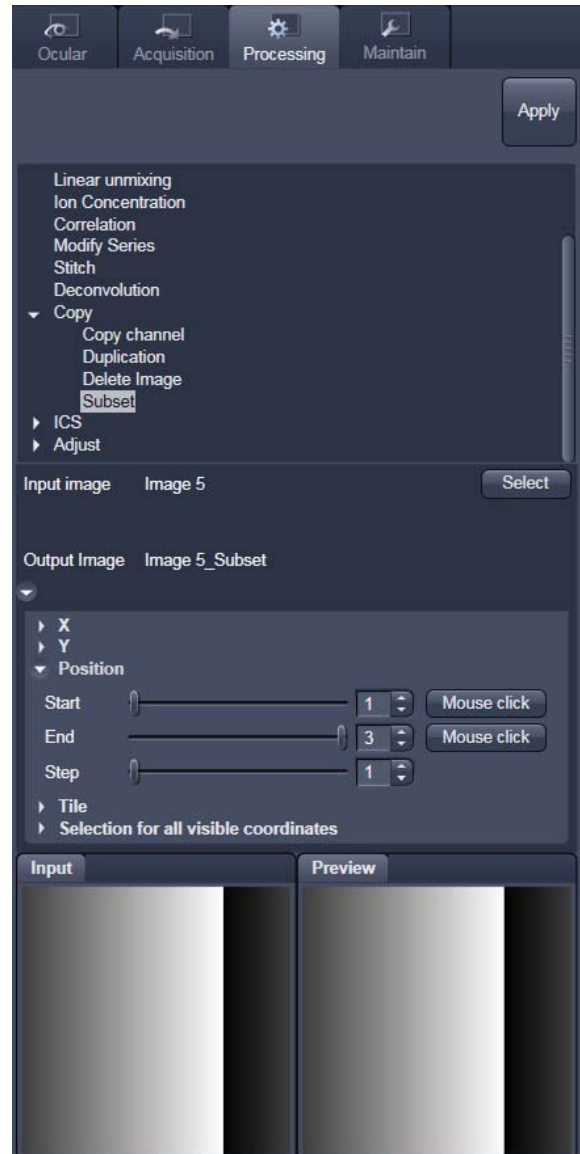
Fig. 4-115   Subset tool with loaded Multiposition Tile-Scan
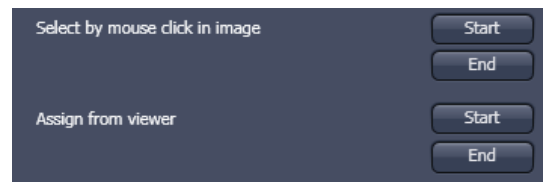
Fig. 4-116   Subset panel for "visible"
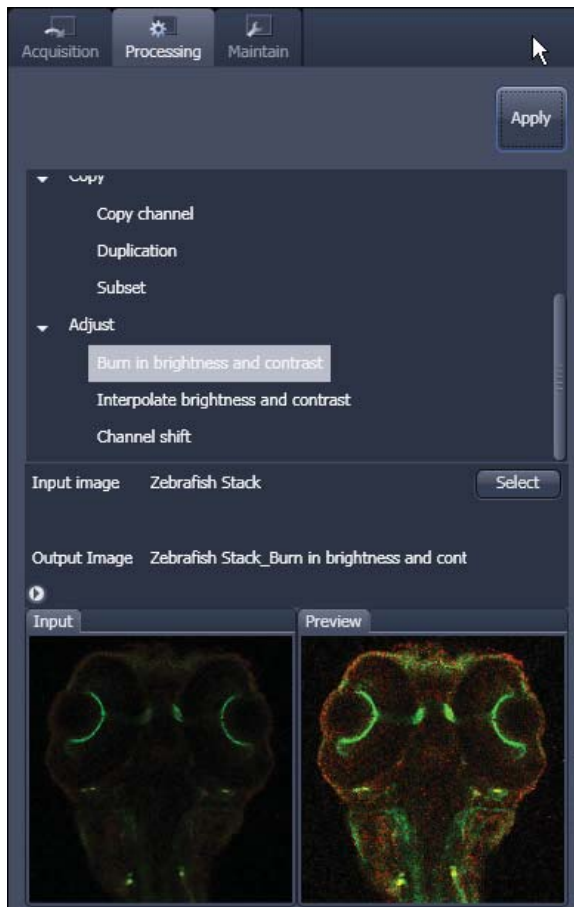
Fig. 4-118   Burn in Brightness and contrast tool

Fig. 4-119   Interpolate brightness and contrast tool

4.5.10.13   Adjust

Burn in Brightness and Contrast

The **Burn in brightness and contrast** function creates a new image document in which the current brightness and contrast settings from the **Display View Options** control block (see section 4.6) are permanently written to the image file (Fig. 4-118).

The [Select] button selects the active image in the **Image Display** as the Input Image to be processed.

- After adjusting the brightness and contrast in the **Display View Options** control block, click **Apply** to create the new image document with the burnt in contrast and brightness settings.

Interpolate Brightness and Contrast

This function permits a continuous contrast and brightness adjustment in a Z-Stack or Z-Stacks over time by interpolation between the starting and end values of the respective image series. This permits a post-acquisition compensation of signal loss in, for example, thick tissue imaging where excitation and detection efficiency decrease significantly. Interpolation can be defined for the entire image or only for individual channels. In addition, a set of coordinates for which intensities are kept fixed during the interpolation calculation can be defined (Fig. 4-119).

The [Select] button selects the active image in the **Image Display** as the Input Image to be processed.

In the **Interpolation** panel (Fig. 4-120) the parameters for the interpolation procedure are set.

- In the **Coordinate** pull down, select the dimension for the interpolation. Depending on the image data type, x, y, z and time are the possible options.

- Use the [List size ___ 4 ⬍] spin box to set the number of fixed data points. The table in the **Interpolation** panel will be extended to the selected number of rows.

- Highlight a row of your choice by clicking on it. Then use the **Brightness, Contrast and Z** (or t) slider to set the values for this fixed data point. The numbers in the table will be updated according to the slider position. Then highlight the next row, set the parameters and keep setting the fixed data points for all rows.

- Use the available channel buttons [All] [Ch1-T1] [Ch2-T2] to select the channel for interpolation or click on the **All** button if the entire image is to be interpolated.

- Having set the parameters, click on the **Apply** button. Interpolation will be performed in a new **Image Display** window.

- The newly created image (series) can be stored using the **Save As** function.
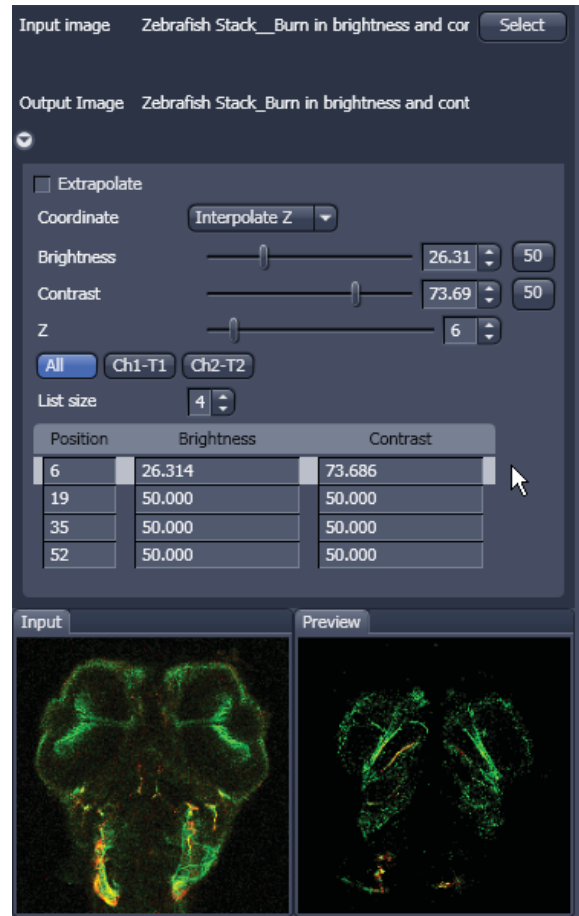
Fig. 4-120   **Interpolation brightness and contrast tool with loaded Z-Sstack**

☞   If you de-activate the **Extrapolate** check box, only the slices lying between the first and last fixed data point (table rows) will be taken into account for interpolation. Otherwise, brightness and contrast will also be changed for the other slices.
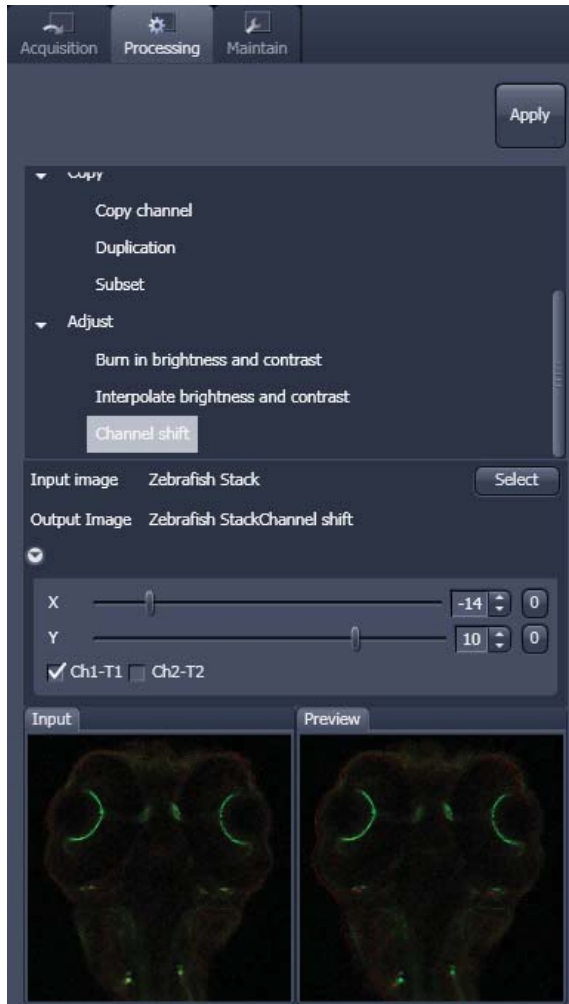
Fig. 4-121   Channel Shift window

Channel Shift

Channel Shift is used to produce a congruent image with relation to the pixels of the various channels (Fig. 4-121).

This pixel correction function is particularly important in UV applications.

☞   This is a manipulation of your data that has to be considered very carefully when co-localization analysis is part of your experiment.

The [Select] button selects the active image in the **Image Display** as Input Image.

The image has to be a multi-channel image. In the

- Select the channels you want shift by ticking the **Ch1** or **Ch2** check box: [✓ Ch1-T1 ☐ Ch2-T2]

- And use the sliders

  or the spin-boxes to select the pixel shift in the horizontal and vertical direction. Clicking the Zero next to the spin-boxes resets the shift to the original position.

  – A preview is automatically generated at the bottom of the Channel Shift tool in the Processing tab of the Left Tool Area and at the same time. A large preview is shown in the Preview view tab in the Image display in the Centre Screen Area.

- Click on the **Apply** button to generate a new image with the set pixel shift.

Shading Correction

Shading Correction is a tool to manipulate images which are unevenly illuminated over the field of view. This works with single images, time series, Z-Stacks or tiled images. Click on Shading correction to open the interface for using that function.

Select the image (or image series, tiled image or Z-Stack) for which the shading correction should be applied by clicking **Select** next to **Input image**. When clicking **Apply** the system calculates a reference image from the input image(s) which is used to correct the input image(s). The corrected image is displayed in the center area screen.

In addition the calculated **reference image** can be stored and used for further shading corrections of other images. Therefore the check box in the lower part of the tool box muss be checked. Specify the folder where the image should be stored in the text box next to **Path**. **Browse** opens the windows folders from which you can select. A suggestion for the name of the image is made based on the original name of the image. Change this if needed.

This reference image can then be loaded and selected as reference image for the shading correction of subsequent images, which are then processed much quicker.

The reference image must be taken from an image that was acquired with identical settings for objective, zoom, image format and orientation.
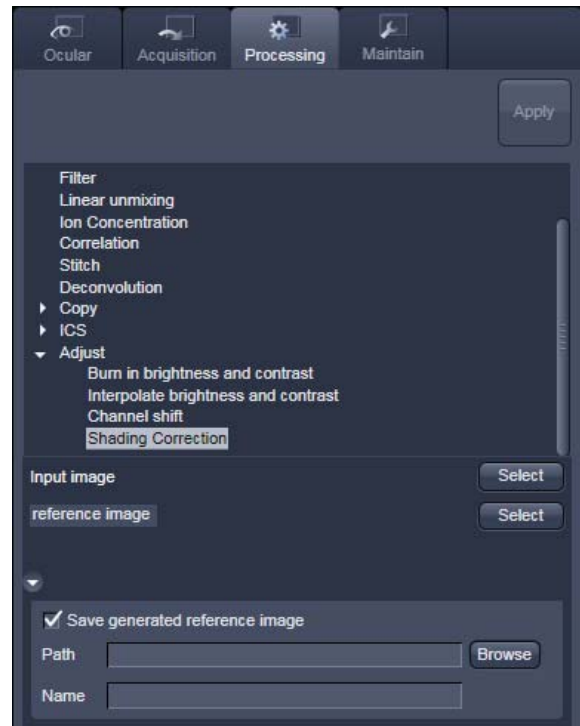
Fig. 4-122   Shading Correction interface

4.5.11 Maintain

The **Maintain** tool group on the **Maintain** tab provides functions to adjust hardware components of the system.

4.5.11.1 Maintain Tab: Adjust Pinhole and Collimator

This function permits pinholes and collimators to be optimally aligned and adjusted to the used beam path. The function can be called up in the **Maintain** panel in the **Adjust Pinhole and Collimator** tool of the **Maintain** tool group (see Fig. 4-123).

The position of the pinhole (X-Y-Z-coordinates) in relation to the detector makes a major contribution to image optimization.

In all existing standard configurations, the pinholes have already been adjusted at the factory. These settings are taken over for active operation when a standard configuration is loaded.

If you want to create a setting that differs from the standard configurations, adjust the pinhole as follows.
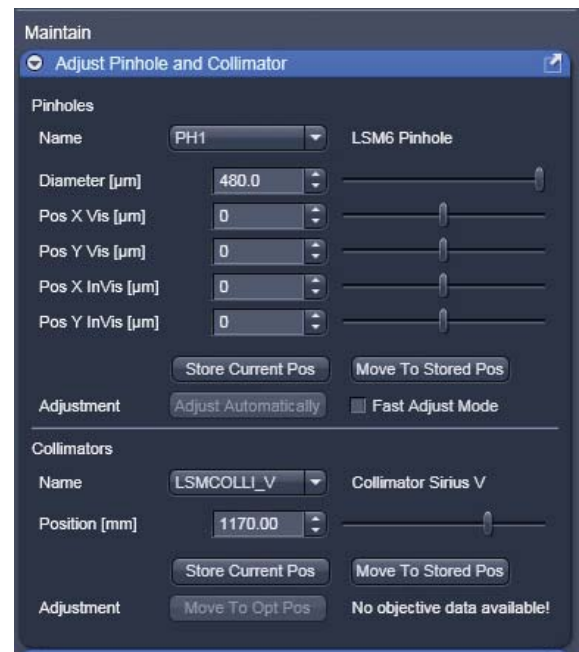
Fig. 4-123   Adjust Pinhole and Collimator tool

(1)   Pinholes Section Panel

The **Pinholes** section panel has the following options (see Fig. 4-123):

Description field: Display of the relevant active channel.

Diameter [µm] slider   Setting of diameter, X-, Y- and Z-position of the pinhole in relation to
the beam path (Z-position can be set only for PH1) using the slider or
Position X Vis [µm] slider   arrow buttons

Position Y Vis [µm] slider   Status display for setting procedure: green for ready and red for busy.

Position X InVis [µm] slider

Position Y InVis [µm] slider:

Stored Pos button: Pinhole setting is reset to the position last stored.

Adjustment: Adjust   Automatic pinhole adjustment (not available in some systems).
Automatically button:

Fast Adjust Mode check box: If this check box is activated, the pinhole adjustment is only performed
in a limited area. Used for readjustment (not available in some
systems).

No further software function can be activated and executed while automatic pinhole adjustment is
running.

(2)   Collimators Section Panel

The **Collimators** section panel has the following options (Fig. 4-124):

Name drop down menu	Selection of the collimator (IR / VIS or UV / VIS) to be adjusted via **the drop down menu.**
Description field:	Display of the relevant active Collimator
Position [mm] field:	Setting of collimator position using the slider or arrow buttons; the display to the left of the slider indicates the current position
	status display for setting procedure: green for ready and red for busy.
Current Position button:	Stores the current collimator position.
Stored Pos button:	Sets the collimator to the stored value.
Adjustment Move to Opt Pos button:	Starts the automatic collimator adjustment. Available for some objectives.
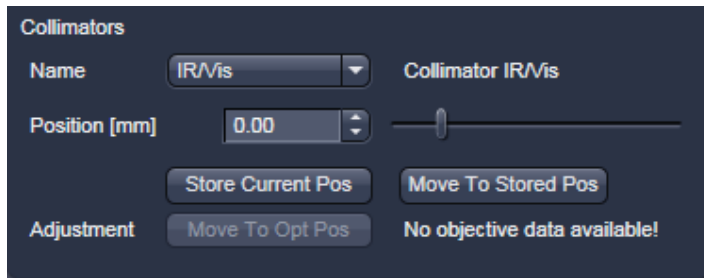
Fig. 4-124   Collimator section panel

(3)   Adjust the Pinhole

Adjustment of pinholes can be performed manually or automatically.

If several channels are used to produce the image, all the used pinholes must be adjusted separately.

Temporary optimization of the adjustment:

The position of the pinhole relative to the detector in terms of X-Y-Z coordinates contributes substantially to image optimization.

Requirements to make the influence of pinhole position changes on image brightness immediately visible:

– The image must be scanned by the continuous scan method.

– Select a fast scanning speed.

– Measurement with Average Number 1 only (no averaging of several measurements).

– On the **Channels** tool in the LSM **Online Acquisition** tool group select the pinhole diameter so as to have the best possible image contrast (see Fig. 4-125).

- Open the **Adjust Pinhole and Collimator** tool in the **Maintain** tool group in the **Maintain** panel.

- Select the pinhole to be adjusted from the **Name** list box.

- Use the **Diameter** slider to set the smallest possible size which produces a good, high-contrast image.

– This setting changes the pinhole diameter.

– The **Optimal Interval** box of the **Optimize Sectioning and Step** submenu of the **Z-Stack** tool in **Show all** mode within the **Multidimensional Acquisition** tool group simultaneously displays the depth resolution corresponding to the pinhole diameter (see Fig. 4-126).

☞   Image optimization can be effected with the **Range Indicator** or in the **Line-Scan**   mode.
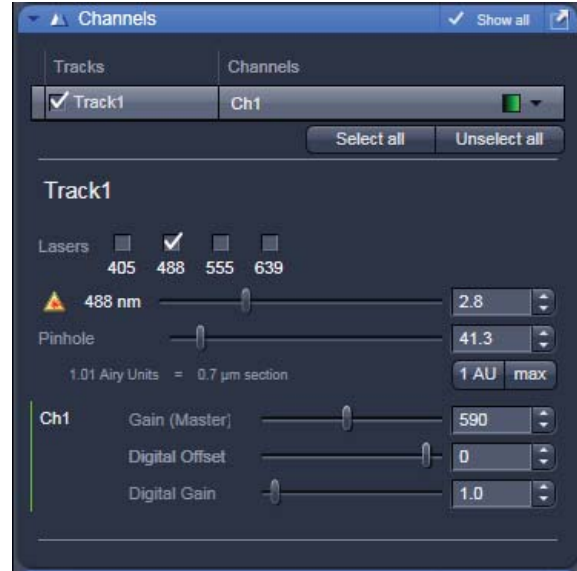
Fig. 4-125   Channels tool
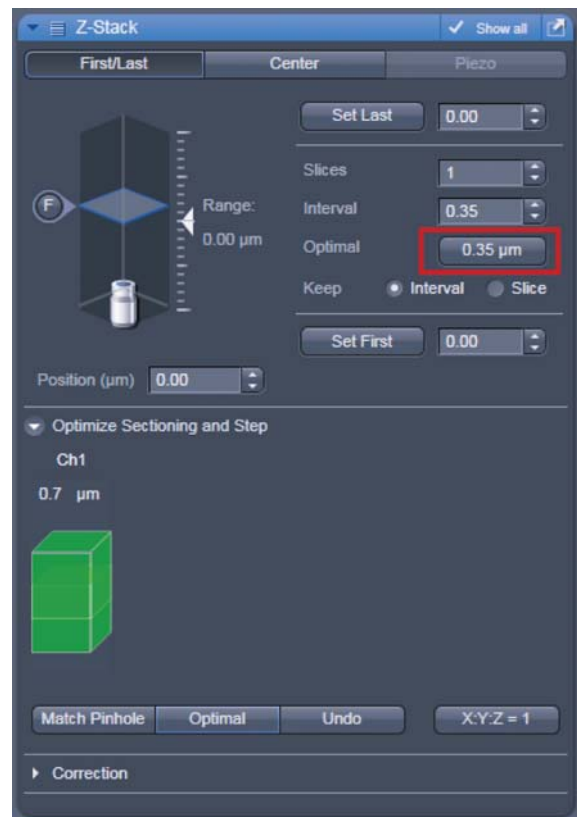
Fig. 4-126   Z-Stack tool

- Click the LSM **Continuous** action button to start a continuous scan. Optimize the pinhole position in X, Y and Z (Z only for PH1) relative to the PMT using the **Position X [µm]**, **Position Y [µm]** and **Position Z [µm]** sliders to maximum image brightness (see Fig. 4-127).

- Moving the **Position [mm]** slider in the **Collimator** panel allows the collimator to be adjusted to maximum image brightness. Optimum collimator adjustment obtained in this way can be stored by clicking on the **Current Pos** button. The Collimator is pre-set from the factory and care should be exerted by changing its position.

- Click the **Stop** action button to stop the continuous scan.
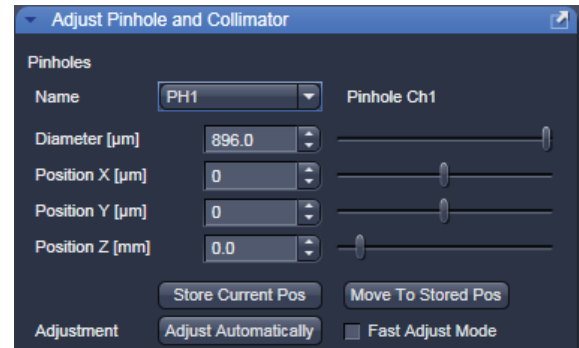
Fig. 4-127   **Manual pinhole adjustment**

Automatic pinhole adjustment:

The automatic adjustment allows the LSM 510 pinholes to be used with any combination of beam splitters and more than one active channel.

☞   Please do not make any program manipulations while the automatic pinhole adjustment is running (status display is red - busy).

- Click on the **Adjust Automatically** button.

  – The **Requirements for Adjustment** window will then appear.

- Meet the requirements listed in the **Requirements for Adjustment** window (Fig. 4-128). Press **Cancel** to abort the automatic pinhole adjustment. Press the **OK** button to proceed.

  – Pinhole adjustment will then run automatically and the Scan window will appear (see Fig. 4-129). The adjusting procedure takes approx. 3 min.

  – The determined data are stored automatically and will be available for all further examinations using the same configuration.
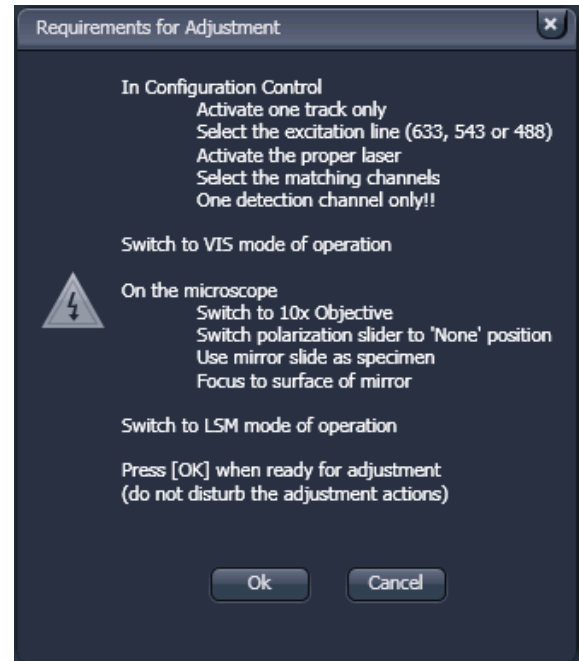
Fig. 4-128   **Requirements for Adjustment window**

☞ A change of the pinhole diameter made manually in the **Pinholes** section panel will update the pinhole diameter in the **Channels** tool and vice versa.

☞ Pinhole adjustment should be done on a regular basis (monthly, depending on system usage).
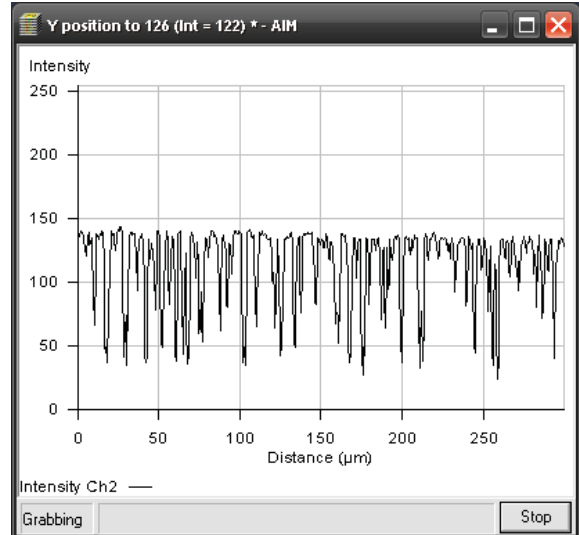
Fig. 4-129   Pinhole adjustment scan window
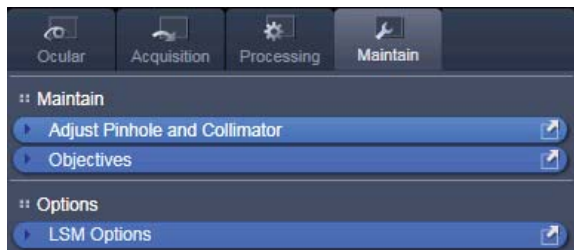
Fig. 4-130   Maintain and Options tool groups

4.5.11.2   Maintain Tab: Objectives

This function permits changed objectives to be activated and the parfocality to be set without having to exit the software. The function can be called up in the **Maintain** panel in the **Objectives** tool of the **Maintain** tool group (see Fig. 4-130).

Fig. 4-131   Objectives tool

(1)   Change Objective

- Change the required objective in the nosepiece.

- Click on the **Objectives** tool in the **Maintain** tool group of the **Maintain** panel.

    – A list of available objectives is displayed with position and name (Fig. 4-131).

- Click on the graphical button of the relevant nosepiece mount (**Position**).

    – An expansion (**Change Objective**) window appears.
    All available objectives are listed in the **Potential Objectives**, **User defined Objectives** or **Favorite Objectives** directories of the expanded window (see Fig. 4-132).

- Select the new objective by highlighting it from the appropriate **Objectives** directory. Press the **Set As New Objective** button to assign the objective to the selected **Position**.

- Click anywhere outside the expansion window to close it.
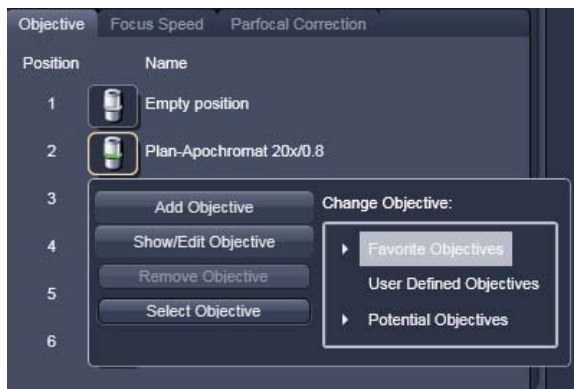
Fig. 4-132   Change objective window

(2)   Add Objective

This function permits new objectives to be added to the database.

For this, proceed as follows:

- Click on the **Add Objective** button on the **Change Objective** window (Fig. 4-133).

  – An expansion (**Create new Objective)** window is opened (Fig. 4-134).

- Enter the data of the new objective in the appropriate display boxes of the expansion window, and then click on the **Apply Add** button.

The new objective is stored in the database in the objective directory under **User defined Objective**.

Fig. 4-133   Add Objective window – Potential Objectives

☞   If you have activated the **Non Zeiss** check box, objectives from other manufacturers can also be included in the database.
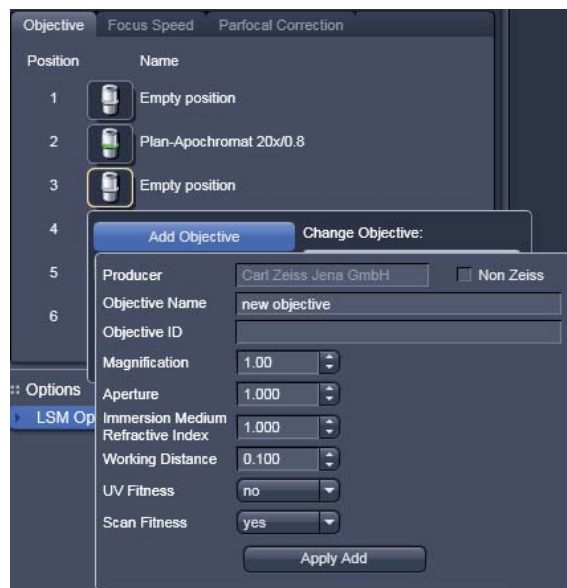
Fig. 4-134   Editing a new objective

Fig. 4-135   Remove an objective

(3)   Remove Objective

You can remove user-defined objectives. Hence, all objectives in the **User Defined Objectives** directory can be deleted (see Fig. 4-135).

- To remove an objective from the database, select it with a click of the mouse in the expansion (**Change Objective**) panel and then click **Remove Objective**.
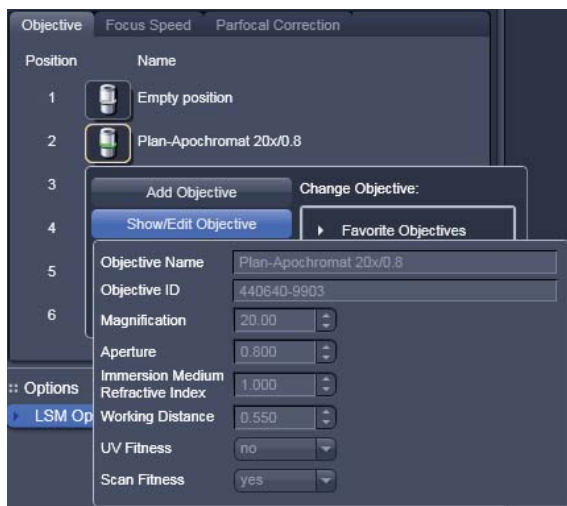
Fig. 4-136   Edit an objective

(4)   Edit Objective

You can edit user-defined objectives. Hence all objectives in the **User Defined Objectives** directory can be edited.

- To edit an objective from the database, select it with a click of the mouse in the expansion (**Change Objective**) panel and then click on **Edit Objective**. An expansion (**Edit user Defined Objective**) panel will open and the parameters of the Objective can be edited. Only those parameters are accessible that are allowed to be changed. Other parameters are grayed.

- Press the **Apply Edit** button to store the new annotation.

(5)   Focus Speed

The focus Speed can be changed for every objective present in the nosepiece via the **Focus Speed** tab in the **Objectives** tool. Use the speed sliders to set the speed and the **Write** button in order to take over the changes also for manual Z-control on the microscope.
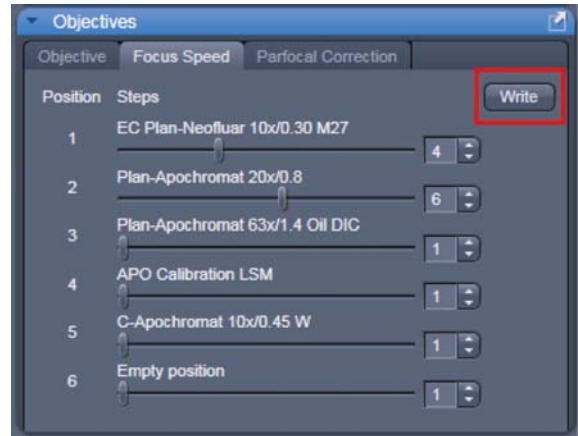
Fig. 4-137   Focus speed setting

(6)   Parfocal Correction

Parfocal Correction for different Objectives can also be done within the **Objectives** tool.

To activate the parfocal correction, tick the tick box **Parfocal correction active** ✔ Parfocal correction active .

For adjusting the individual objectives press the Start button. The first Objective will be used. Focus on a thin sample (Cells or grid) and press Next . The next Objective will be used. Continue this procedure for all objectives present on your system.

⚠ **CAUTION**
If Parfocal Correction is in use, a motorized stage can damage your objective!
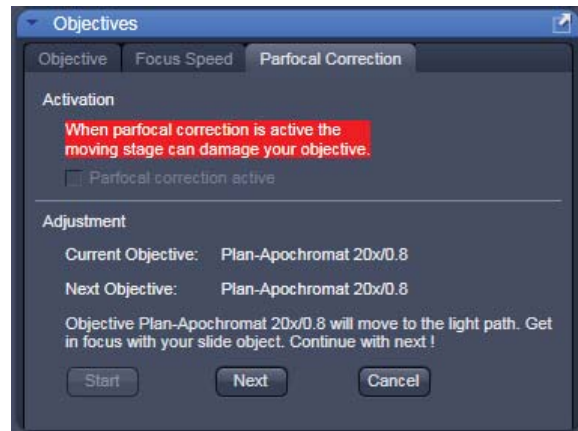
Fig. 4-138   Parfocal correction

4.5.11.3   Maintain Tab: LSM Options

In the **LSM Options** tool the following user-accessible program **Settings** of the ZEN software are available (see Fig. 4-139):

- – **Load configuration**
- – **Re-use**
- – **Hardware**
- – **Image display**

- Click on the respective register to display the panel.
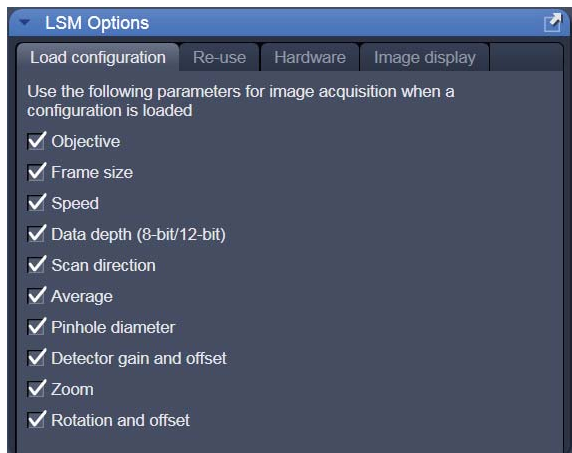
Fig. 4-139   Options tool with load configurations panel opened

(1)   Load configuration

Load configuration (see Fig. 4-139) lists parameters to be taken into consideration (if checked active) for loading an imaging configuration.

Fig. 4-140   Options tool with re-use panel opened

(2)   Re-use

Within the **Re-Use** panel (see Fig. 4-140) you can determine whether the settings of the objective, the collimator and the incubation parameters, which are stored with the image, should be taken over when the **Reuse** function is used.
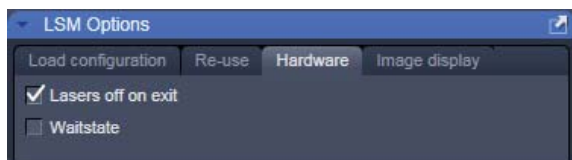
Fig. 4-141   Options tool with hardware panel opened

(3)   Hardware

The **Hardware** tab (see Fig. 4-141) allows you to set some hardware defaults.

By activation of the **Lasers off on Exit** check box the lasers are automatically switched off when the ZEN software is closed. The lasers are allowed to cool for five minutes before they are switched off.

Waitstaite is on as default setting. This function delays the image acquisition during time series or Z-Stacks in case vibrations of the stage occur and are registered at the beginning of individual images. The delay is set to 350 ms. This can occur for specific image acquisition settings and is dependent on the speed, the stage and the objective in use.

(4)   Image display

Any changes in the **Image Display** will per default not affect the original image data. When the check box **Apply brightness and contrast to diagrams and tables** (see Fig. 4-142) is marked, any changes in brightness and contrast of the image will also affect the image data shown in diagrams or tables.

The **Temporary Files Directory** can be set manually. For large datasets it is advisable to use a drive **other** than the C-Drive of your computer.

Interpolate: Interpolates the signal between pixels and thus smoothens the image. This interpolation is not saved with the original image data and can be active also during acquisition.

For the options **Invert x**, **Invert y**, **Invert z**, **Invert time** and **Exchange x and y** the original data are also not changed when those options are applied.
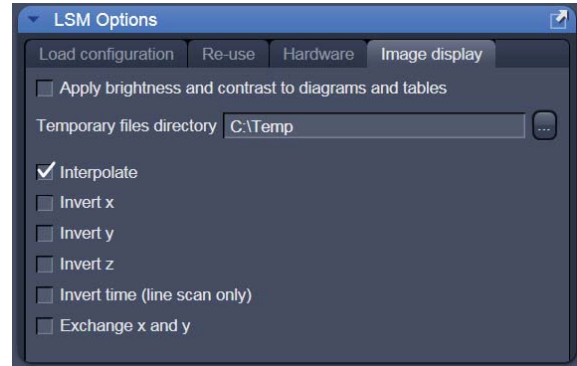
Fig. 4-142   **Options tool with image display panel opened**

4.5.11.4   Maintain Menu Bar

The **Maintain** menu from the **Menu bar** contains additional modules to check and guarantee the interference-free operation of all the software and hardware components of the LSM 510. In the **Menu bar**, click on **Maintain**. This opens the Maintain list (Fig. 4-143).

Fig. 4-143   **Maintain list**

The maintain list is bipartite. On upper section lists user accessible tools, the lower section lists tools reserved for service personnel, they are password protected.
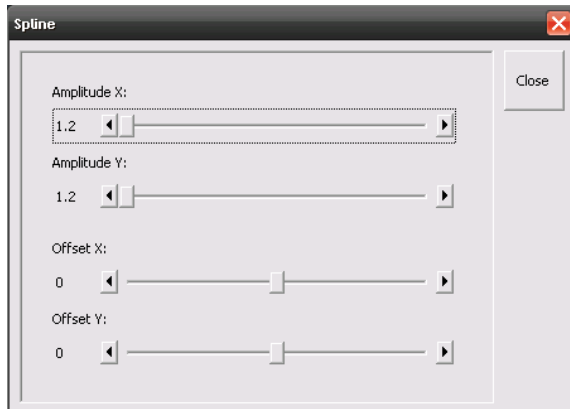
Fig. 4-144   Spline window

4.5.11.4.1 Set Spline

This function permits calibration of the Scanner position signals. This is required for the use of spline curves in the **Line** scanning mode.

- Click on **Set Spline** in the **Maintain** list.
  - The **Spline** window appears on the screen (see Fig. 4-144).

- You can set the required **Amplitude X**, **Amplitude Y**, **Offset X** and **Offset Y** values by using the sliders.

- Click the **Close** button to leave the window.
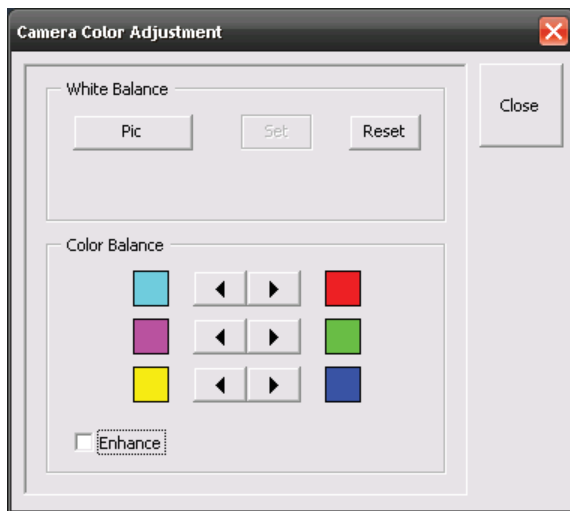
Fig. 4-145   Camera Color Adjustment window

4.5.11.4.2 Camera

This function allows the user to adjust the white balance and color balance of a connected camera.

- Click on **Camera** in the **Maintain** list.
  - The **Camera Color Adjustment** window appears on the screen (see Fig. 4-145).

Clicking the **Pic** button allows to set the white balance using the mouse cursor in the camera image. The **Set** button will become active. Press set to store new settings, press **Reset** to return to the old settings.

Use the arrow buttons to adjust the color balance of the camera.

4.5.11.4.3 Hardware Administrator

The **Hardware Administrator** function is for servicing purposes and may only be used by authorized service personnel. Its access is therefore password-protected.

4.5.11.4.4 Test Grid

The **TestGrid** function is for servicing purposes only and may only be performed by authorized personnel. Its access is therefore password-protected.

4.6   Center Screen Area / Image Containers - Display and Image Analysis

4.6.1   Structure and Functional Concept of the Central Screen Area and the Image Display Container

4.6.1.1   General Structure

In this section, the Center Screen Area of the **ZEN Main Application** window is described. The **Center Screen Area** can be set up to hold 1, 2 or 3 **Image Containers**. Fig. 4-146 shows the layout with one or two Image Containers.
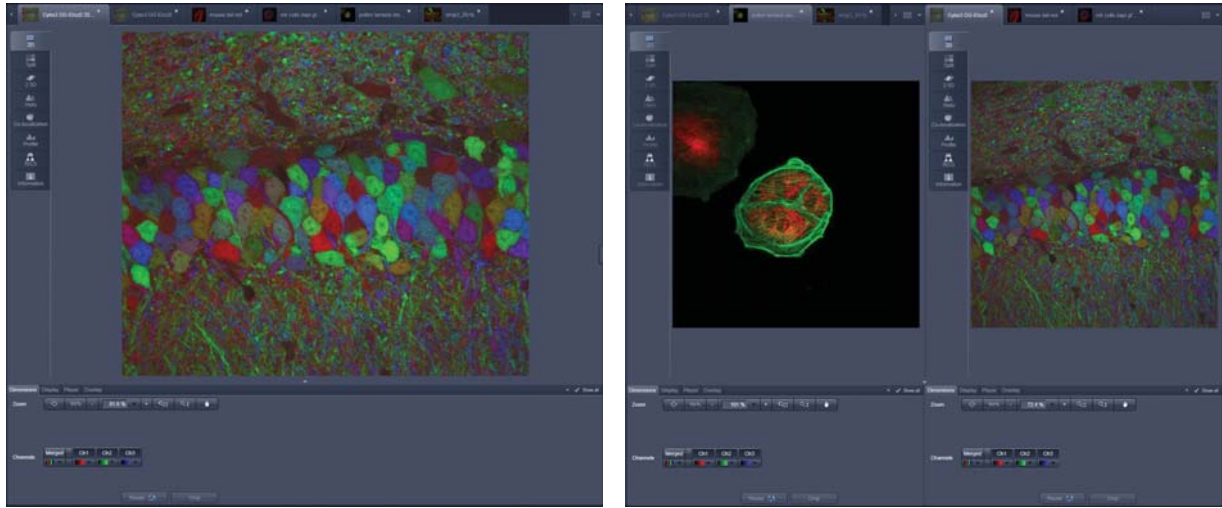
Fig. 4-146   The Centre Screen Area of the ZEN Main Application window – Left: 1 Image Container, Right: 2 Image Containers
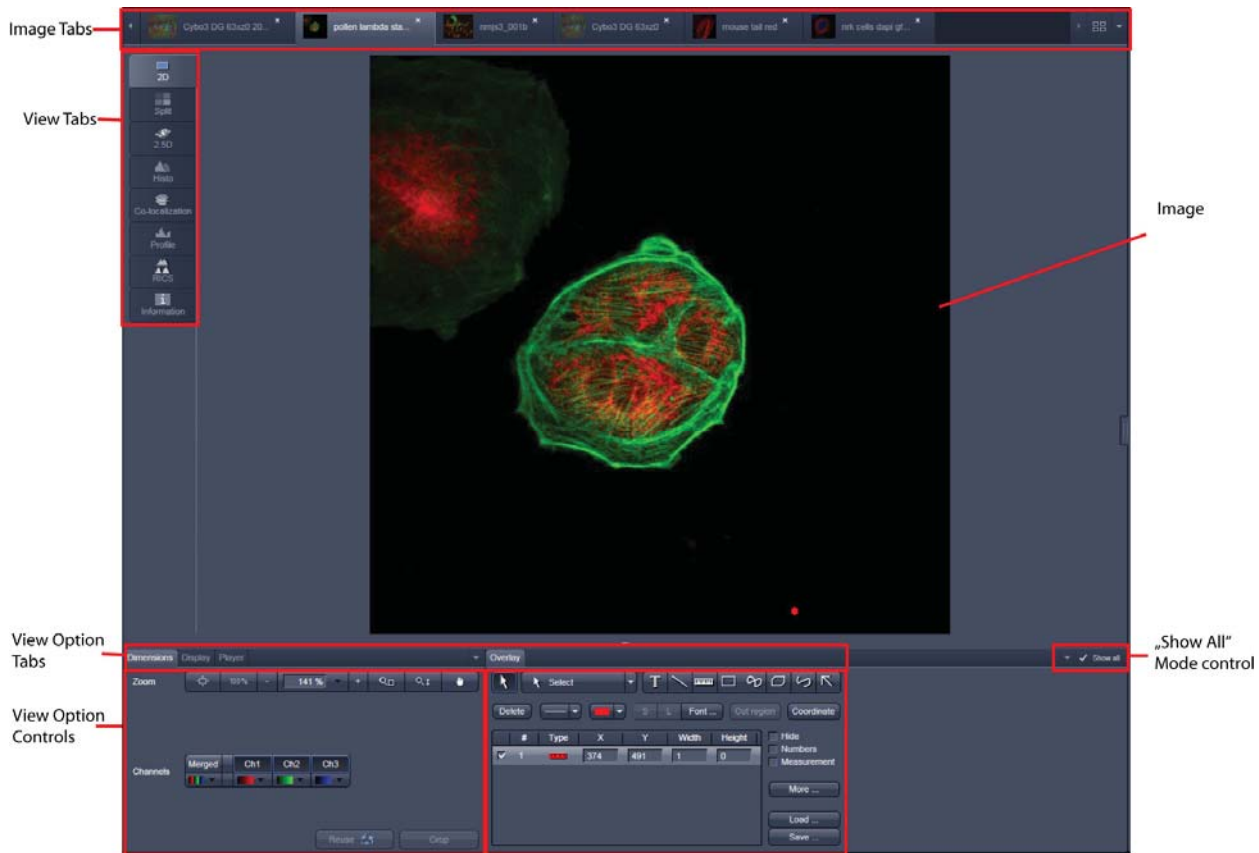
Fig. 4-147   Image Display window; Select - Overlay

View tabs:

The View tabs make all viewing options and image analysis functions directly available from the main view. Switching from one View tab to another changes the view type for the currently active image, keeping the image in the foreground. This avoids several display windows for different analysis tools and keeps all information always right at hand.

☞   After switching between several open images, upon returning to a previously activated Image tab, the image document "remembers" which view type was activated before and displays the same parameters upon return to this image.

Image tabs:

The Image tabs show every opened window so they are easy to find and access even when the **Open Images** panel in the **Right Tool Area** is hidden/minimized. This way of organizing open images avoids the problem of many opened image display windows lying on top of each other. The **Image Container** concept allows large numbers of images open at the same time and having them all easily accessible while keeping the workspace organized and tidy. **Image** tabs can show the image file name only (Fig. 4-148/**a**), the name and a small image thumbnail (Fig. 4-148/**b**) or the file name and a larger image thumbnail (Fig. 4-148/**c**). This can be configured in the Container Context menu (see Fig. 4-150).

Fig. 4-148   Image Display window; Select - Overlay

View Option control tabs:

These tabs allow individual activation / deactivation of the available **View Option** control blocks by clicking on the tabs. Available but hidden View Option Control tabs are grey. Active tabs are displayed in front of the others.:
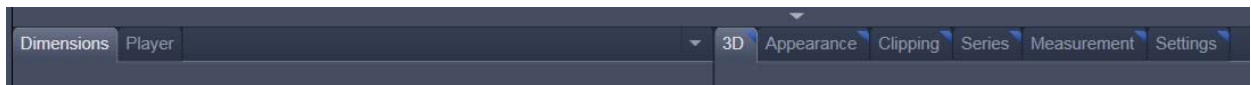
The **View Option** control tabs:

The **View Option** control tabs are placed in the area under the image display. Each block hosts functionally related tools for image analysis, display modification and data manipulation. There are two groups of **View Option** control tabs: A general one which contains the blocks **Dimensions**, **Display**, **Player** and **Overlay**. The second group consists of **View** tab–specific tool tabs hosting tools that are functionally linked to only one view type. The View tab Specific control tabs are marked with a blue triangle on their upper right corner. The control tabs of the general group and their respective tools are described in the section for **2D View** (the first and default **View** tab).

Tab mechanics:

All tabs of the two groups can be dragged out of their groups and placed freely in the **View Options Area**. Where ever it is possible to drop a grabbed tab – the underlaying area is displayed in blue:
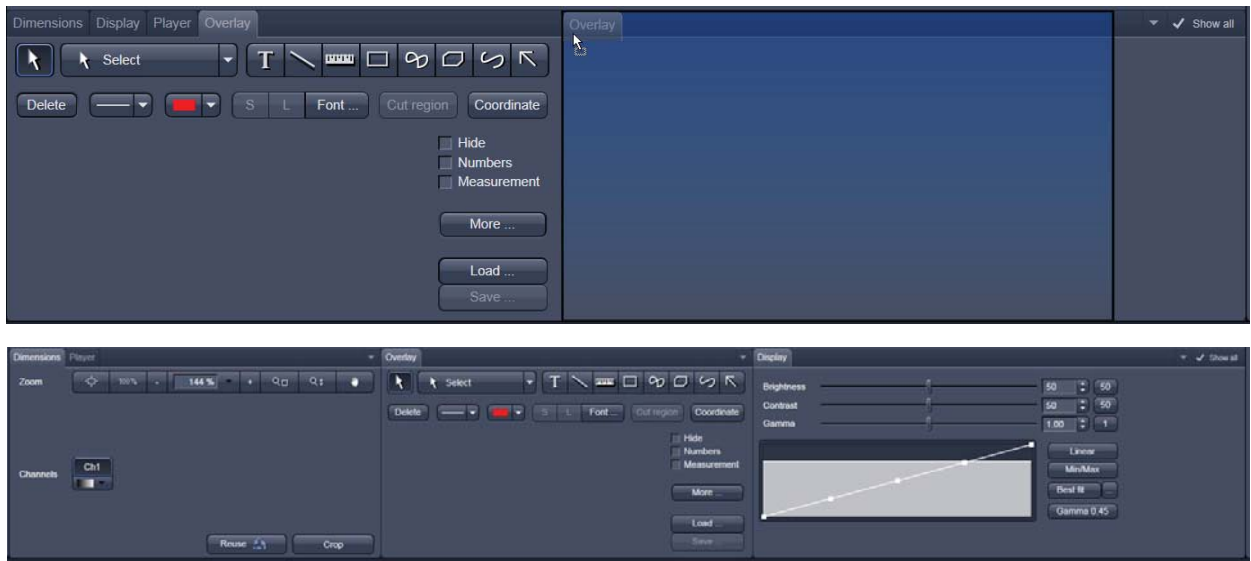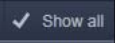
Fig. 4-149   General View Options control blocks

In order to reset the tab layout – use the entry **Reset Tab Layout** in the context menu or click on the

triangle to access the View Options Area menu.

Show all mode of the View Options Area:

Activating the **Show all** mode of the View Options Area has two effects:

a) In every view options control tab all the available tools are shown; de-activating the **Show all** mode hides less often used tools.

b) All available view options control tabs are shown; de-activating the **Show all** mode hide less often used tabs.

Any changes done with these tools have immediate effect on the image display but not on the saved data. To permanently store your results you have to save the data as described in section 4.5.10.12 and/or 4.5.11.3.

Fig. 4-150   Open Image pull down and context menu of the Central Screen Area

Image Handling and Container Layout Configuration:

This little control area (Fig. 4-150) on the top right corner of the Image Display is for scrolling and listing the Image tabs which can not be displayed if too many images are opened. This is just an alternative way of handling the open images. The main tool for this purpose is the Open Images Panel (see section 4.7). A right click opens the context menu for the layout of the central screen area.

The Image Display:

The Image Display contains and displays the image data or –depending on the active view type – a combination of image data, overlays, graphs and tables. The content is automatically maximized to the available image or display size.

Expose Mode:

Clicking the **Expose Mode** button [icon] in the top right corner of the Image Display opens a field of thumbnails in the image display with one large thumbnail for each open image. Whenever you lost track of your open images, open the Expose Mode and click once in the image you are looking for and it will be immediately brought to the foreground of the Image Display (Fig. 4-151).
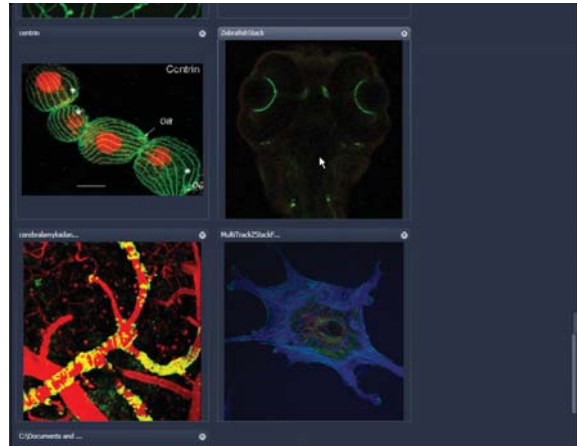
Fig. 4-151   Expose Mode Display

4.6.1.2   Container Configuration with the Container Context Menu

Clicking on the container background with the right-mouse button opens a context menu for:

– setting the view for the Image tabs (see above),

– choosing to split the Center Screen Area in 1, 2 or 3 containers and setting the automatic container layout

– choosing between separate or shared view controls between the containers.

The same menu is available in the main menu bar in the **View** part of the menu.

Fig. 4-152   Container Context Menu

Splitting the Centre Screen Area in several containers has the advantage that side-by-side comparison of image data becomes very easy.

The disadvantage is that the individual container necessarily becomes smaller. Up to 3 containers can be chosen. The "Automatically layout container" option is switched on by default and fixes the container width in layouts with multiple containers. In two-monitor setups it also fixes the right edge of the first container to the monitor edge.

If **Separate view Controls** are chosen, each container has its own set of View Options control blocks. If a **Shared view Controls** is chosen, the set of View Options control tabs spans the whole area under the containers. The tools and functions always apply to the currently active container / image.

4.6.2　2D View

This function allows to

 – display a single image in frame mode,

 – display multiple channel images in superimposed mode.

The following **View Option** control blocks apply to this View Type:

Dimensions, **Display**, **Player** and **Overlay**.

In the 2D view, only the general **View Option** control blocks is displayed. These view option blocks are also available in those other view options in which the functions are applicable.

The general view option control blocks are only described in this section.

Fig. 4-153　　**View Options control block - Dimensions**

4.6.2.1　Dimensions

The **Dimensions View Options** control block holds the tools to modify the image display with respect to the (multiple) dimensions of the image data set.

The sections (slices) in the dimensions can be scrolled with sliders (z-position and Time in Fig. 4-153 and also directly addressed with setting numbers in the spin-boxes next to the sliders.

The small button on the right hand side of the spin-boxes starts and stops an animation in the respective dimension. This basic animation can not be customized. For all other player-functionality refer to the **Player View Block.**

(1)　Zoom

In the Dimensions View Options control block there are 5 buttons related to changing the zoom factor of a displayed image:
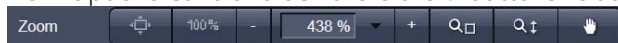
Any changes on these buttons affect the displayed image immediately. The zoom function can be performed online.

The functions of the individual buttons are:

Zoom Normal: The image is fitted automatically to size of the **Image Display** window which is always the originally displayed size.

Zoom + enlarges the image.

Zoom - reduces the image.

Zoom 100 % displays one pixel of the image also as one pixel on the screen.

Zoom-Mouse allows you to enlarge / reduce the zoom factor of an image using the left / right mouse button, provided that the cursor is inside the image.

All (only in **Gallery** or **Split** view) When active it allows you to zoom all images of a gallery to the same extent without changing the display image format.

Zoom selection via mouse: draw the area to zoom in directly on the image.
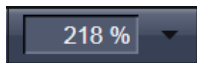
Display box: type in your magnification value in percent directly.

Slider
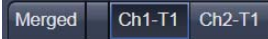
The zoom factor can be set by moving the slider. The display box displays the current zoom factor in %. 100% corresponds to the original size.

Fig. 4-154   Image Display - Dimensions - Channels

(2)   Dimensions – Channels

The **Channel(s)** buttons are designed to switch on/off channels or the display of the merged image as well as to assign color look-up tables (LUTs) to the individual channels:

Clicking on the [Merged | Ch1-T1 | Ch2-T1] buttons switches the respective channels on/off in the display.

Clicking on the [buttons] buttons switches forth and back between the assigned LUT and the **Range Indicator** .

Clicking on the little black arrow on the [buttons] buttons opens a selection of color look-up tables to assign a color or LUT to the respective channel (see Fig. 4-154). Any changes on these buttons affect the displayed image immediately.

(3)   Reuse

Clicking the [Reuse] button transfers **ALL** acquisition parameters (exception: objective and collimator, see below) from the stored image data to the **Microscope Hardware Settings / Control tools** and applies those parameters directly to the system.

The acquisition parameters of an image are displayed in the **Information View** (section 4.6.18).

In the **Maintain** tab (section 4.5.11.3) (**LSM Options** - **Reuse** tab), it can be set whether the objective and the collimator settings are also "re-used" and applied to the system. **Reuse** of the microscope objective only works in microscopes with motorized objective revolvers.

(4)   Dimensions – Crop

This function allows to interactively define the size and orientation of a rectangular scan area on the image displayed in the **Image Display** window.

The defined area is synchronized with the **Zoom, Offset and Rotation** parameters in the Scan Area panel (partially **Show all** mode) in the **Acquisition Mode** tool (section 4.5.2.5).

Click on [Crop] will display the **Crop Rectangle** in the **Image Display** (Fig. 4-155). Any changes done with the **Crop Rectangle** are setting the scan parameters immediately. On the next execution of a scan (**Auto Exposure**, **Live**, **Continuous**, **Snap**) these new scan parameters will be applied. To reset the crop function and use default values set **Zoom=1**, **Offset=0** and **Rotation=0** in the **Scan Control** window in the Scan Area panel in the **Acquisition Mode** tool (or click "reset all" in the same panel). When the [Crop] button is de-selected, the scan parameters are reset as well.
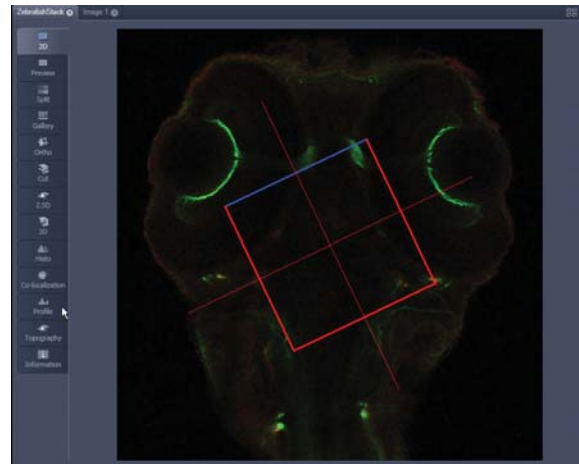

Fig. 4-155   Image Display - Dimensions - Crop

The **Crop Rectangle** is controlled via the following functional elements:

Offset   ⊕   • Click into the crop rectangle, keep the left mouse button pressed and drag the crop rectangle to the required position. Release the mouse button.

Zoom   ⤡   • Click on a corner of the crop rectangle, keep the left mouse button pressed and set the required size. Release the mouse button.

Rotation   ⟳   • Click on one end of the crosslines, keep the left mouse button pressed and set the required rotation angle. Release the mouse button. The first line scanned is highlighted in blue.

Side ratio   ⇔   • Click on any of the intersection points between crossline and crop rectangle, keep the left mouse button pressed and change the side ratio as required. Release the mouse button.

(5)   Dimensions – Positions

When clicking Positions a crosshair will be displayed at the cursor position in the image. With a mouse click the position of the crosshair in the image will be added to the Positions list in the Stage tool and the Positions tool.

(6)   Dimensions – Stage

Stage will show the cursor as a rectangle with a crosshair in the centre. With a mouse click the stage (and therefore the sample itself) will be repositioned. The image position with the cross hair will become the centre position of the stage and therefore the centre position of a next image

Fig. 4-156   View Options control block - Display

4.6.2.2   Display

In the **Display** View Option control block, **Brightness**, **Contrast** and **Gamma** of the displayed image can be adjusted (Fig. 4-156). With the [All] [Ch1-T1] [Ch2-T2] buttons, the effect of the slider settings can be restricted to an individual channel. By default, the settings apply to all channels simultaneously. The parameters can be changed with sliders or spin-boxes – or directly by typing in numbers in the number-field. With the [50] - [50] - [1] buttons, the original settings are easily re-set.

☞   The settings in this View control clock apply only to the Image Display and are NOT changed in the original data set. To write the changed settings to the image data set, use the **Burn in Brightness and Contrast** function in the **Processing** tab (section 4.5.10).

4.6.2.3   Player

This function allows to

– animate frames of a Z-Stack or a time series

– specify animation parameters such as range and animation speed

When the image displayed in the **Image Display** window is neither a Z-Stack nor a time series this View Option control block is empty.

Fig. 4-157   View Options control block - Player

The **Player View Option** control block is controlled by the following elements:

The current position slider is displayed in the View Option control block **Dimensions** and can also me moved manually or by entering the slice number in the input box. The slider can be accessed only, when the player animation is off.

Start and **End** slider: The setting of the **Start** sliders limits the number of slices to be used for the animation. Slices before **Start** and after **End** are not taken into animated. These sliders can be changed during automatic animation.

Starts the forward motion of the automatic animation. After the last slice has been passed, restart is made at the first slice.

Starts backward motion of the automatic animation. After the first slice has been passed, restart is made at the last slice.

Starts the combined forward / backward motion of the automatic animation, i.e. when the last slice has been reached, the backward motion is activated, and the forward motion is activated again on reaching the first slice.

Stops the automatic animation.

Move to the first slice.

Move to the last slice.

Speed can be set through a slider or spin-box (selection box).

Multidimensional animations can be selected by including individual dimensions by checking tick boxes.

Fig. 4-158   View Options control block - Overlay

4.6.2.4   Overlay

This function allows to

– select from a set of drawing functions such as rectangles and arrows,

– add a scale bar to the image, as well as text annotations,

– use a set of interactive measurement functions for length, angle, area and size,

– add a text box that displays the coordinate of a hidden dimension (e.g., the z-position in a Z-Stack or the timestamp in a time series).

(1)   Functional Description

The overlay function uses a plane separate from the image plane (the graphics plane) and therefore does not change the content of the image(s).

The overlay view option control block is **available in all View Types except 2.5D, Lambda Coded and Preview**.

Any changes done with this function are effective immediately.

The overlay graphics can be stored in the images or separately in a file from where they can be re-loaded.

- Activate the **Overlay** view control block by clicking on [Overlay] in the control bar above the view controls. If the overlay view option control block is displayed, the [Overlay] button is highlighted in blue. Click the button again to hide the control block.

- **Note** that if the display of the overlay elements has not been deactivated by selecting the **Hide** tick box, the created elements will still be displayed in the **Image Display** after deactivation of the **Overlay** view control block.

(2)   Available functions in the Overlay View control block

Fig. 4-159   Overlay view control block

The following list describes the most common functions in the **Overlay** view control block. More can be found in the [Select pull down menu icon] pull down menu.

Select tool: Activation of the mouse cursor for selection, resizing or movement of an overlay element in the **Image Display**.

Resizing: Click on the handle and hold down the mouse button, drag the handle, release the mouse button.

Movement: Click on the line and hold down the mouse button, move the entire element, release the mouse button.

Line tool: Creation of a straight line in the **Image Display**.
Click and hold down the mouse button, draw a line in any required direction, release the mouse button to end the procedure.

Rectangle tool: Creation of a rectangle in the **Image Display**.

Click and hold down the mouse button, draw a rectangle in any required direction, release the mouse button to end the procedure.

Closed polyline tool: Creation of a closed polyline figure in the **Image Display**.

The first click sets the starting point, each additional click adds a further line, a click with the right mouse button closes the figure and ends the procedure.

Open Bezier tool: Creation of an open bezier figure in the **Image Display**.

The first click sets the starting point, each additional click adds a further line, a click with the right mouse button ends the procedure.

Ellipse tool: Creation of an ellipse in the **Image Display**. This tool is available through the pull down menu.

The first click sets the center point, the displayed line permits the determination of the first dimension, the second click sets the first dimension, the second dimension and rotation direction can then be determined, the third click sets the second dimension and direction and ends the procedure.

Line with arrow button: Creation of a line with arrow in the **Image Display** window.

Click and hold down the mouse button, drag the line in any required direction, release the mouse button to end the procedure.

Scale bar: Creation of a horizontal or vertical scale bar with default increments in the **Image Display**. Click and hold the mouse button for the starting point, drag horizontal or vertical scale, release the mouse button to end the procedure.

Gray tones / color shades button: Generates a rectangle with a display of gray tones or color shades in the image.

Text (Annotation) tool: Creation of a text box in the **Image Display**.

After clicking on **T**, the **Text** window will be displayed, and text can be entered via the keyboard. The **Font ...** button enables you to select the font style and size in the **Font** window. The entered text will be displayed in the left upper corner of the **Image Display** window after clicking on **OK** and can be moved to the required position using the mouse.

The **Text** window can also be activated with a double-click on a created text box, and the entered text can be edited subsequently.

Insert opens up a further window which allows you to annotate coordinates, time and Z-position with either automatic or user definable units and precision. This annotation is updated during image acquisition and can be exported with the image. The annotation can be stamped into already existing images.

Delete button: All the overlay elements and dimensions in image are deleted. If one overlay element was marked before, only this element will be deleted from the image.

Measurement of the overlay element in the **Image Display** window. On activation of the **Measure** button, the selected overlay element and all the elements created afterwards are measured and assigned with a measuring value. The measuring value can be shifted without regard to the overlay element. If of importance, the length and perimeter of a line figure, the area of a closed figure and the inclination angle of a single line will be displayed. On deactivation of the **Measure** button, the measuring value of the selected element is no longer displayed, and all the elements created afterwards will not be assigned with a measuring value.

Line thickness pull down: sets the line thickness of the selected element.

Cut Region tool: The region of a Z-Stack or 4D-image surrounded by an **Overlay** element is extracted and displayed separately in a new **Image Display**. This function is only active if the active **Overlay** element defines a closed contour or volume.

Color selection pull down: In this pull down, different colors can be assigned to the overlay elements with a click of the mouse. A selected color is automatically assigned to the currently selected overlay element and to all elements created afterwards.

To insert a **Coordinate Label** in the image display, click the `Coordinate` button. The coordinate settings dialog (Fig. 4-160) offers the available settings and upon clicking **OK**, the selected coordinate is displayed as a text field in the image display. This text field is always immediately updated when the slice/section/time point is changed in the display by changing settings in the **Display View Option** control block or, for example, starting the **Player** animation. This feature is particularly useful to display time or z-position in an exported animation movie for presentations (see Fig. 4-159).

Fig. 4-160   View Options Overlay - Coordinate

`More ...`**: The More** options (Fig. 4-161) allow toggling the:

Creation of Overlay Mode between

–   **Switch to selection mode:**
    default setting, always switches back to the **Select** tool after creation of an overlay element.

–   **Multiple Elements:**
    allows to create one overly element after the other with the selected overlay element type, without need to re-select the respective tool.

–   **One element only:**
    In this mode, only one element in the overlay plane is allowed. Creating a new one deletes the previous one.

Color Mode between

–   **Individual colors**:
    The color of each element can be selected with the color selection pull down.

–   **Automatic assignment**:
    The color of the overlay elements are assigned automatically

–   **Common color**:
    All elements have the same color.
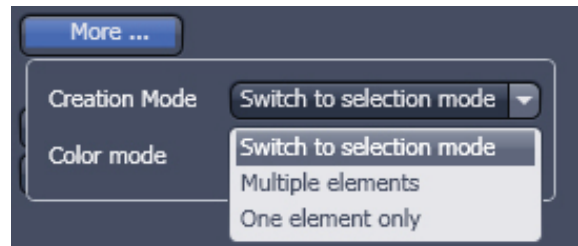
Fig. 4-161   View Options Overlay - More

Activation of the tick box `Hide` keeps all overlays of the image document hidden.

Activation of the tick box `Numbers` displays the number of every overlay element in the image display

To load / save overlays from / to a file use the **Load / Save** buttons in this View Options control block. Naming and location in the file system can be set individually.

4.6.2.5   Preview View

The Preview View Type is an enlarged copy of the thumbnail Previews described in the **Processing** tab section of this manual (section 4.5.10).

4.6.3   Split View

This function allows to

– display the individual channels of a multi channel image as well as the superimposed image.

The settings of the **Dimensions**, **Display**, **Player** and **Overlay** view options control blocks apply with the following additional features:

– The **Dimensions** View Options control block shows the **Merged** tick box to activate / deactivate the display of the channel overlay and a **Zoom All** button is added synchronize zooming to the Dimensions view control block.

– Overlay elements are always displayed in all channel displays.
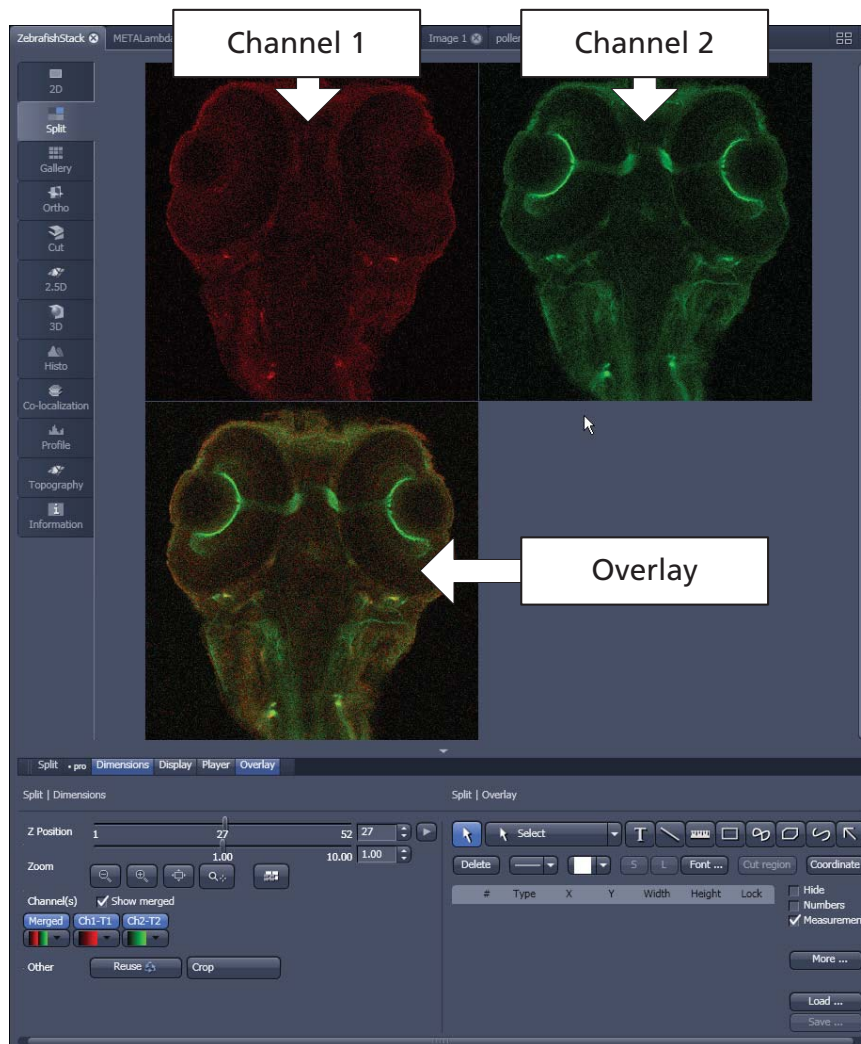
Fig. 4-162   Image Display, Split view type

☞   This function is useful to optimize the individual channels in a multi channel image acquisition together with the **Range Indicator** palette.

4.6.4   Gallery View

This function allows to

- display images (Z-Stack, time series, combination of both) side by side in a tiled fashion,
- add data relevant to the displayed images (Z-Stack slice distance, time of acquisition or wavelength),
- extract a subset of images from the original stack and store the result as a new image (controls for this function are in the **Processing** tab function **Copy → Subset** (see section 4.5.10.12).

The settings of the **Dimensions, Display**, **Player** and **Overlay** View Options control blocks apply.

The additional view-specific **Gallery** View Option control block includes a tick box to switch on and off the display of the slice coordinates in the Gallery display. If this text is activated, transparent mode and color can be selected in this control block too (see Fig. 4-163).
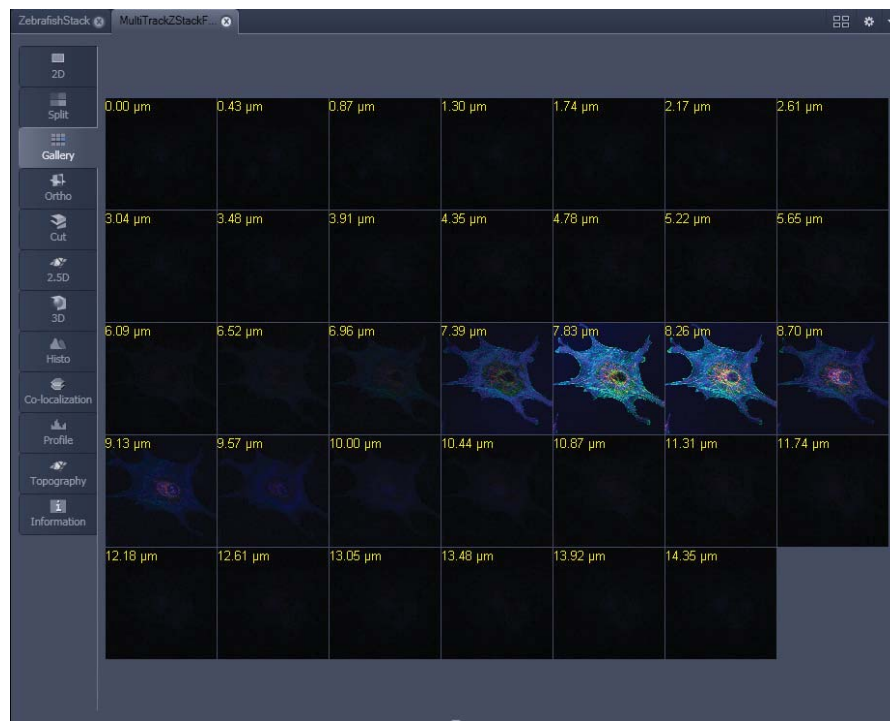
Fig. 4-163   Image Display window - Gallery display
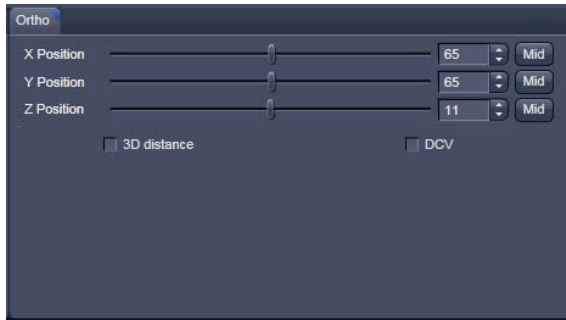
Fig. 4-164   View Options control block - Ortho

4.6.5   Ortho View

This function allows to

– display a Z-Stack of images in an orthogonal view

– measure distances in three dimensions

The settings of the **Dimensions, Display**, **Player** and **Overlay** view options control blocks apply.

In addition to the 4 general View Option control blocks, the view-specific **Ortho View Option** control block is available (Fig. 4-164).

In the **Ortho** View, section sliders appear in the **Ortho View Option** control block together with orthogonal projections in the image (Fig. 4-165).
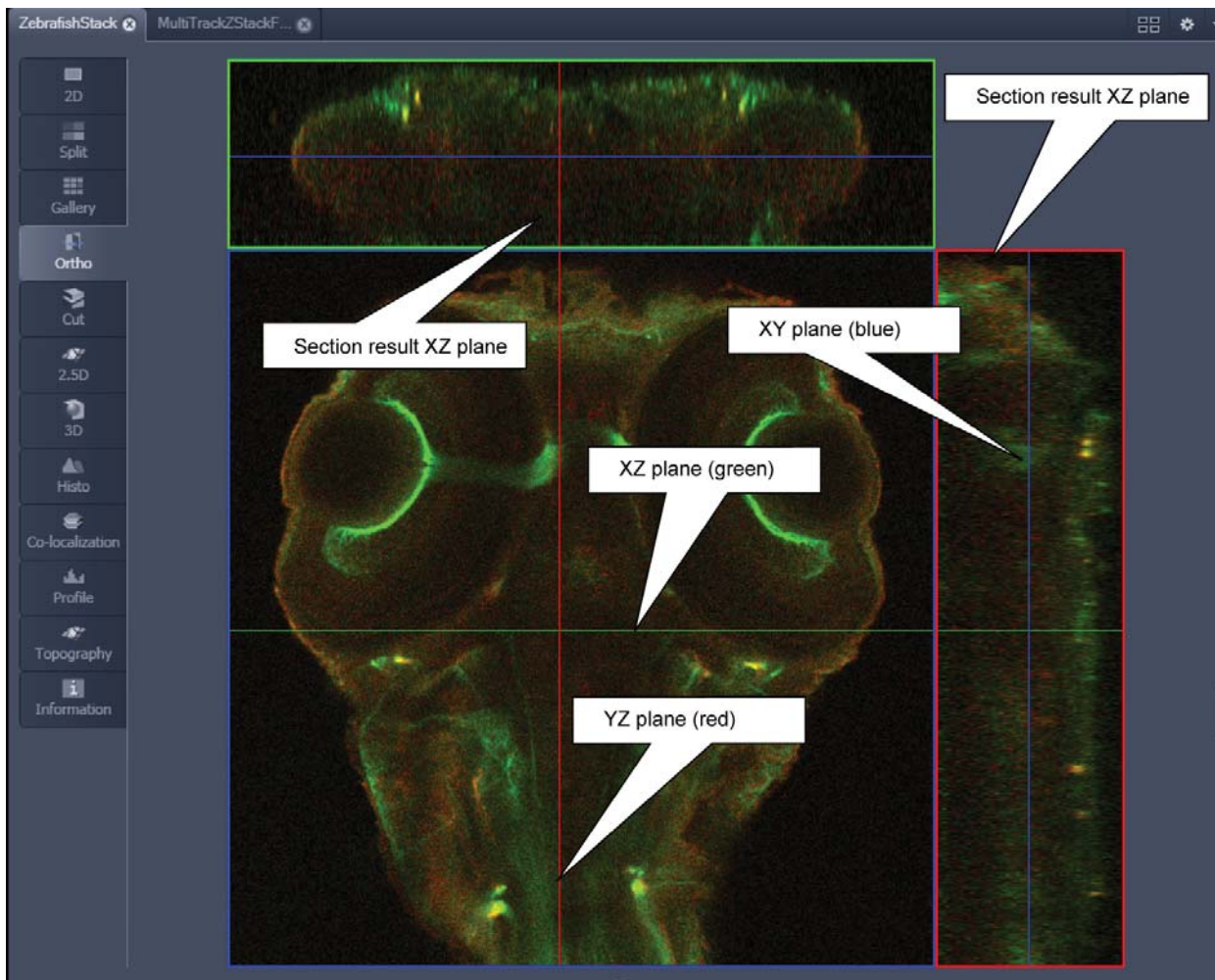
Fig. 4-165   Image Display - Ortho View

4.6.5.1   Ortho - Select Function

- By changing the parameters X, Y and Z in the **Ortho** View Option control block, the section plane can be positioned at any XYZ coordinate of the Z-Stack.

The position of section planes can be changed in various ways:

- By moving the sliders on the **Ortho** View Option control block.

  – X and Y settings may range from 1 up to the maximum number of pixels scanned (in the example shown: 512).

  – Z settings may range from 1 to a maximum of n, with n standing for the number of slices produced in the stack.

- By directly entering the relevant number value in the X-, Y- or Z-input box and pressing the **Tabulator** or **Enter** key.

- If you move the cursor into the **Image Display** window, it changes into a crossline symbol $\oplus$. By positioning this symbol with the mouse you can move the XZ and YZ section planes to any point of intersection with the XY plane. A click with the left mouse button places the intersection to the desired position.

- If you move the crossline symbol $\oplus$ onto the intersection of the red and green section planes, it changes into the: symbol. If you now press the left mouse button and keep it pressed you can reposition both section planes **simultaneously**.

- If you move the crossline symbol $\oplus$ onto the green section plane, it changes into the symbol. If you now press the left mouse button and keep it pressed, you can reposition the (green) XZ section plane.

- You can reposition the (red) YZ plane in the same way using the symbol.

The result of an orthogonal section is visible at the image margin.

  – Section of the XZ plane (green line) through the stack: above the XY image.

  – Section of the YZ plane (red line) through the stack: right of the XY image.

  – Section of the XY plane (blue, slice plane of the stack): center image.

4.6.5.2   Ortho - Distance Function

- Activating the ✓ 3D distance tick box permits length measurements in 3D space.

- Click on the Mark button to set the first XYZ-point for the measurement of the spatial distance.

- Set the second XYZ-point for measurement by moving the X-, Y-, Z-sliders or by moving the green, red and blue lines in the image.

  - The projections of the spatial distance are shown in the image planes by yellow lines (Fig. 4-166).

    The actual spatial distance is calculated and shown in µm next to the Mark button.
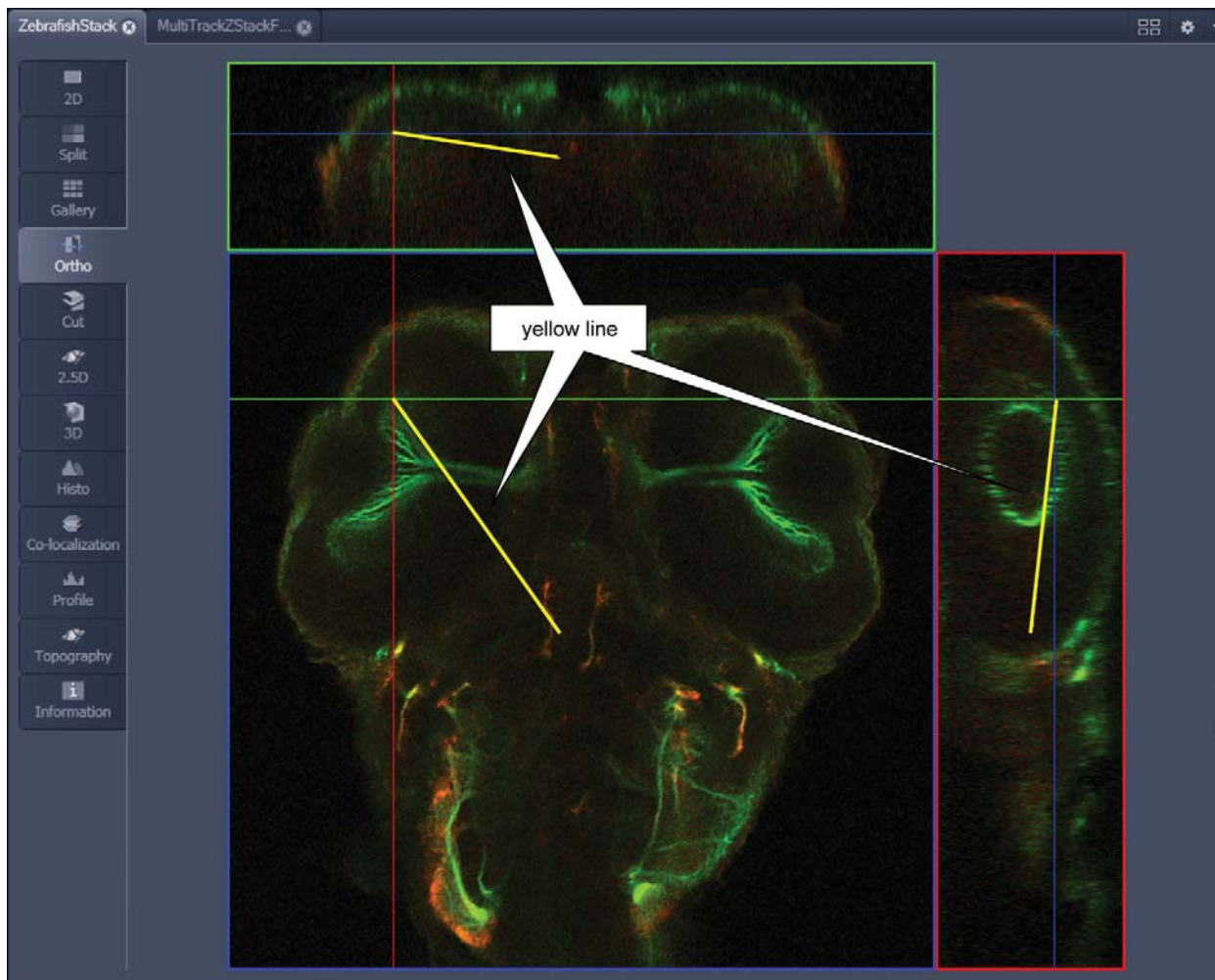
Fig. 4-166   **Image Display – Ortho View - 3D Distance Measurement display**

4.6.6   Cut View

This function allows to display a user defined section plane (= cut plane) of a Z-Stack. It automatically improves the image of the section plane by trilinear interpolation.

The settings of the **Dimensions, Display**, **Player** and **Overlay** View Options control blocks apply.

Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily hidden while the toolbar is displayed.

Fig. 4-167   Cut View – control block

- By varying the parameters **X**, **Y**, **Z**, **Pitch** and **Yaw**, you can position a section plane of any orientation within the stack volume.

- Clicking the Mid Mid Mid 0 0 buttons restores the original position.

In order to place the Cut View Plane interactively use the interactive graphic in the cut view control tab.

4.6.7   2.5 D View

This function allows to

– display the two-dimensional intensity distribution of an image in an pseudo 3D mode,

– show the intensity values in profile, grid or filled mode,

– show separate distribution for each channel in a multi channel image.

☞   The **2.5 D** button can also be used online during scanning.

The settings of the **Dimensions** View Options control blocks apply. The settings of **Display**, **Player** and **Overlay** View Options control blocks do not apply.

Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily hidden while the toolbar is displayed.

The image display in the **2.5 D View Type** is the same as in the **3D** (VisArt*plus*) View Type. For a description of the controls in the display window see section 4.6.8.8.

In brief, the viewing plane of the **Image Display** window can be rotated, tilted either directly with the mouse or by the scroll bars on the right-hand side and the bottom of the **Image Display** window.

a)

2.5 D Profile View

b)

2.5 D Grid View

c)

2.5 D Filled View

d)

2.5 D Surface View
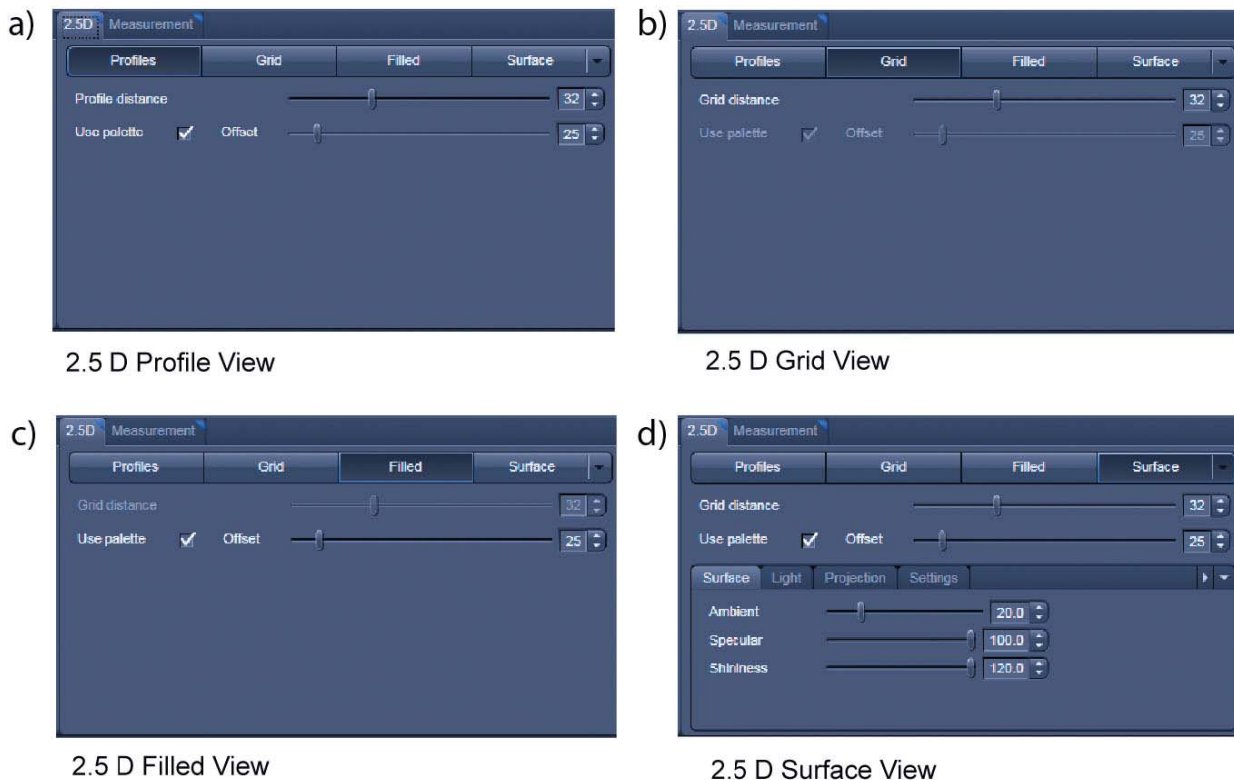
Fig. 4-168   2.5 D View Option control tabs

2.5 D Profile View

2.5 D Grid View

2.5 D Filled View

2.5 D Surface View
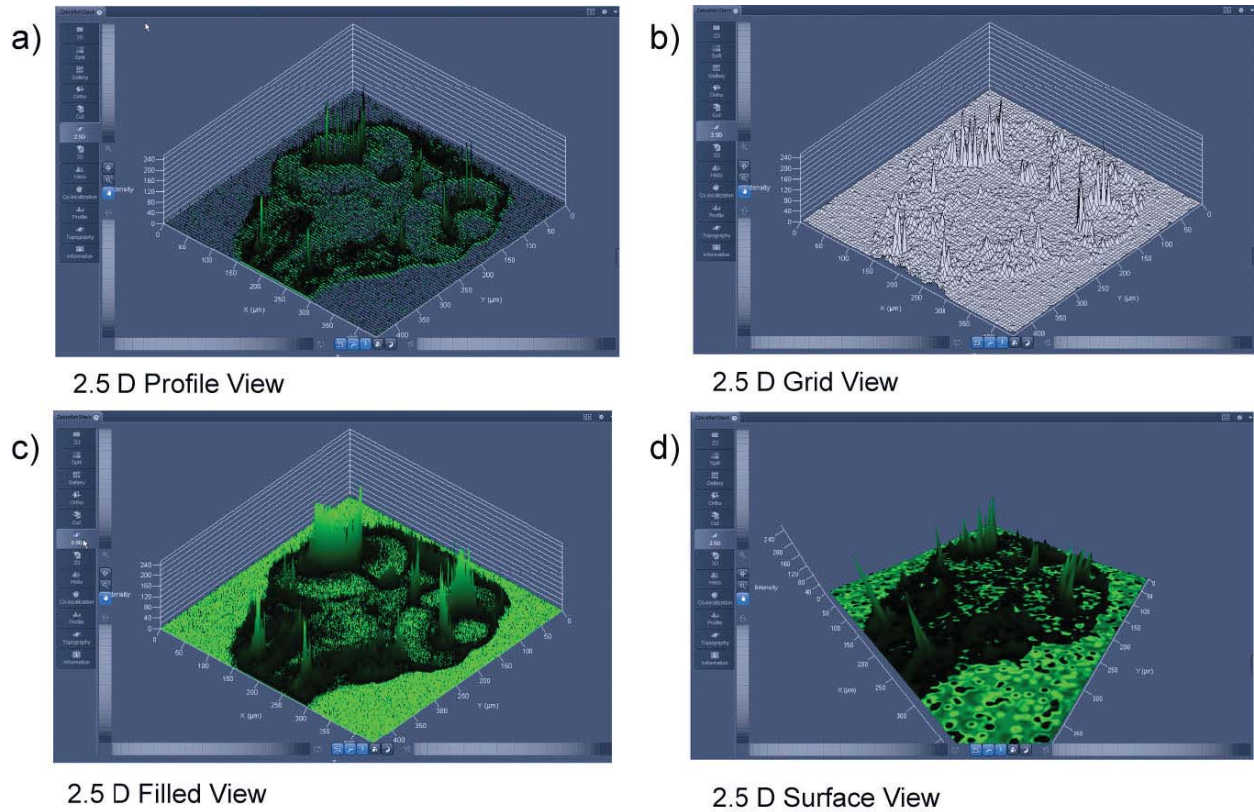
Fig. 4-169   2.5 D Image Display

The **2.5 D** view control tab toolbar contains the following function elements:

Profile button   Profile display (vertical polygon display). Setting of the **Profile Distance** between 1 and 20 using the slider.

Grid button   Grid display (horizontal grid display). Setting of the **Grid Distance** between 1 and 20 using the slider.

Filled button   Color diagram (filled 3D diagram). Selection between the **Mono**, **Rainbow** and **Six Step** color palettes.

4.6.8   3D View (Image VisArt*plus*)

With Image VisArt*plus* the Z-Stack of images generated by using the LSM systems can be displayed in three dimensions. Display and rendering of Z-Stacks with up to eight color channels are supported and the data can be a time lapse series (5D Image Stack).

The parameters of the **Channels** (including the selected LUT) set in the **Dimensions** View Options are applied. All other parameters from the **Dimensions, Display**, **Player** and **Overlay View Option** control blocks are not applied.

A click on **3D** will display the **3D** View Controls. Any changes done within these controls are effective immediately.

☞   The **3D** View can also be used online during scanning.

Fig. 4-170   Image Display window - 3D display

The **3D** view controls contain the following functional elements:
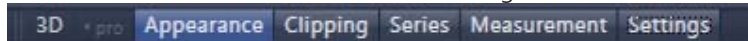
They are displayed when the tab is pressed in the order shown above.

3D:
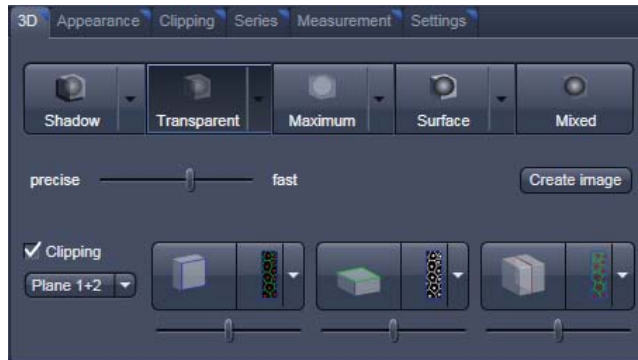
On the **3D** View control panel the different render modes are accessible:
Shadow, **Transparent**, **Maximum**, **Surface** and **Mixed**. The rendering quality can be adjusted by using the ⎡precise ——○——————— fast⎤ slider. The **Create image** button ⎡Create image⎤ opens a new image window and produces a 2D image of the currently used render mode. It uses the screen resolution unless it is otherwise defined in the **Settings** tab (see there).

Clipping ☑ Clipping activates the Clipping Plane functionality. 3 different Clipping Planes are displayed and can be operated individually via 3 buttons:
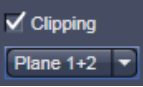
The Cut button toggles between different clipping behaviors of the respective planes.

On the Clipping Planes different surfaces can be chosen from a pull down list:

The Position of the clipping planes can be adjusted by the **Position Slider**.
Further functionality is available on the **Clipping Plane View control tab** → see there.

A wedge between 2 Planes can be clipped using the pull down ⎡Plane 1+2 ▼⎤ underneath the activation check box for the clipping functionality.

Appearance:
On this view control panel the settings for the respective render mode are available.

Clipping:
VisArt*plus* has the option to cut off certain parts of the 3D information by using so called "clipping planes". All the necessary tools to operate the clipping planes are found on this control panel.

Series:

3D Data can be visualized online by simply grabbing and turning the data stack with the mouse. In addition to this interactive way of interacting with the data VisArt*plus* is able to perform render series where the dataset is turned around one of the axis, along a freely defined position list or a freely definable start and end point. The resulting series can be saved as movie files for presentations. A preview of the render series is available in the **position list** mode.

Measurements:

Measurements of distances, angles and polylines can be measured using the **Measure** panel in VisArt*plus*.

Settings:

In the **Settings** panel global settings for the VisArt*plus* Module can be found.

Fig. 4-171   Appearance panel - Shadow button
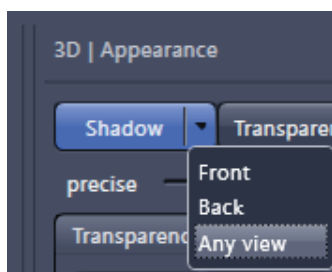
Fig. 4-172   Shadow pull down menu

4.6.8.1   Shadow Projection

In the Shadow render mode the structures in the image are illuminated by a virtual light source.

The volume is viewed as if looking through the eyepieces of the microscope and the light source projects a shadow onto a background plane.

This gives the data a reference in space, making visualization much easier. The rendering effect itself consists of a combination of light reflection and opacity (degree of transparency).

The display in this mode is calculated by the main processor (CPU) of the computer (Voxel mode). The different modes can be accessed by clicking on the pull down symbol at the right hand side of the **Shadow** button (Fig. 4-172).

- **Front** button: Shadow rendering front view

- **Back** button: Shadow rendering back view

- **Any View** button: Shadow rendering with user defined view

With a click on **Front**, the 3D reconstructed image is displayed in a shadow projection where it is illuminated at a 45° angle from the front left.

A click on the **Back** button creates the same projection with illumination from back left.

Detailed settings for shadow projection can be accessed via the **Appearance** tab.
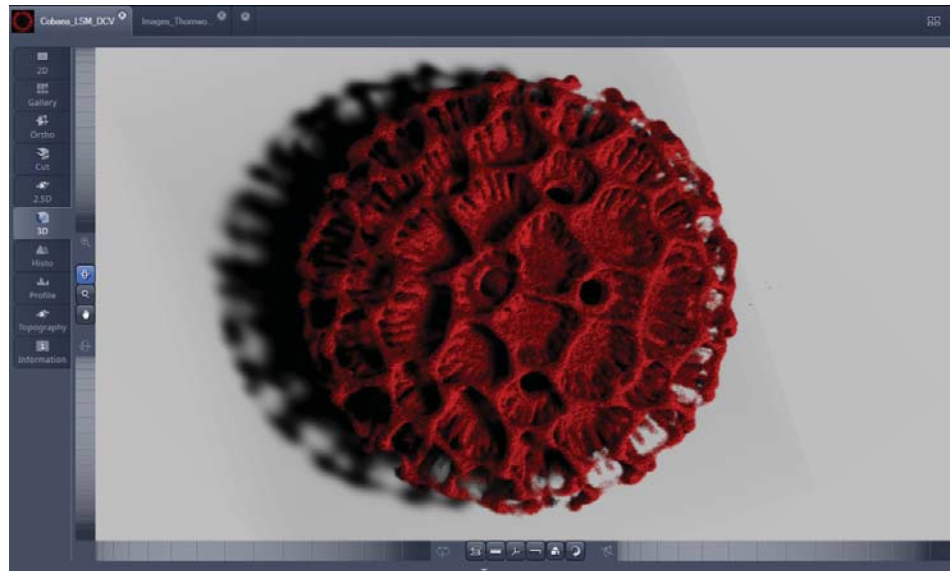
Fig. 4-173   Image Display window, 3D display, Shadow projection, Front view

The zoom wheel to the left of the **Image Display** window allows continuous zooming of the 3D reconstructed image.

A click on the **Any View** button displays the 3D reconstruction image in a shadow projection where the viewing point can be defined. In addition to the zoom setting, the image can be rotated around the three orthogonal axes via the relevant setting wheels.

However, the 3D orientation can also be set directly in the **Image Display** window by clicking, holding and dragging the 3D reconstructed image with the mouse.
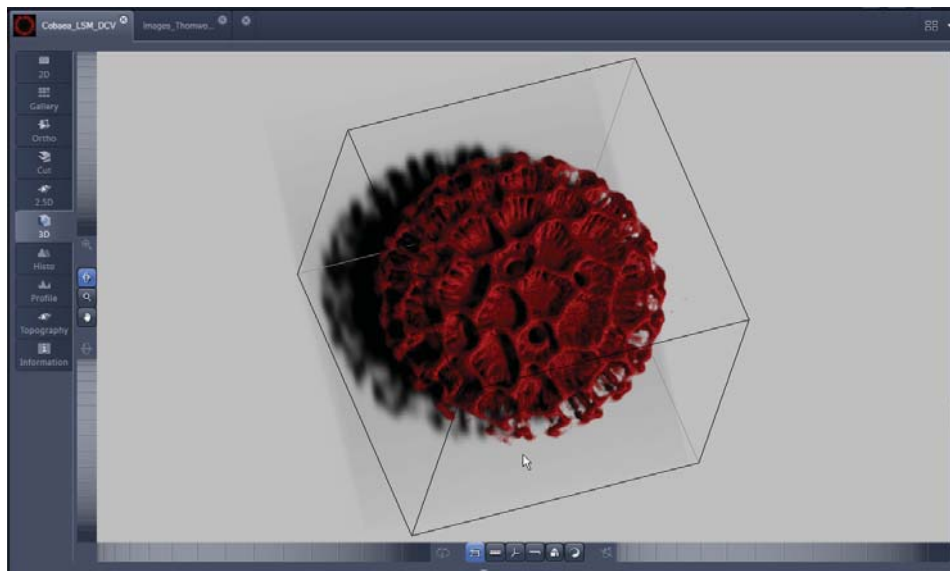
Fig. 4-174   Image Display window, 3D display, Shadow projection, Any View

The following additional buttons are available in the **Any View** shadow projection mode:

- After activation of the **Frame** button (below the image), a bounding box is drawn around the 3D reconstructed image.

☞   Depending on the used mode and hardware configuration, it can take several seconds until the 3D reconstruction is refreshed on the monitor after reorientation.

- A click on the **Coordinate System** button displays a colored coordinate system in the **Image Display** window, where the X axis is displayed in red color, the Y axis in blue and the Z axis in green.

- A click on the **Scale** button display an X-, Y- and Z-scale in the **Image Display** window.

- A click on the **Home** button resets the display parameters to the default values.

A click on the **Any View** button displays the 3D reconstruction image in a shadow projection where the viewing point can be defined. In addition to the zoom setting, the image can be rotated around the three orthogonal axes via the relevant setting wheels.

However, the 3D orientation can also be set directly in the **Image Display** window by clicking, holding and dragging the 3D reconstructed image with the mouse.

- A click on the **Animation** button activates the animation mode. The object can be pushed by dragging in the **Image Display** window and rotates continuously. Any new push with pressed left mouse button changes the rotation direction and speed of the animation.

4.6.8.2   Transparency Render Mode

Principle:

In the **Transparent** mode a three-dimensional image with a transparent effect is calculated. At least two two-dimensional texture stacks (computed from two different angles) are used depending on the volume orientation. In contrast to the Shadow mode the scene here is illuminated by diffuse white light from the rear. By changing the available parameters one can mix different channels and reveal relationships between information in those channels. This view is particularly useful for visualizing the three-dimensional relationships between structures within the volume. This mode can be displayed both in CPU based Voxel mode (**Basic**) and also (with the help of a suitable graphics card) with accelerated calculations in so-called OpenGL mode (**Advanced**).
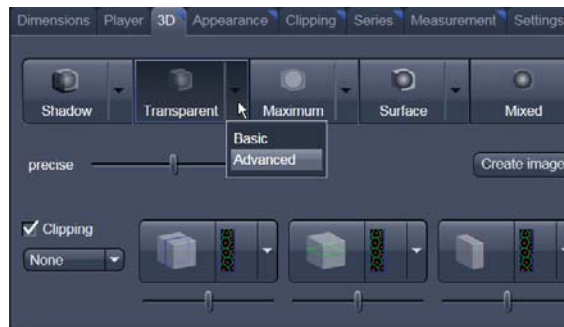
Fig. 4-175   3D View Control Panel, Transparent

Basic   Transparency rendering (voxel based) (CPU)

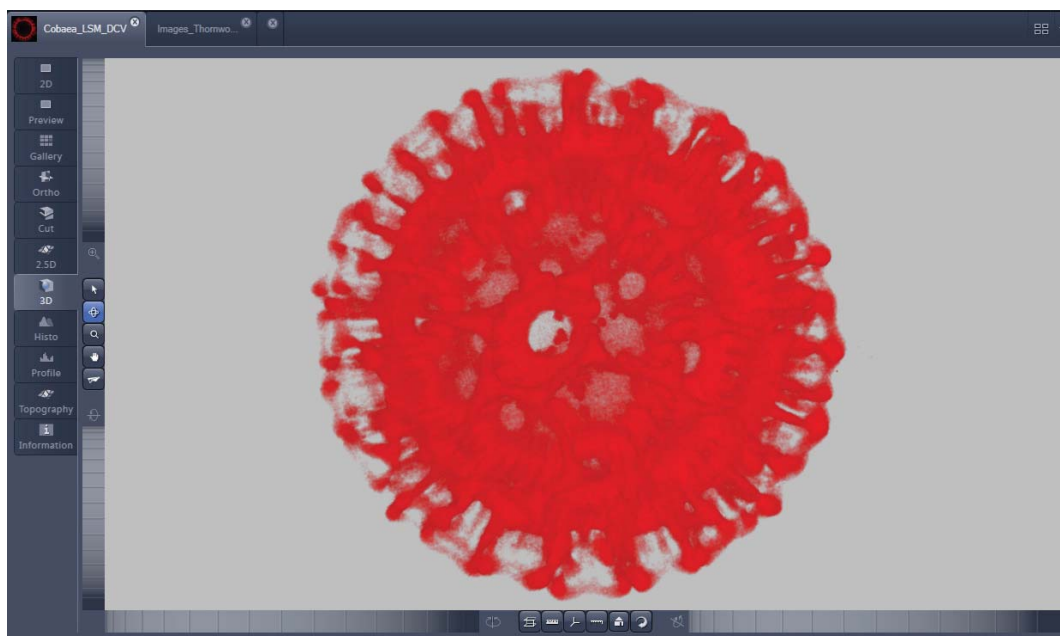**Advanced**   Transparency rendering (voxel based) with textures (GPU)

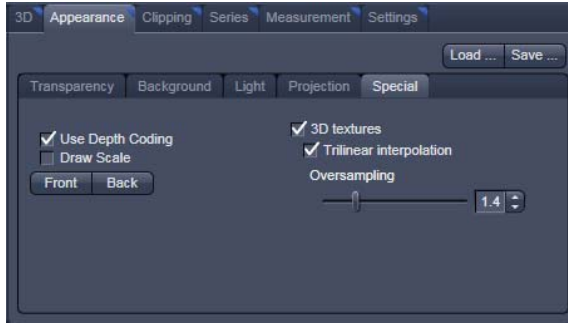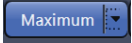**Fig. 4-176   Image Display window, 3D display, Transparency projection, Advanced**

Fig. 4-177   **3D toolbar – Transparent – Depth Coding**

By means of the **Depth Coding** function (Fig. 4-177), the Z-information contained in a sequence can be colored with the colors of the rainbow, in which case "blue" stands for higher values and "red" stands for lower values.

The **3D Textures** option can be used for faster and better rendering of transparency renderings.

☞   Note that this function is only available in Transparency and Maximum Intensity Render Modes.

4.6.8.3   Maximum Mode

Clicking on the [Maximum] button switches to **Maximum projection** mode, which calculates a three-dimensional view of the data by only showing pixels of the highest intensity along the projection axis. This view is particularly useful for displaying three-dimensional images in two dimensions, e.g. in publications, and guarantees a maximum possible degree of transparency. The display in this mode can be calculated by the main processor (CPU) of the computer ("Maximum basic") or the graphics card (Maximum advanced) in OpenGL mode.

Note that this is a rendering. If desired 1:1 Maximum Intensity Projections can also be done in the **Processing** tab in **Maximum Intensity Projections** (see section 4.5.10.2). With these Images exact Intensity Measurements can be performed.

Maximum Basic	Maximum Intensity CPU based
Maximum Advanced	Maximum Intensity GPU based

4.6.8.4   Surface Render Mode

In the **Surface** mode (Fig. 4-178) grey values in the image are computed as solid surfaces (also known as Isosurfaces) instead of soft transparent shades.

This display mode is useful to focus the attention on certain structures (e.g plant cell walls, nucleoli, intracellular vesicles) while potentially hiding additional structures deeper within.
In this mode you also can select between Voxel (CPU) [basic] and OpenGL (graphics card) [advanced] display.

Fig. 4-178   3D toolbar – Surface

The [full resolution] projection is based on a high precision calculation method for 3D information on the basis of triangles with maximum resolution.

Basic button   Surface rendering (voxel based)

Advanced button   Surface rendering (triangle based)

Full Resolution button   High accuracy surface rendering (triangle based)

4.6.8.5   Mixed Render Mode

In **Mixed** mode both, transparency and surface mode, can be mixed in one volume display.

Using this mode one can render small structures within cells such as vesicles or speckles (e.g. FISH signals) as surfaces in one channel of a multichannel image while rendering the surrounding cytoplasm from another channel in transparency mode.

This way one can also visualize more complex relationships convincingly.

Fig. 4-179   3D toolbar – Mixed

The render mode (**Transparent**, **Surface**, **Surf.+Transp.**) can be chosen per channel.

☞   The channels can also be switched off individually by de-activating the channels in the "Dimensions" view control panel.
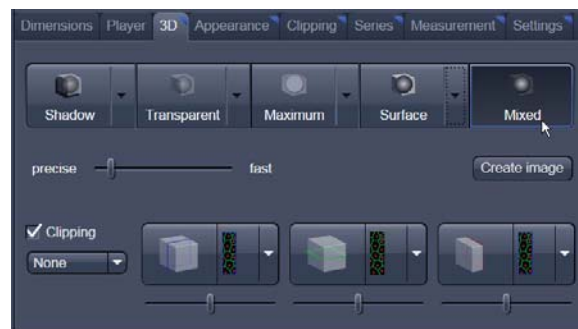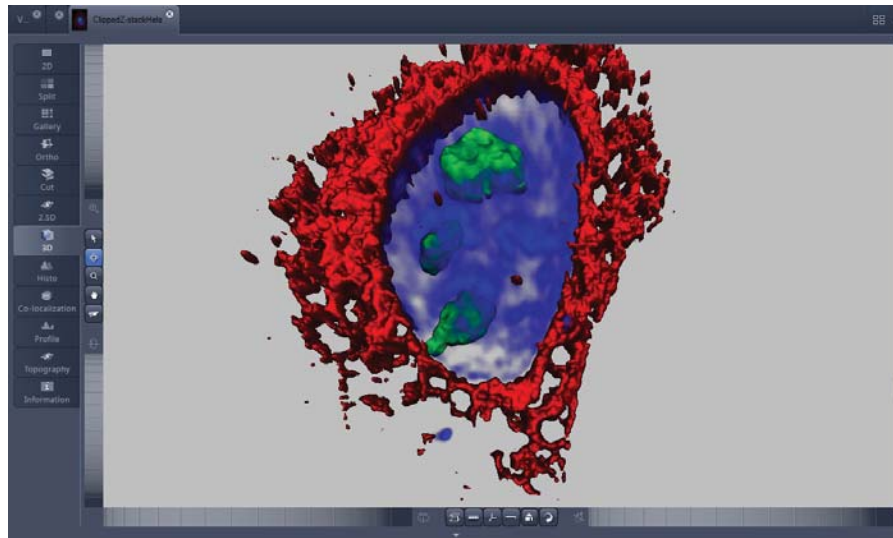
Fig. 4-180   Image Display window, 3D display, Mixed projection

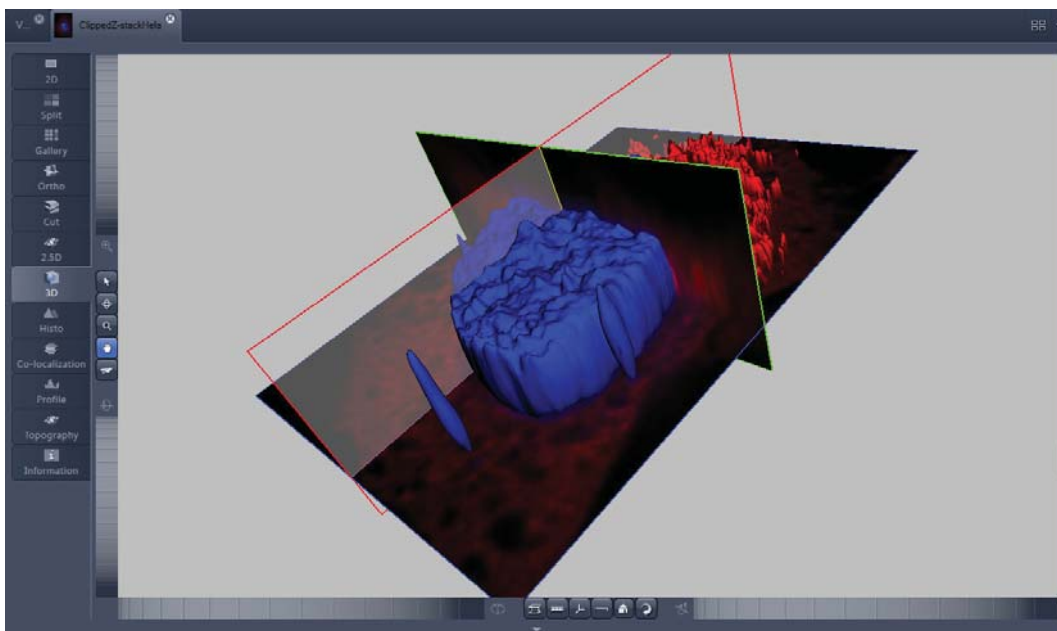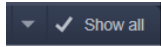### 4.6.8.6   Clipping Planes

Fig. 4-181   Image Display window, 3D display, Clipping planes

VisArt*plus* can display up to three so called **Clipping planes**. These planes can be used to cut the volume open in order to visualize structures which are within a given volume. Clipping planes make most sense when used in the **Surface** and **Mixed** mode but can also be used with limited functionality with the rendering modes **Maximum** and **Transparency** as well as in the Split mode (see below)

The clipping planes can be accessed via the **3D** tab or the **Clipping** tab. On the 3D tab only the most commonly used functionality is available. The full functionality can be accessed on the **Clipping** tab.

Note that the **Clipping** tab is only available with the **Show all** mode active.
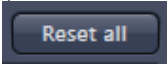

In **Surface** mode clipping planes can be used in a channel specific manner. This means, that in a multichannel image one can selectively cut away portions of the volume in one channel in order to unveil other structures from other channels. This technique is also applicable in **Mixed** mode.

A further possibility is, to use clipping planes in order to display only the grey values of the volume which touch the clipping plane.

All these functions can be used for creating a render series in order to show them and to make spectacular movies for presentations.

There are three clipping planes, which are labeled for ease of use with colored outlines. These outlines do NOT represent the channels. The standard orientations of the planes are **blue**: XY plane, **green**: YZ plane and **red**: XZ plane. This is linked to the default orientation of the volume axis.

Activating the **Clipping** panel displays the clipping planes and displays those with default settings if the image was not opened in the 3D module before. is setting back the appearance of the planes to the following settings:

–   Each plane is placed into the center of the volume and oriented in orthogonal fashion.

–   The planes have an opaque surface and colored outlines.

–   The clipping functionality of the planes is not activated in this state.

Controlling the clipping functionality of the 3 planes

The clipping planes are operated by three buttons on the **3D** tab which are color coded in the same way the respective planes are color coded. The buttons are located below the **Render Mode** buttons and can be activated by ticking the **clipping** tick box.

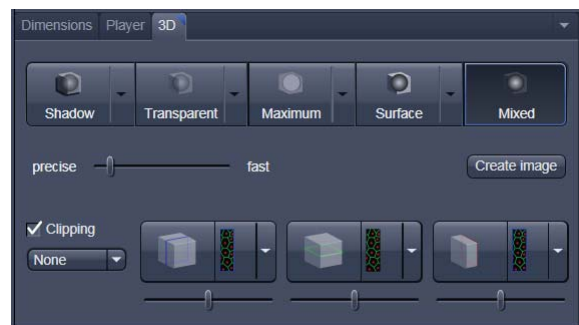☞   Note that full functionality of the clipping planes can be found in the **Clipping View Control** tab!

Fig. 4-182   Toggle buttons

By toggling these

 buttons, the clipping behavior of the planes can be set in the following manner:

– **None:** hides the plane completely

– **Front:** clips away the data on the front side of the plane

– **Back:** clips away the data on the back side of the plane

– **Both:** clips away the data on both sides of the plane

– **Show Plane** only displays the plane – no clipping

These adjustments can be made individually for each plane. The clipping planes can be chosen by selecting the respective button.
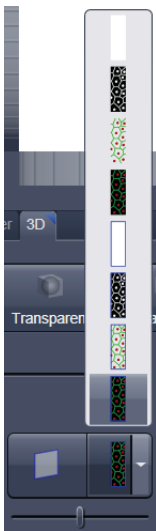

Fig. 4-183   Clipping planes

Appearance of the clipping planes

By default the clipping planes have an opaque surface and a colored outline. This can be changed by using the dropdown in the **3D** View Control tab. In the **Clipping** View Control tab, this function can be found in a pull down.

☞   This function is applicable to all 3 clipping planes separately.

Colored:
white - opaque surface of the plane

Binary:
displays the binary data on the clipping planes

Textured:
Data which are touched by the plane are displayed on the clipping plane as in the render mode **Transparent** but as a two-dimensional projection. This does include the settings (threshold , ramp etc) applying for **Transparent** render mode. Black pixels are transparent.

Textured fine:
Same as above but with 4x times the resolution

Tansparent:
Data which are touched by the plane are displayed on the clipping plane as in the render mode

Transparent but as a two-dimensional projection. This does not include the settings applying for **Transparent** render mode. Black pixels are transparent.

Textured opaque:
as with **Transparent** (including the settings) but in this case black pixels are non-transparent, rendered volume data in the background do not shine through.
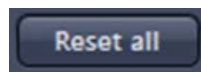
Interactive positioning of the clipping planes

The clipping planes can be moved interactively with the mouse by activating the ▮ button. If hovering over a plane outline of the plane turns into a different color, the cursor turns into a crosshair indicating that the plane can now be moved. Clicking and dragging then moves the plane. The clipping behavior (e.g. "front") is maintained during the movement.

☞   These movements can also be "recorded" using the render series function.

Clipping Planes Settings

Activate or deactivate the clipping functionality globally by the check box **Enable clipping**.

 displays the three clipping planes in the default mode (opaque surface, colored outlines, clipping planes active, no clipping).

With the **Plane** tabs the planes can be addressed individually and their settings can be adjusted independent from each other.

Outline displays or hides the colored outlines. The actual color can be chosen from a color chooser for each plane individually.

Show Plane hides or displays the plane.

Fig. 4-184   Clipping planes settings

☞   A plane can cut away information whilst being "invisible".

☞   For distance or angle measurements on a clipping plane the plane has to be displayed and a color has to be chosen for the clipping plane.

The appearance of the plane can be selected from a drop-down menu (see Fig. 4-183).

Clip names a row of toggle buttons switching between different clipping modes as named on the buttons

.

Using the **Pitch**, **Yaw**, and **Position** sliders each plane can be freely orientated in three-dimensional space.

Fig. 4-185   Sliders Pitch, Yaw and Position

4.6.8.7   Flying Mode

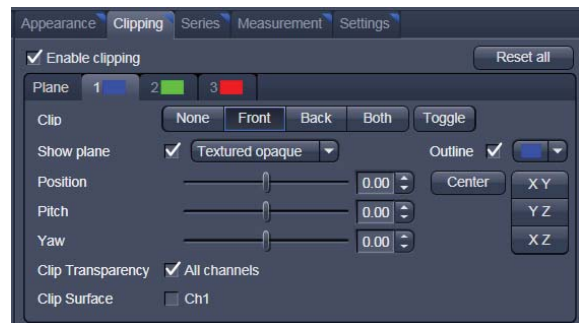The **Flying** mode is an interactive way to explore three-dimensional data. By pressing the mouse wheel you can fly into and through a dataset. Moving the mouse left / right and up / down controls the flyingdirection. Pressing the mouse wheel and the right mouse button at the same time allows for flying backwards.

☞   Once the **Flying** mode is activated, the rotation of the data switches modes: Whereas normally the data turns around an imaginative point in the center of the dataset, in the fly mode the data turns (if moved interactively with the mouse once one stops flying) around an imaginative point "behind" the operator. Pressing the ▢ button switches back into the normal mode.

If a wheel-mouse is not available, pressing the ▢ button turns the left mouse button into the **Flying** button.

☞   The **Flying** mode is only available in graphics card accelerated render modes.
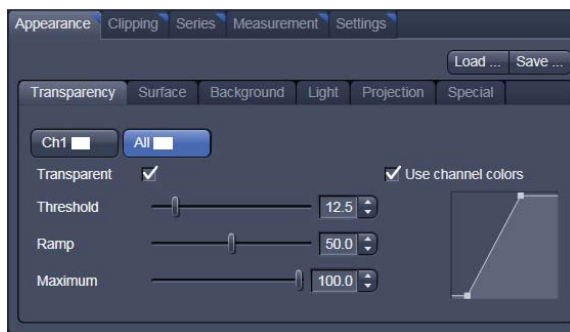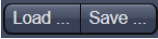

Fig. 4-186   3D Rendering window (e.g. Surface Advanced rendering mode)

4.6.8.8   3D Rendering Settings in VisArt*plus*

Depending on the render mode selected the following adjustments can be applied to the datasets:

All settings can be saved and re-applied to other datasets by using the ▢ Load ... Save ... ▢ buttons.

– **Threshold** specifies a lower threshold as a percentage of the gray values displayed. This decides which data enter the rendered image. If one fluorescence channel contains a homogeneous fluorescence signal with high signal strength, one would set the threshold higher in order to "protect" signals from weaker channels from being quenched.

– **Ambient** describes a diffuse, non-directed light source, which lets both bright and dark structures appear darker or brighter in an equal fashion (values on a scale between 0 and 100).

– **Specular** sets a directional brightness (values on a scale between 0 and 100). Influences the differences between dark and bright structures, which are strongest at a value of 0.

– **Shininess** changes the surface gloss (on a scale between 0 and 100).

– **Ramp** controls the degree of the transition from completely transparent to completely opaque.

– **Maximum** influences the degree of opacity.

The settings are combined into a histogram ▢. The x axis corresponds to the gray values and the y axis to the degree of opacity. You can also use the mouse to interact with the histogram curve.

- **Roughness** (for Shadow mode only): You can also influence the roughness used to display the surface of the image structures. This changes the plasticity of the display (relative scale between 0 and 1). This setting quickly can produce artifacts if pixels reach saturation. The degree of roughness should be reduced accordingly.

- **Brightness** can be controlled via input field or slider bar.

- **Distance** determines the distance between light source and volume. At a value of 0.1 the light source is close by, shadow effects etc. are minimal. The further away the light source, the more prominent the shadow effects become ("evening shadows are longer").

- **Azimuth** describes the angle of the light source above the virtual horizon and can be set by entering values directly or by slider (value range: 0° to 90°).

- **Elongation** describes the horizontal direction of lighting and can be set by entering values directly or by slider (value range: -180° to +180°).

- **Channels**: The settings are entered separately for each channel using sliders or by entering a numerical value in the corresponding input field. To select a channel click on the corresponding button (labeled with the channel color and number).

Fig. 4-187   **Channels**

- **Distance** (for Shadow mode only) sets the distance between the 3D object and the virtual background on a scale from 0.5 to 3.0.

- **Color**: The color of the background can be freely chosen from a table which appears when clicking the color chooser button .

- **Viewing angle / Scale Z**: In hardware accelerated modes, you can change the projection angle used to view the scene anywhere between 10° and 80°. Use the slider or enter the **Angle** into the input field. The effect of this on the display perspective is similar as if you were viewing the 3D image through a telephoto or wide-angle lens.
The Z-Scale of the dataset can be altered interactively by using the slider. The slider ranges from a factor of 0.1 to a factor of 5.

Fig. 4-188   **Viewing angle**

- **Background:** The background color can be altered by using the pull down menu. For some 3D Objects it can be beneficial to display a **Structured background** for better orientation within the dataset.

Fig. 4-189   **Background**

Fig. 4-190   Clipping planes settings

4.6.8.9   Clipping Planes Settings

Activate or deactivate the clipping functionality globally by the check box **Enable clipping**.

 displays the three clipping planes in the default mode (opaque surface, colored outlines, clipping planes active, no clipping).

With the **Plane** tabs the planes can be addressed individually and their settings can be adjusted independent from each other.

Outline displays or hides the colored outlines. The actual color can be chosen from a color chooser for each plane individually.

Show Plane hides or displays the plane.

☞   A plane can cut away information whilst being "invisible".

☞   For distance or angle measurements on a clipping plane the plane has to be displayed and a color has to be chosen for the clipping plane.

The appearance of the plane can be selected from a drop-down menu (see Fig. 4-183).

Clip names a row of toggle buttons switching between different clipping modes as named on the buttons.
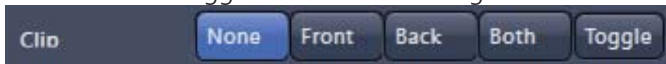
Fig. 4-191   Sliders Pitch, Yaw and Position

Using the **Pitch**, **Yaw**, and **Position** sliders each plane can be freely orientated in three-dimensional space.

4.6.8.10   Series

The **Series** tab (Fig. 4-192) displays the **Render Series** control panel. This panel allows settings for the axis to be used for rotation of the 3D reconstructed images. This feature is applicable to all render modes available in VisArt*plus*.

Fig. 4-192   Series button

There are four different modes for producing render series (see Fig. 4-194).

Depending on the activated mode, directly set the parameters for animation in the **Render Series** window and the position of the 3D image in the **Image Display** window (zoom, rotation axes, rendering parameters).

Fig. 4-193   Render Series window (e.g. Turn around X mode)

Click on **Apply** to start the animation

The animation is performed in a separate **Image Display** window, which permits the animation to be saved afterwards.
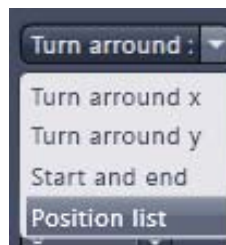
Fig. 4-194   Render Series modes

(1)   Turn around X and Turn around Y mode

In this mode, the image is turned around the X-axis or the Y-axis exclusively.

The values for **Number of Views**, **Difference Angle** and **First Angle** can be selected accordingly).

(2)   Start and End mode

In the **Start and End** mode, the image is reconstructed between a start position and an end position.

The rotation angles for X, Y and Z and the distance (zoom) can be determined using the sliders.

The value for **Total frames** can be varied. The frames are distributed equally between the start and end points (Fig. 4-195).
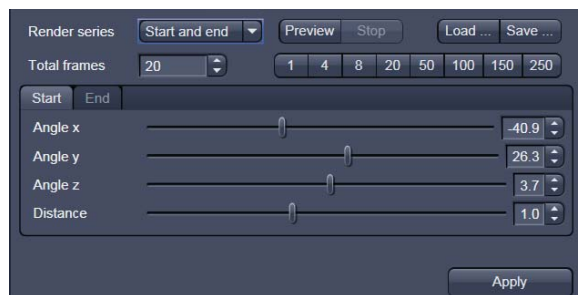
Fig. 4-195   Start panel in the Render View control block

**Fig. 4-196   Render Series window -
                  Position List mode**

(3)   Position List mode

In the **Position List** mode, the image is reconstructed between any required number of interim positions to be determined individually. Just rotate the reconstructed image with the mouse or do any other adjustment and add this as a "position" in the position list. The module will create a movie using those positions as a guideline. It will interpolate all the parameters ticked in the list.

The rotation angles for X, Y and Z and the zoom can be determined directly in the image.

Every required interim position is included in the list of the **Render Series** window with a click on the **Add** button.

Remove permits the contents of the list to be deleted. **Insert** inserts a position above the highlighted list entry.

The value for **Total frames** can be varied. A value of 20 produces a render series with 20 frames in total.

Interpolate lists variables that are interpolated during the Render Series.

Pressing the button creates a short and low resolution (no interpolation!) preview of the rendering using the positions currently in the list. This serves as a help to quickly review the rendering without actually starting the process (which might be time consuming in cases of long position lists.

* Click on the **Apply** button calculates a spline along all the defined positions from the list and starts an animation along this spline track in space.

4.6.8.11   Interactive Measurements

VisArt*plus* features three tools for interactive measurements in Euclidian space. Measurements can be performed in all graphic-card accelerated modes (**Transparency**, **Surface**, **Maximum** and **Mixed**). The measured tools are shown as shapes in the rendered volume and can be changed there interactively. Measurement values can be exported as a table to the clipboard for downstream processing in other programs.

First a suitable tool has to be chosen. In order for measurements to work, the mouse cursor must "recognize" a structure. In the **Surface** mode this is a uniquely identifiable surface structure, for the transparent modes the tools look for the highest local gray level maximum.

As a sign, that the mouse cursor has "found" a structure, the cursor shape changes from into .

The found measurement point is drawn as a blue circle .

Interactive 3D-Measurement

The measurement dialog contains a toolbar with the available measurement tools.

The **Line** tool measures the length along a line in µm. First click on a starting point and move the mouse to the desired end point while keeping the left mouse key depressed. The measurement is concluded upon releasing the left mouse key.

Fig. 4-197   3D Measurement View control block

The **Angle** measurement tool defines an angle between two connected line segments. First define the starting point by clicking, and then draw the first line segment until you click on the first endpoint. Now draw the second line segment and conclude the measurement by clicking onto the second end point. This measures the angle in degree.

The **Curve** tool measures the total length (µm) along a variable multi-segmented polygon line. Please click from point to point and finish the measurement by clicking with the right mouse key.

Measurement values are compiled in a list. In order to delete a measurement, select it in the list and press the **Remove** button. One can also delete all measurements by clicking on **Remove all**.

The measurements can be copied into the clipboard with the **Copy** button.

Measurements are only transiently drawn into the volume and are not stored automatically with the image.
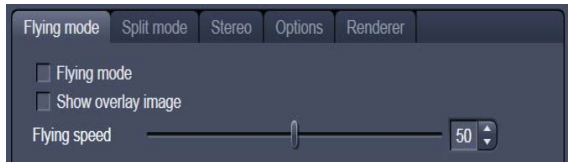
Fig. 4-198   3D Renderer Settings – Flying mode

4.6.8.12   Settings

Flying mode

Ticking the tick box **Flying** mode activates the mode, unticking deactivates it.

To show or hide an overview image tick the **Show overlay image** box.

The fly-speed can be adjusted in a range from 1 to 100.
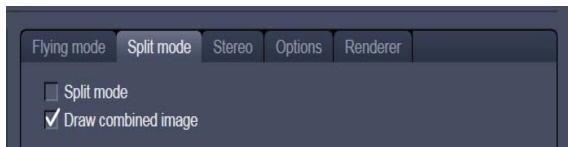
Fig. 4-199   3D Renderer Settings – Split mode

Split Mode

Ticking this tick box activates a channelwise 3D split view of the data. This is only available in surface render mode only. An overview image with all channels combined can be shown optionally by ticking **Draw combined image**.
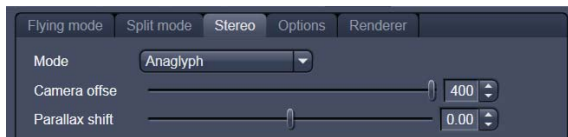
Fig. 4-200   3D Renderer Settings – Stereo

Stereo

Two Stereo views can be chosen from this sub-menu:

Anaglyph is a view in which the data can be examined in 3D using red/green glasses. The image is built up twice (once each for the red and green colors), resulting in a stereoscopic image.

The stereoscopic effect can only be seen with the aid of red/green 3D goggles. The red lens is to be used for the right eye and the green lens for the left eye.

Camera offset: Defines the distance of both cameras from each other (units in meters).

Parallax shift: Determines the degree of shift which is necessary to correlate both camera views. The slider should be adjusted to maximize the stereo effect. Please note, that this setting will be different for each observer.

4.6.8.13   Options

XY Textures only: Instead of working with three texture stacks per dataset in transparent render mode, the system will only use one texture stack. This will result in faster renderings. Looking at the dataset from aside although will make the individual z-planes visible.

Inner surface lighting: If checked, illuminates the inside of a surface rendered volume. If unchecked, the inside is dark. If one views the inside of a volume by flying or zooming into it, the inside illumination is helpful. This option is only available for the **Surface** mode.

Fig. 4-201   3D Renderer Settings – Options

Lit front and back side: With activated **Inner surface lighting**, this checkbox colors the inside lighting with a bluish hue. This makes distinguishing inside from outside easier. This option is only available for the **Surface** mode.

Draw border triangles: When displaying the data touching the six sides ("borders") of the Z-Stack in 3D, the triangles used to do this are all located in one plane and orientation. This can cause artifacts, therefore the display of those border triangles can be switched off.

Texture switch angle: This parameter influences the volume angle, upon which the next set of 2D-Textures will be loaded into the graphics card (value range 1 to 100 degree). Depending on the object orientation, the number of z-slices and the chosen render settings this loading process can be visible and may be disturbing especially when rendering a series for movie export. Changing this angle can postpone or even avoid this switch during a series rendering. This option is only available for **Transparency** and **Maximum** modes.

Image Rotation with mouse: Activates a method to rotate the dataset interactively with the mouse. In addition to rotating it freely, when clicking and dragging at the sides of the image window the dragging rotates the data around one axis only. Just like the wheels on the 3D window frame.

Mouse Sensitivity: Influences the mouse sensitivity (value range: 1 to 200). Small values make the mouse actions precise but slow, large values less precise but faster.

Texture Opacity: With this slider the α-values of the dataset can be adjusted. The rendering is getting more or less transparent using this setting.

Reset: Sets all render settings back to the factory standard settings.

Renderer Options:

Renderer: OpenGL Software is rendering the 3D data with the CPU; OpenGL Hardware uses the Graphics card to do this. Especially when having trouble with non Zeiss-certified graphics cards it is sometimes useful to switch to a pure CPU based rendering.

Fig. 4-202   3D OpenGL Renderer settings

Palette textures: This feature is an optimization feature not supported by modern graphics cards any more. It used to optimize display of single channel images.
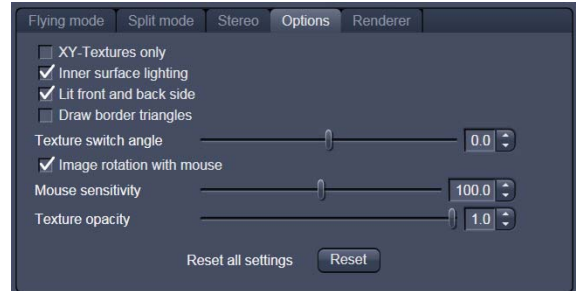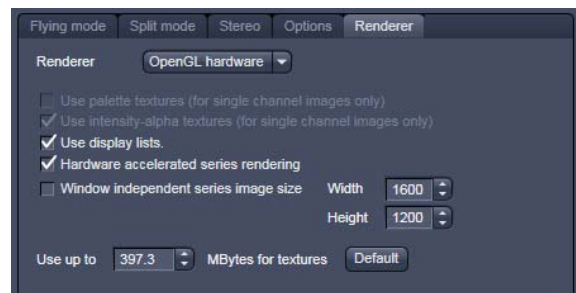
Intensity Alpha textures: This feature optimizes the usage of the graphic ram with single channel images.

Display Lists activates a batch processing of the operations the graphics card is doing. It results in faster rendering.

Hardware accelerated series rendering: Un-ticking this feature uses the Computer Processor for render series. In case of trouble with non-certified graphics cards render series can be performed with the CPU without risk. The use of memory of the graphics card can be adjusted in a dialogue box.

Window independent series image size: Using this feature creates render series (see section 4.6.8.10) with the output size indicated in the two input boxes. The maximal resolution is 4096 x 4096 pixels. This feature is available in all Render Modes (CPU and GPU accelerated). This applies also to the "**Create Image**" button on the **3D View Options** tab.

Allocate Graphics Card RAM: The amount of Graphics card RAM can be freely defined in the last Item on the **3D | Settings** view control panel. **Default** sets this value to ~ 80 % of the available RAM of the currently used graphics card.

4.6.8.14   3D View – Basic

Without an active license for VisArt*plus* ZEN offers the following 3D functionality:

The available basic 3D options are described in section 4.6.8. The following features are available:

– Shadow view - Front view,

– Transparency render mode - Basic, rotate around x or y render series,

– Maximum brightness projection – Basic Mode only, only rotate around x or y, or start to end movies,

– Surface - Basic Render Mode, rotate around x or y movies,

– Stereo Anaglyph Mode.

4.6.9   Histogram View

The **Histogram** function (**Histo View**) allows to

– display a histogram (distribution of pixel intensities) of an image or Region of Interest,

– show the histogram values in table form,

– copy table to clipboard or save as text file,

– measure area and mean gray value and standard distribution in an area.

The settings of the **Dimensions, Display**, **Player** and **Overlay** view options control blocks apply. The additional view-specific **Histo View Option c**ontrol block is shown in Fig. 4-203.

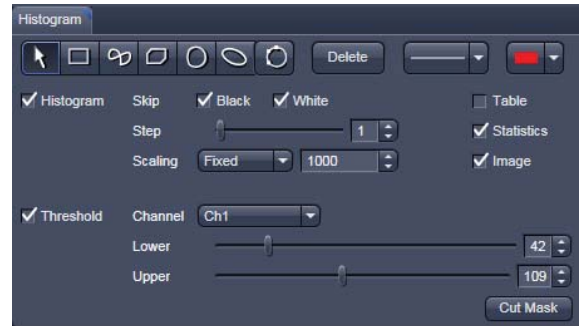Any changes done with this View Option control block are effective immediately.
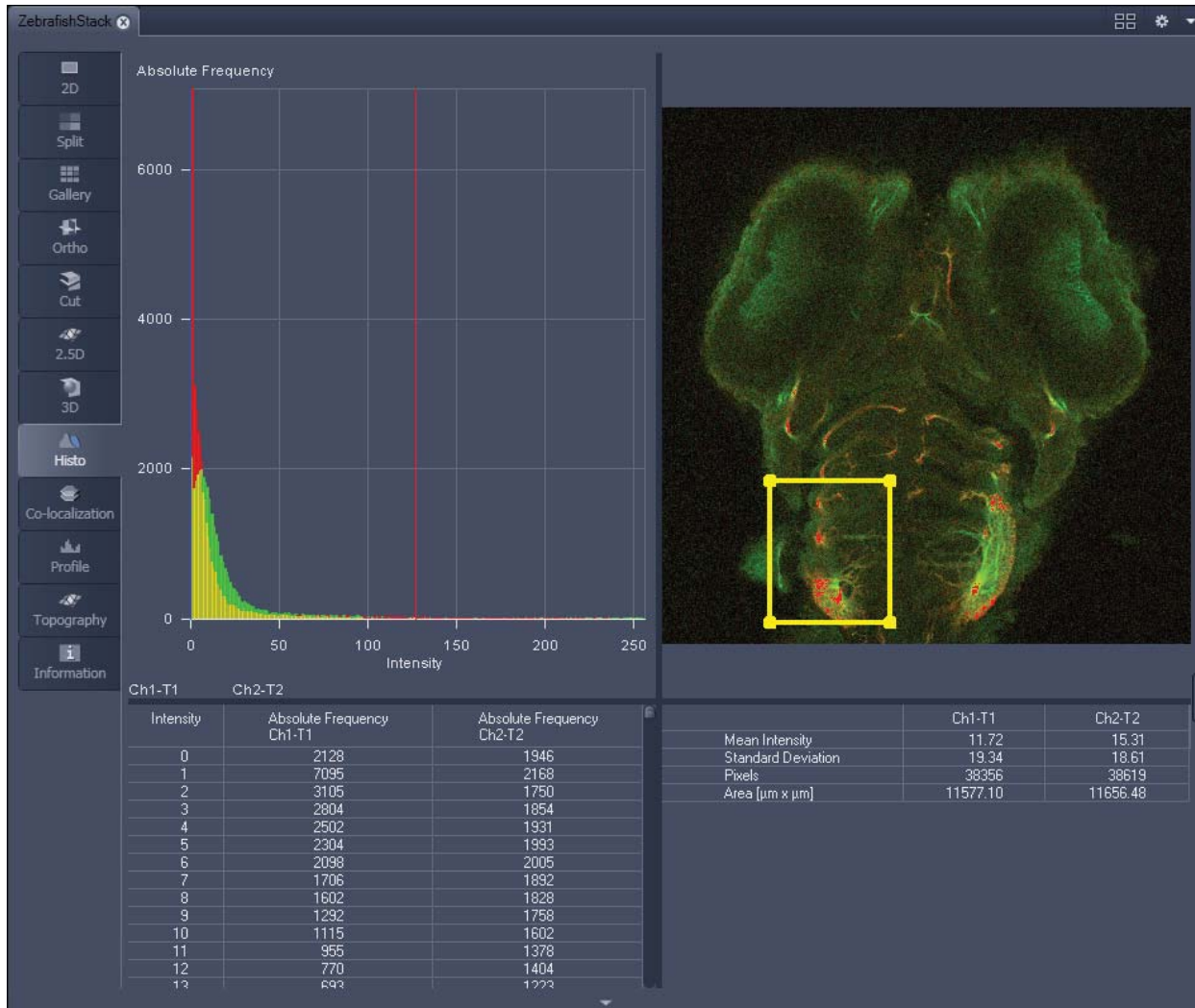

Fig. 4-203   View Option control block - Histo View

Fig. 4-204   Image Display, Histogram view

☞   The **Histo** button can also be used online during scanning.

Histogram functions:

Skip Black and **Skip White** tick boxes: Ignore black pixels (gray value 0) and Ignore white pixels (gray value 255 or 4096) in the histogram.

Step slider and spin box: Sets the number of intensity steps ("trays") of the histogram display. Step 1 corresponds to 256 intensity steps, Step 64 to 4 intensity steps (for 8 bit images). Reduction is made by averaging.

Scaling pull down menu: Sets the histogram graph scaling to either dynamic (adjusting to the maximum value) or fixed
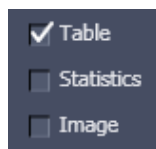
Show Table tick box: A table of the intensity values is shown in the **Image Display** window.
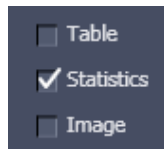
Show Statistics tick box: Displays statistical parameters (Mean Intensity, Standard Deviation, Number of Pixels and Size of Area) in an additional table. Area measurements of very small areas (<10 pixels) give only approximate values.
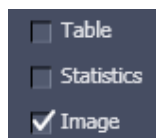
Show Image tick box: Shows the image in the **Image Display** window with the histogram graph.
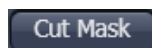
The **Cut Mask** tool creates a new image document which sets every pixel outside ROIs to Zero. Within the ROIs, only the pixels with values between lower and upper threshold are transferred to the new image.

☞   Tables can always be saved or copied to the clipboard by right-mouse clicking on the table display!

Histogram functions and Overlays:

The functionality of the **Overlay View Option** control block fully applies to the **Histo View** Type. If a ROI is marked/selected in the Histo View Image display, only this ROI will be taken into account for the histogram display, the table and the statistics. If there are several ROIs, but none is marked/selected, the histogram display, the table and the statistics analyze all the pixels included in all ROIs.

Fig. 4-205   Histo View Option control block - Threshold

Histogram Threshold:

When the Threshold tick box is selected, the controls to set a lower and upper threshold for the histogram calculation are displayed (Fig. 4-205). The threshold can be set for each channel (channel selection pull down menu).
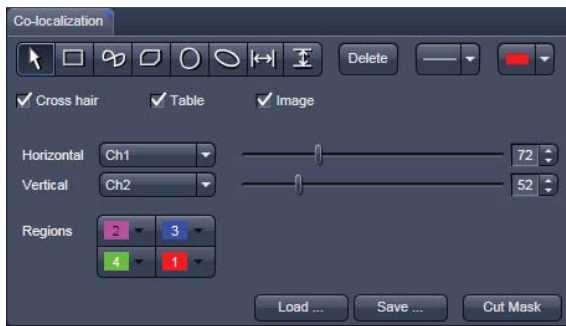
Fig. 4-206   Colocalization View Option control block

4.6.10   Colocalization View

The **Colocalization** function permits interactive analysis of two channels of an image by computing a scatter diagram (co-localization).

The settings of the **Dimensions, Display**, **Player** and **Overlay** view options control blocks apply. The additional view-specific **Colocalization View Option** control block is shown in Fig. 4-206.

Any changes done with this View Option control block are effective immediately.

The Image Display in the Colocalization View shows 3 panels: the scatter diagram, the data table and the pseudo-colored image display (see Fig. 4-207 and Fig. 4-208).

☞   Colocalization is defined by the presence of two or more different molecules at the same location in a specimen. However, in the context of digital imaging, the term colocalization refers to colors emitted by fluorescent molecules detected by the same pixel in the image. It is important to be aware of the fact that colocalization can not be analyzed for fluorophores with similar emission spectra. Accurate colocalization analysis is only possible if the fluorescence emission spectra are well separated between fluorophores and the correct filter sets (or spectral detection bands) are used for data acquisition. If spectral bleed-through artifacts are present because of spectral overlap between the fluorophore emission spectra, or due to the use of incorrect filter sets, colocalization measurements will be meaningless. To avoid this, the fluorophores must be carefully chosen and matched to the excitation laser lines to obtain the maximum excitation efficiency while still maintaining a useful degree of separation between emission wavelengths. The choice of fluorophores is crucial for colocalization analysis.

Fig. 4-207   Image Display, Colocalization view

Fig. 4-208   Scatter diagram and threshold with crosshair

How a scatter diagram is generated
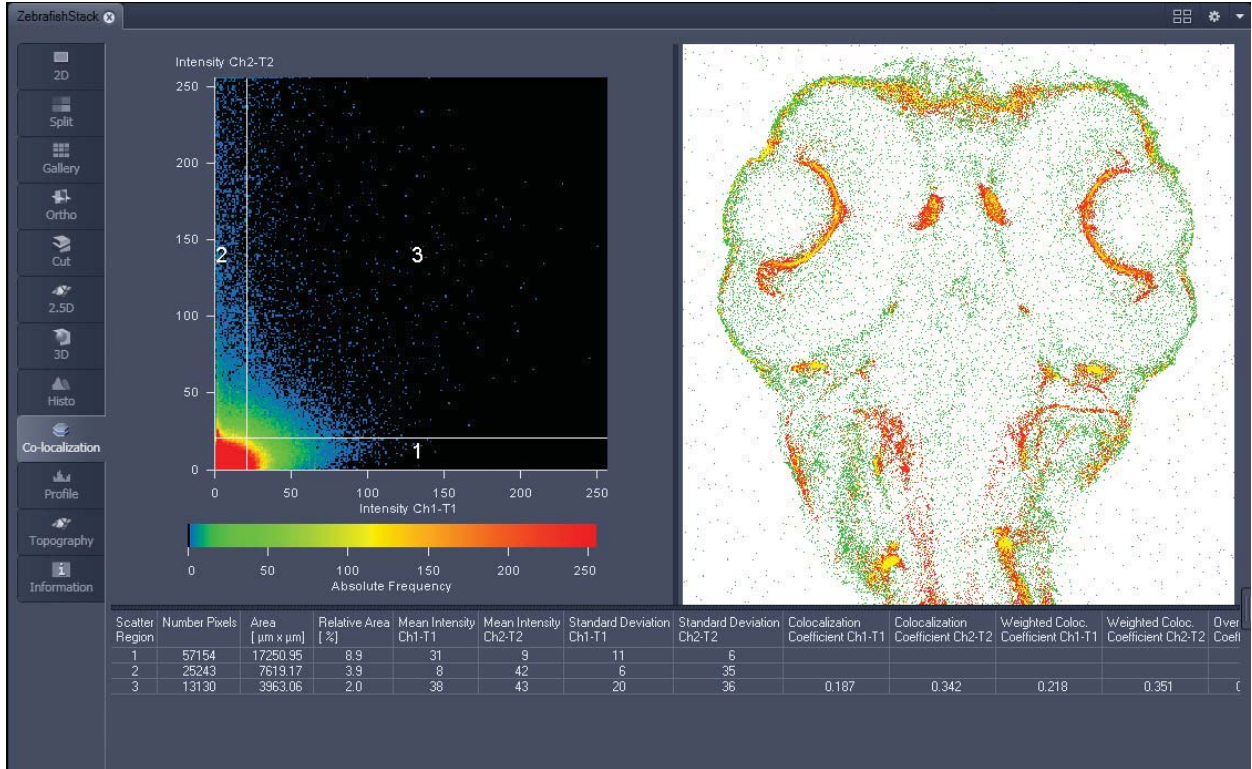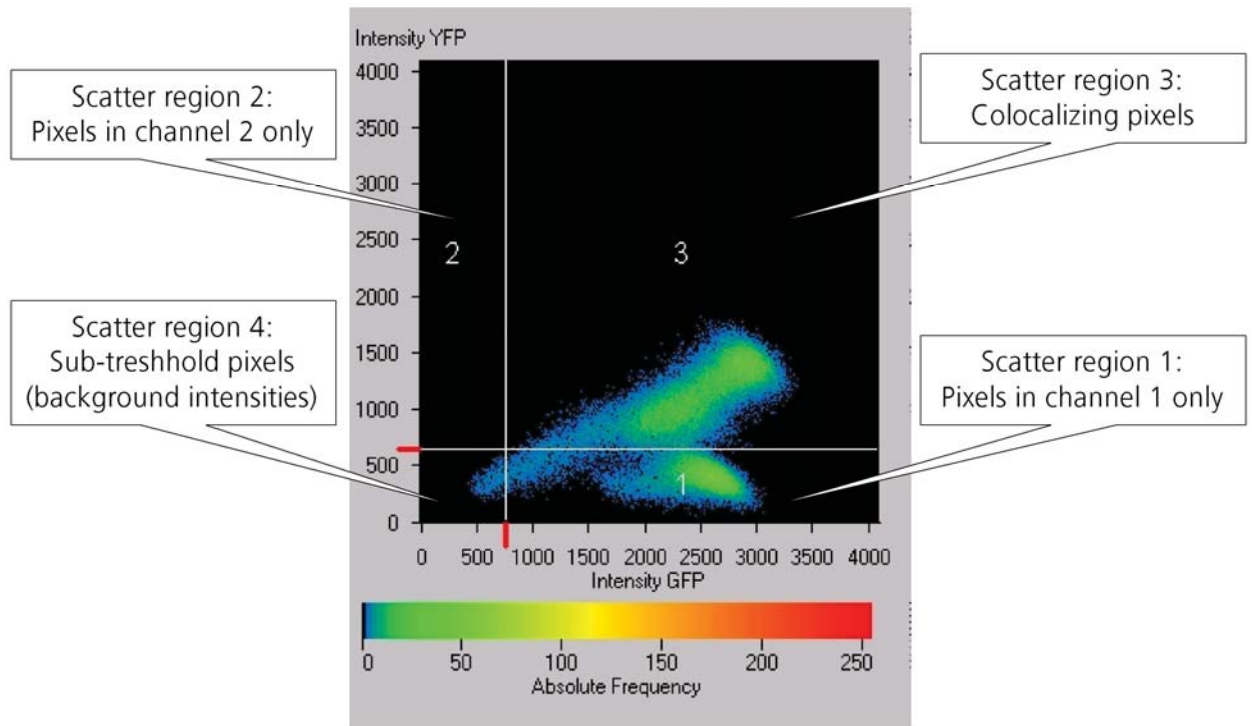
All pixels having the same positions in both images are considered a pair. Of every pair of pixels (P1, P2) from the two source images, the intensity level of pixel P1 is interpreted as X coordinate, and that of pixel P2 as Y coordinate of the scatter diagram. The value of the pixel thus addressed is increased by one every time, up to the maximum number of pixels used. This way, each pixel of the scatter diagram is a value that shows how often a particular pair of pixels has occurred.

☞   Differences between the images cause irregular spots in the scatter diagram.

Identical images produce a clean diagonal line running from bottom left to top right, because only pixel pairs (0,0), (1,1), (2,2) with the same intensity can occur. Differences between the images cause an irregular distribution in the scatter diagram.

☞   Scatter diagram, image display and data table are interactively linked and immediately updated when settings are changed.

Available Controls:

Drawing tools

With the **drawing tools** the colocalization analysis can be restricted to a region of the image. Tools in the **Colocalization View Option** control block and in the **Overlay View Option** control block work the same and can be combined.
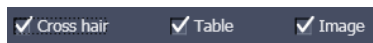
The **Cross hair, Table** and **Image** selection tick boxes: when selected, the respective element is displayed in the Image Display area. If off, the element is hidden.
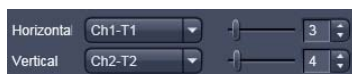
Scaling pull down menu: Sets the histogram graph scaling to either dynamic (adjusting to the maximum value) or fixed
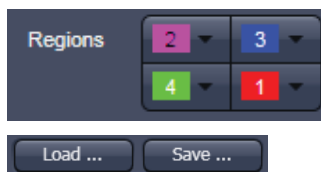
Set the color LUTs for each quadrant in the image display to improve visibility of colocalized pixels.

The Load and Save buttons can be used to save colocalization settings (like color settings for the quadrants and crosshair-positions) to a *.col file and re-load it when analyzing a different data set. This allows using the same parameter for the colocalization analysis of different images.
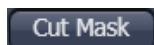
The **Cut Mask** tool creates a new image document which sets every pixel outside Quadrant 3 (not colocalized) to Zero. ROIs don't play a role for this **Cut Mask** function (as opposed to the **Cut Mask** in **Histo View**).

☞   Tables can always be saved or copied to the clipboard by right-mouse clicking on the table display!

Quantitative Colocalization Parameters shown in the data table:

- **No. of pixels** in image ROI or scatter region

- **Area / relative area** of image ROI or scatter region

- **Mean intensities / SD** within image ROI or scatter region

- **Colocalization coefficients**

- **Weighted colocalization coefficients**

- **Overlap coefficient** after Manders

- **Correlation coefficients** (R and R^2)

Colocalization coefficients

$$c_1 = \frac{pixels_{Ch1,coloc}}{pixels_{Ch1,total}} \qquad c_2 = \frac{pixels_{Ch2,coloc}}{pixels_{Ch2,total}}$$

- Relative number of colocalizing pixels in channel 1 or 2, respectively, as compared to the total number of pixels above threshold.

- Value range 0 – 1 (0: no colocalization, 1: all pixels colocalize)

- All pixels above background count irrespective of their intensity.

Weighted colocalization coefficients

$$M_1 = \frac{\sum_i Ch1_{i,coloc}}{\sum_i Ch1_{i,total}} \qquad M_2 = \frac{\sum_i Ch2_{i,coloc}}{\sum_i Ch2_{i,total}}$$

- Sum of intensities of colocalizing pixels in channel 1 or 2, respectively, as compared to the overall sum of pixel intensities above threshold and in this channel.

- Value range 0 – 1 (0: no colocalization, 1: all pixels colocalize)

- Bright pixels contribute more than faint pixels

Correlation coefficient, Pearson´s correlation coefficient

$$R_p = \frac{\sum_i (Ch1_i - Ch1_{aver}) * (Ch2_i - Ch2_{aver})}{\sqrt{\sum_i (Ch1_i - Ch1_{aver})^2 * \sum_i (Ch2_i - Ch2_{aver})^2}}$$

- Provides information on the intensity distribution within the colocalizing region

- Value range -1 to +1
    -1,+1: all pixels are found on straight line in the scatter diagram
    0:     pixels in scattergram distribute in a cloud with no preferential direction

Overlap coefficient, overlap coefficient after Manders

(Manders, Verbeek and Aten, J. Microscopy 169:375-382, 1993)

$$r = \frac{\sum_i Ch1_i * Ch2_i}{\sqrt{\sum_i (Ch1_i)^2 * \sum_i (Ch2_i)^2}}$$

– Another parameter used to quantify colocalization in image pairs
– Insensitive to differences in signal intensities between the two channels, photo-bleaching or amplifier settings

Value range 0 – 1 (0: no colocalization, 1:all pixels colocalize)
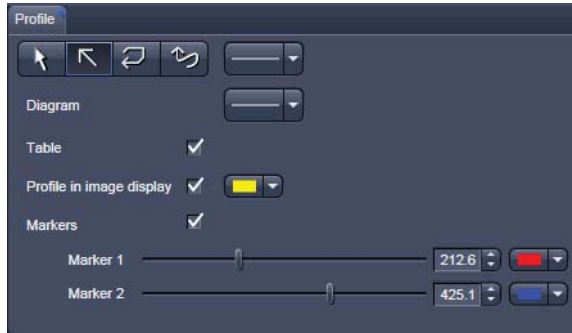
Fig. 4-209   View Option control block - Profile View

4.6.11   Profile View

This function allows to

– display the intensity distribution of an image along a straight or curved line,
– show the intensity values in table form and copy table to clipboard or save as text file,
– show separate profiles for each channel in a multi channel image.

The settings of the **Dimensions, Display**, **Player** and **Overlay** view options control blocks apply. The additional view-specific **Profile View Option** control block is shown in Fig. 4-209.

Any changes done with this View Option control block are effective immediately.

The content of the Overlay plane is temporarily hidden while the toolbar is displayed.

☞   The **Profile** View can also be used online during scanning.

• Draw a Profile line in the image display to define a profile. The intensity curves are shown in a separate graph. (see Fig. 4-210).

• In the **Profile** View Option control block you can select the width and type of the profile line. The color setting is taken from the **Overlay** View Option control block

• You can place two markers on the profile line to measure differences in intensities and distances in the XY plane.

• Tick the **Profile in Image Display** block to see an intensity graph superimposed on the image. (see Fig. 4-211).
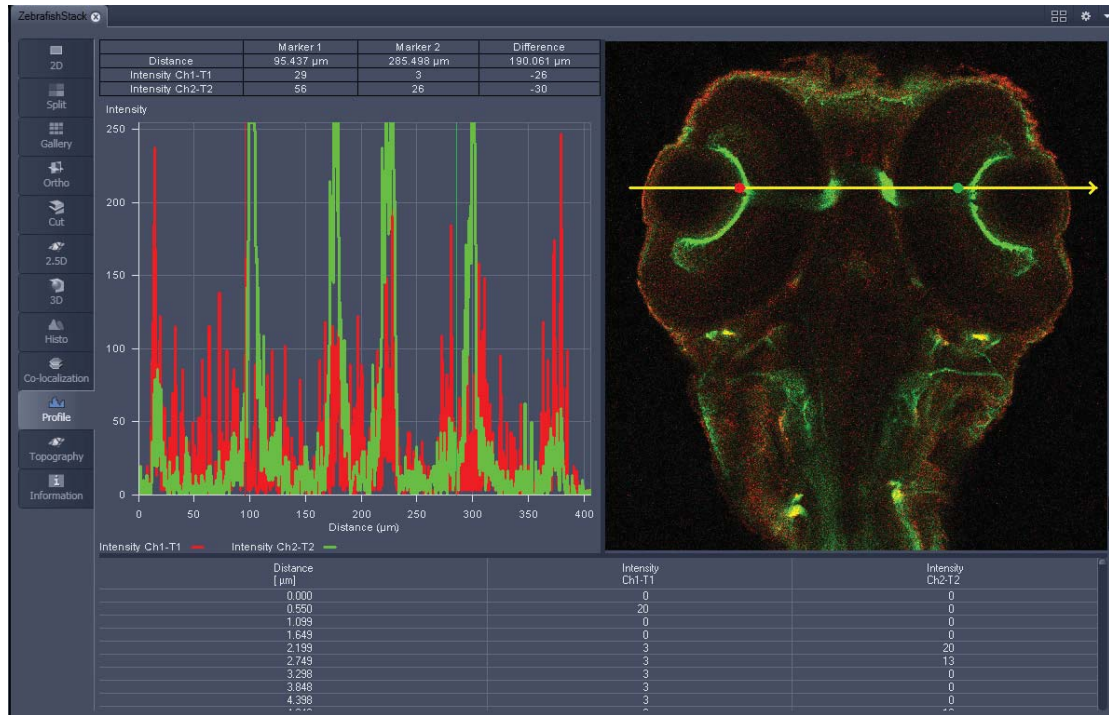
Fig. 4-210   Image Display - Profile View, Line Profile with markers
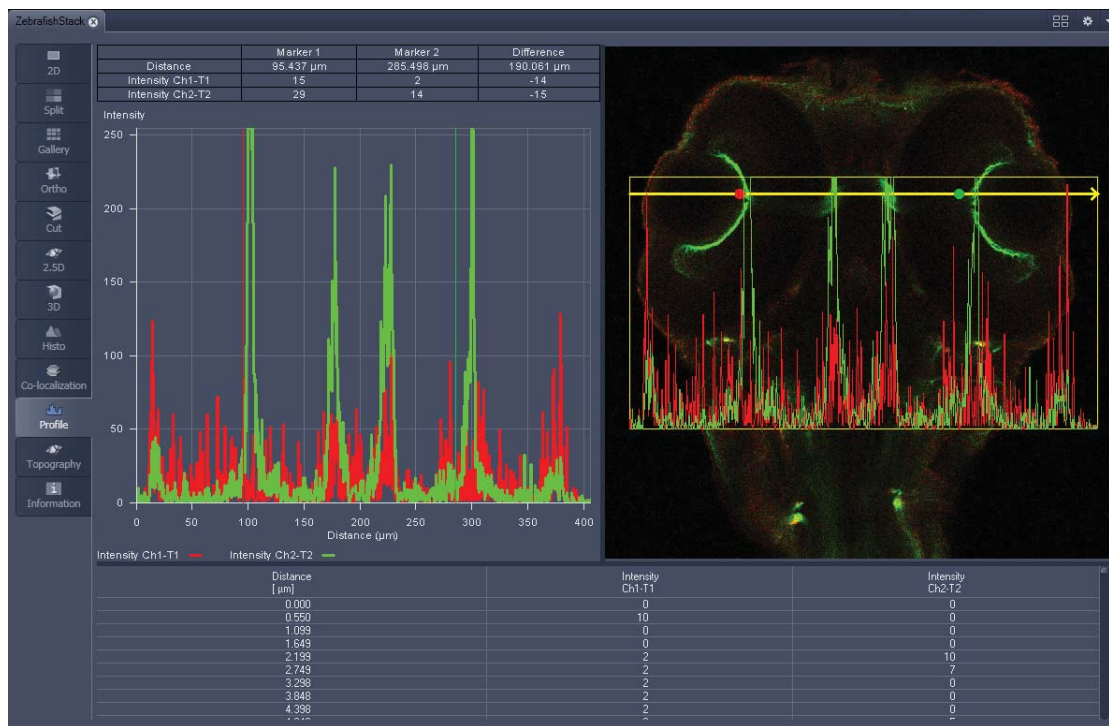
Fig. 4-211   Image Display - Profile View, Profile displayed in Image

Control block tools

The **Profile** toolbar contains the following tools:

Select button: Activates the mouse button for selection, resizing or movement of the profile line in the **Image Display** window.

Resize: Click on handle and hold down the mouse button, move the handle, release mouse button.

Movement: Click on line and hold down the mouse button, move the entire line, release mouse button.

Line with arrow button: (open arrow): Activates the straight profile drawing mode.

Click into the image and hold the mouse button, drag a line in any direction and release the mouse button to end the procedure.

Open polyline arrow button: Activates the open polyline drawing mode.

The first click into the image sets the starting point, each additional click adds a further line, right mouse click ends the procedure.
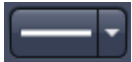
Line button: This button allows you to determine the line thickness of the profile line. It has no influence on the way the intensity profile is generated.

Show Table button: The profile diagram is displayed as a table at the bottom of the **Image Display** window.

☞ Tables can always be saved or copied to the clipboard by right-mouse clicking on the table display!

4.6.12 Topography View

This optional function allows to

- process, display and measure topographic information.
- use frame Z-Stacks
- and frame Z-Stacks over time

The **Topography** function is mainly used for applications in material research and industry.

The settings of **Channels** and **Zoom** of the **2D Dimensions** control block are applied. The **channel color** settings are applied in some 3D display modes. Also the channel for generation of the topography is selected in **2D Dimensions**.

The content of the **2D Overlay** control block is temporarily hidden while the Topography view is displayed.

The topography of a Z-Stack is displayed in the **Center Screen Area**. The parameters used at the last exit of the Topography function are applied.
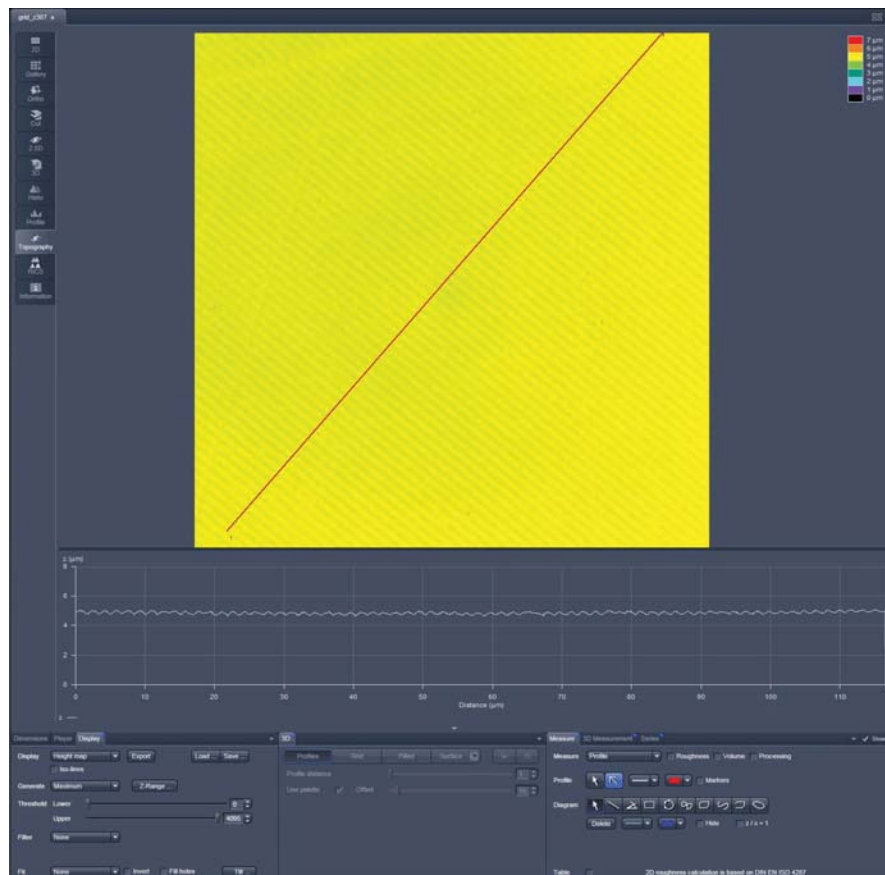
Fig. 4-212   Center Screen Area, Topography view

The **Topography view** contains the following **image view control blocks**:

Display   Set 2D or 3D, kind of generation, thresholds, filters and fits

3D   Set options of different 3D views.

Measure   Includes all measurement functionality, like profiles, roughness etc.

3D Measurement   Includes additional measurement functionality for 3D views.

Series   Includes different kinds of series generation.

4.6.12.1   Control Block: Display

- Select **2D** (**Height map**, **Maximum intensity**) or **3D**.

  – In case of Height map and Maximum intensity and ticked Iso-lines checkbox two additional values can be changed:
    Distance: Changes the distance of the Iso-lines.
    Offset: Setting of the height level where the Iso-Lines display starts.

- Additional settings for 3D are chosen in the 3D-block

- Define the kind of topography generation.

- These views can also be exported as image. Height maps can be exported as lsm-file and later opened by ZEN and used as a topo image. As raw data will be not saved in this kind of export changes of topography as described below can almost not be made any more.

Maximum

- Click on the **Maximum** button to calculate the topography surface by finding the maximum intensity value. If the optical section with the highest intensity value is found, the intensity values of both neighboring slices are also taken into account, so that a 3 point maximum fit is calculated.

- In case it happens, that the maximum possible intensity value is present in more than one optical slice for a given pixel (saturation), the mid section of all saturated intensity slices is chosen as a reasonable approach.

Center

- Click on the **Center** button to calculate the topography surface by using the center of gravity of all summed up intensities of the stack for a given xy print.

☞   This mode provides better result for smooth surfaces of low intensity or nearly transparent surfaces. The receiver gain and offset has to be properly tuned and MarkFirst- / MarkLast-positions of the stack should be located approximately in the same distance from the real surface.

First / Last maximum

- Click on the **First maximum** button to calculate the topography surface by using the first maximum coming from the top.

- This mode provides better result for surfaces of semitransparent materials with inclusions of higher reflectivity or transparent multilayers with subsurface layers of higher signal intensity.

- Click on the **Last maximum** button to calculate the topography surface by using the last maximum coming from the top.

First / Last threshold

- Click on the **First threshold** button to calculate the topography surface by using the first slice coming from the top, where the intensity reaches the value defined by the lower intensity threshold.

Load / Save gives you the possibility to save / load settings for:

  - **Generate**,

  - **Threshold**,

  - **Filter**,

  - **Fit**.

Fig. 4-213   Image view control block - Display

- Click on **Z-Range…** to calculate the topography surface by using the lower and the upper height thresholds for image display. Use of this function is recommended to get rid of unwanted peaks and valleys taken into account for parameter calculation. All topographic data with height values less or higher than the thresholds set are ignored for the display and parameter calculation. This threshold applies both for 2D as well as for 3D topography display modes.

Fig. 4-214   Z-Range pop up to define height thresholds

- By clicking on the **Peak - valley** button the surface topography is displayed in that way that it is automatically normalized to the lowest and highest Z value of the current 3D topography. Ticking the checkbox **Automatic** means, that this procedure is continuously performed during further changes, like application of different filters.

- Set the **Threshold** to define the lower and upper intensity thresholds used for calculation of the topography surface. Use of this function is recommended to find the real surface in the case of images with pronounced noise. All image pixels with intensity less or higher than the thresholds set are ignored for the surface calculation.

- Chose a **Filter**:

  – **None**

  – **Median**

  – **Low pass**

  – **High pass**

In case of **Low** and **High** pass, **Gauss** and **Butterworth** FFT filters can be chosen below. This function performs a Fast Fourier Transformation (FFT) in the frequency range, applies highpass or lowpass filtering in the frequency range and performs the inverse FFT. Additional **Cut off** and **Degree** should be defined. The **Cut off** frequencies ranges from 1/1000 of the X dimension of the stack to four times of the X dimension of the stack. The dimensions of filtering are given in units of µm. Select a position of the **Degree** slider. The filter functions can be calculated from 1st order to 5th order accuracy.

Changing the topography geometry:

Fit button:

None

> **Plane:** The topography is tilted in such a way that the mean deviation value plane is calculated.

> **Sphere:** A spherical form is eliminated; determination of micro roughness on spherical surfaces can be performed.

> **Cylinder:** A cylinder form is eliminated; determination of micro roughness on cylindrical surfaces can be performed.

☞   You can display the exact values of the **Cylinder / Sphere** fit by ticking the checkbox **Processing** in the **Measure** block.

> A **3-point-tilt** is available using Height map or Maximum intensity with a right mouse click within the image.

Inverse check box:

  – Inverse surface. Depths change to heights, and vice versa.

Fill holes check box:

  – Intensity of a missing pixel of a hole has to be interpolated by the distance-weighted intensity of all surrounding pixels.

  – Fill hole algorithm is optimized for short calculation times.

  – Click on **Tilt…** and change **Pitch** and **Yaw** manually.

4.6.12.2   Control Block: 3D

If 3D is chosen within the Display block, following settings can be applied in the 3D control block (Fig. 4-215).

In the first row **Profiles**, **Grid**, **Filled** or **Surface** can be chosen.

Parameters that can be set:

- **Profile distance** slider: Setting of the distance of profiles.

- **Grid distance** slider: Setting of the mesh value of the grid.

- **Offset** slider: Used to push through a color LUT Look Up Table (e.g.: if the **Rainbow 2** is used as channel color)

Fig. 4-215   **Image view control block - 3D**

Using **Profiles** or **Grid**, the Profiles/Grid (x, y, z matrix) and x, y, z-triples (x, y, z table) can be copied to clipboard and exported by a right mouse click (Fig. 4-216).

☞   Please make sure that the amount of exportable data is adequate to the maximum importing size of the following software package. To lower the amount of data points, use the profile distance slider.
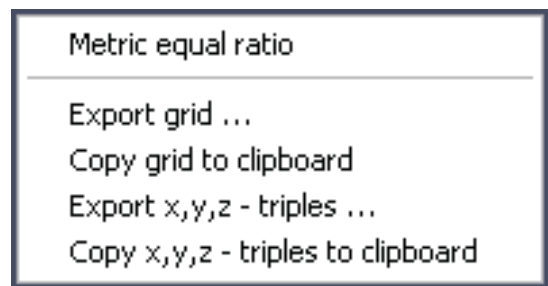
Fig. 4-216   **Context menu using 3D Grid**

In case of **Surface** the renderer item can be set, clicking into the right part of the Surface button (Fig. 4-217

OpenGl - software

The graphics calculation is performed using the installed software.

OpenGl - hardware

The graphics calculation is accelerated by using the installed graphics processor.

Fig. 4-217   **Surface renderer items**

Additionally the shading model can be adjusted using the **Surface**, **Light** and **Projection** tabs:

Fig. 4-218   **Surface renderer: Surface tab**

Ambient/Specular: Material properties; how many % of the light component are projected by the material into which spectral ranges.

Shininess: Suitable to specular light. Shininess equal to 25 % determines diffuse light
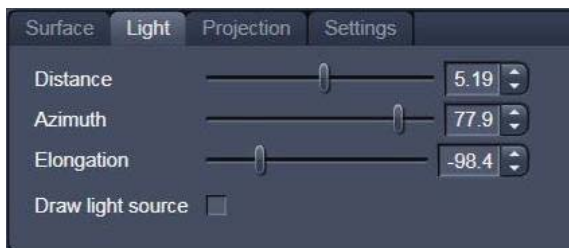
Fig. 4-219   **Surface renderer: Light tab**

Distance: Suitable to diffuse and **specular**, see visualization.

Azimuth: See visualization. Rise angle of the "sun".

Elongation: See visualization. North-south / east-west direction of the "sun".

Visualization: Ticking the **Draw light source** checkbox shows you the "sun" within the image tab.

Fig. 4-220   **Surface renderer: Projection tab**

View angle: Determines the perspective, 0.0 degree: parallel projection, else: central projection.

Scale Z: Determines the zoom factor for the Z / height dimension.
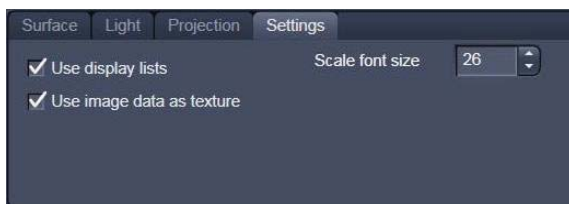
Fig. 4-221   **Surface renderer: Settings tab**

Use display lists: Activates a batch processing of the operations the graphics card is doing. It results in faster rendering.

Use image data as texture: The height data as shown in height map view are used as texture of the 3D model, i. e. only truly measured points are shown.

Scale font size: Sets the font size for the 3D model.

Load / Save (see Fig. 4-215) gives you the possibility to save / load all settings chosen in this block.

The image viewing angle, zoom and axial ratios are set as follows (setting directly in the image):

- Click in the image and hold down the mouse button. The perspective is changed by moving the mouse button in horizontal or vertical direction.

Setting via scrollbars: see section 4.6.8 on **3D View (Image VisArt*plus*)**.

☞   You can set the x, y and z scales to an identical ratio by opening a context menu in the **Image** with a click of the right mouse button and selecting the **Metric equal ratio** function.

☞   The **Profiles** and **Filled** display modes permit a color palette (e.g.: **Glowscale**, **Rainbow** or **User defined**) for the channel to be loaded or redefined.

4.6.12.3   Control Block: Measure

The topography measurement functions are available in the **Measure** control block (Fig. 4-222). The measurement functions can be performed in Height map, Maximum intensity and 3D display modes.
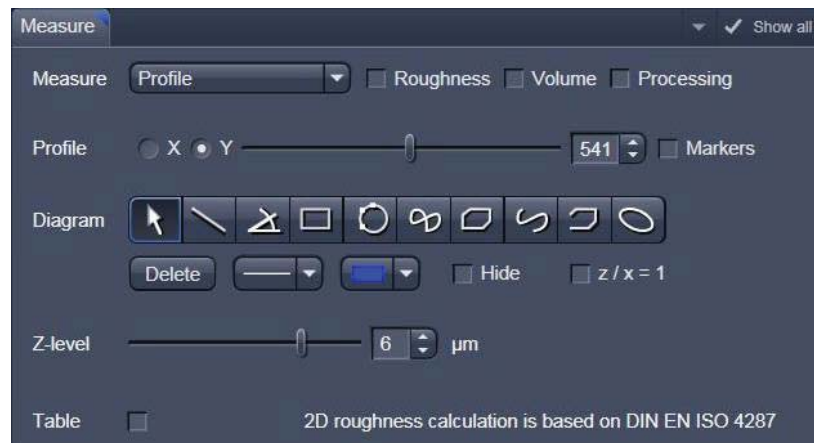
Fig. 4-222   Image view control block: Measure

Automated convention in height statistics analysis:

Topo Filters	None, median,	FFT High	FFT Low
Data formats	**P**rimary profile	**R**oughness	**W**aviness
2D profile	Pxx	Rxx	Wxx
3D topography	PSxx	RSxx	SWxx

The following measurement functions are available in the **Measure** drop down menu:

– **No diagram**

– **Profile**

– **Z-histogram**

– **Bearing area ratio**

Also these checkboxes can be ticked, to perform the measurements:

• **Roughness**: Calculation of the roughness parameters

• **Volume**: Calculation of the volume parameters.

• **Processing**: Shows the processing parameters, i. e.:

  – Generation (calculation mode: Max, Center etc.)

  – Threshold (applied intensity threshold)

  – Filter

  – Fit (plane, cylinder / sphere parameters)

(1)   Profile measurement

• Chose **Profile** in the **Measure** line.

If **Height map or Maximum intensity** is chosen in the Display block, the following tools are available to set the profile line within the image:

	Drag and move a created profile line within the image
	Create a profile line within the image
	Define the thickness of the profile line within the image
	Define the color of the profile line

If **3D** is chosen in the Display block, the following tools are available to set the profile line within the image:
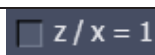
With the slider the profile line can be move on the 3D topography, in X and Y direction.

In order to use **Markers** to measure distances within the profile diagram, tick this checkbox:

These tools are available for measurements within the profile diagram:

	Move the markers. **Zoom** within the diagram by clicking the left mouse button, drawing a rectangle around the interesting area, release the left mouse button. To resize the diagram, just click the right mouse button within the diagram field.
	Creation of a **straight line**. Display of distance, inclination angle, dxdy and dz. Click and hold down the mouse button, drag the line in any required direction, release the mouse button to end the procedure.
	Creation of a **free angle**. Display of the enclosed angle (max. 180 °). The first click sets the starting point, the second and third clicks define the angle and the end point.
	Creation of a **rectangle**. Display of distance, area, height and width. Click and hold down the mouse button, drag the rectangle in any required direction, release the mouse button to end the procedure.
	Creation of a **circle**. Display of radius and area. Clicking three times to define 3 points on the profile. A circle fit is automatically applied on the profile
	Creation of a **closed Bezier** figure. Display of the length of the line figure. First click sets the starting point, each further click adds another line, a click with the right mouse button closes the figure and ends the procedure.
	Creation of a **closed polyline** figure. Display of the perimeter of the figure. First click sets the starting point, each further click adds another line, a click with the right mouse button closes the figure and ends the procedure.
	Creation of an **open Bezier** figure. Display of the length of the line figure. First click sets the starting point, each further click adds another line, a click with the right mouse button ends the procedure.
	Creation of an **open polyline** figure. Display of the length of the line figure. First click sets the starting point, any further click adds another line, click with the right mouse button ends the procedure.
	Creation of an **ellipse**. Display of the area. First click sets the center point, the displayed line permits the determination of the first dimension, second click sets the first dimension, the second dimension and rotation direction can now be determined, third click sets the second dimension and direction and ends the procedure.
Delete	**Deletes** all drawing elements or the one just selected.
	Change of the **line width** of the drawing elements.
	Clicking on the **Color button** opens a color selection box where the color of the drawing element can be selected with a click of the mouse.
Hide	Ticking this box will **hide** all drawing elements, but not the markers.
z / x = 1	Sets the **z/x** ratio in the profile diagram to the value **1**. Check: the following creation of a circle using the relevant tool really results in a circle in the profile display. Measured angle values correspond to the actual slope of the line displayed.

Tick **Table** in order to see the profile diagram data within a table (below the diagram). A right mouse button click within the table allows to copy and save this table as a text file (ASCII).

(2) Z-histogram measurement

- Chose Z-histogram within the Measure dropdown menu. The Z-histogram will be shown as a diagram below the image.

(3) Bearing area ratio measurement

Chose Bearing area ratio within the **Measure** dropdown menu. The bearing area ratio diagram will be shown below the image.

Before determination of the top bearing portion, individual peaks (noise, steep slopes) must be eliminated. The **Median** filter or a suitable longpass filter can be used for this purpose.

Shifting the two cursor crosses within the diagram permits two bearing portions to be given in percent (e.g. Smr1 = 10 %; Smr2 = 90 %) as default values for which the height difference Rdc is determined automatically.

(4) Roughness measurement in 2D (Profile) and 3D

- Chose **Profile** in the **Measure** line to get 2D roughness values.

- Tick **Roughness** checkbox: **☑ Roughness**

  - The roughness parameters are calculated and displayed on the right side of the image / profile. All roughness parameters calculated from a 2D profile are named with **R**, from the 3D surface **RS**.

  - A right mouse click within the roughness value table permits the roughness parameters to be copied to the clipboard and imported to another program (e.g.: MS Word or MS Excel) via the **Paste** function.

2D Amplitude parameters (Profile Roughness):

	Mean height z	Rc	Pc	Wc
Dispersion	Arithmetic mean deviation	Ra	Pa	Wa
	Root mean square deviation	Rq	Pq	Wq
Asymmetry	Skewness	Rsk	Psk	Wsk
Sharpness	Kurtosis	Rku	Pku	Wku
Extremes	Highest peak	Rp	Pp	Wp
	Lowest valley	Rv	Pv	Wv
	Absolute peak to valley	Rt	Pt	Wt
	Averaged peak to valley	Rz	Pz	Wz
	Maximum peak to valley	Rmax	Pmax	Wmax
If chosen filters are		FFT High	No, M	FFT L

Calculation of roughness parameters:

The 2D roughness calculation is based on DIN EN ISO 4287. The following roughness parameters are calculated (e.g. for a Y-section)

– Mean height of all profile height values R_c

$$- R_c = \frac{1}{N_y} \cdot \sum_{j=1}^{N_y} \cdot z(x, y_j)$$

N_x, N_y ... number of pixels in X- or Y-direction

Arithmetic mean deviation of all profile height values R_a

$$- R_a = \frac{1}{N_y} \cdot \sum_{j=1}^{N_y} \cdot \left[z(x, y_j) - R_c \right]$$

– Quadratic mean deviation of all profile height values R_q

$$- R_q = \sqrt{ \frac{1}{N_y} \cdot \sum_{j=1}^{N_y} \cdot \left[z(x, y_j) - R_c \right]^2 }$$

– Skewness of the distribution of all profile height values R_{SK}

$$R_{SK} = \frac{1}{N_y \cdot R_q^3} \cdot \sum_{j=1}^{N_y} \cdot z^3(x, y_j)$$

– Kurtosis of the distribution of all profile height values R_{KU}

$$R_{KU} = \frac{1}{N_y \cdot R_q^4} \cdot \sum_{j=1}^{N_y} \cdot z^4(x, y_j)$$

– Maximum peak height R_P

$$R_P = z_{max} - R_c$$

– Maximum valley depth R_V

$$R_V = R_c - z_{min}$$

– Maximum roughness depth R_t (= Peak to Valley / PV)

$$- S_t = z_{max} - z_{min}$$

maximum height difference of the overall topography along a profile.

– Classification of topography in 5 equal area elements (rectangles in the 2D mode)

– average roughness depth R_z:

$$- R_z = \frac{z_{max1} - z_{min1} + z_{max2} - z_{min2} + z_{max3} - z_{min3} + z_{max4} - z_{min4} + z_{max5} - z_{min5}}{5}$$

Averaging of R_t-values of all the 5 single area elements. When combined, both parameters provide information about the homogeneity of the surface. Big differences are indicative of pronounced inclination of the overall area or of spikes.

Developed Surface Area Ratio: Σ (surface area$_{ij}$) / Σ (projected area$_{ij}$)

The percentage of the 3D surface area (sum off all triangles formed by adjacent data points) to the 2D surface area produced by projecting the 3D surface onto the threshold plane.

– maximum roughness depth R_{max}:

$$- R_{max} = Max\ (z_{max\,1} - z_{min\,1},\ z_{max\,2} - z_{min\,2},\ z_{max\,3} - z_{min\,3},\ z_{max\,4} - z_{min\,4},\ z_{max\,5} - z_{min\,5})$$

maximum of R_t-values of all the 25 single area elements.

☞   Both the roughness parameters and the Z-histogram can be changed by using filters!

3D Amplitude parameters (Topography Roughness):

	Mean height z	RSc	PSc	WSc
Dispersion	Arithmetic mean deviation	RSa	PSa	WSa
	Root mean square deviation	RSq	PSq	WSq
Asymmetry	Skewness	RSsk	PSsk	WSsk
Sharpness	Kurtosis	RSku	PSku	WSku
Extremes	Highest peak	RSp	PSp	WSp
	Lowest valley	RSv	PSv	WSv
	Absolute peak to valley	RSt	PSt	WSt
	Averaged peak to valley	RSz	PSz	WSz
	Maximum peak to valley	RSmax	PSmax	WSmax
If chosen filters are:		FFT High	No, M	FFT L

Calculation of roughness parameters:

The following roughness parameters are calculated:

– Mean height of all surface height values S_c

$$- RS_c = \frac{1}{N_x \cdot N_y} \cdot \sum_{i=1}^{N_x} \sum_{j=1}^{N_y} \cdot z(x_i, y_j)$$

    N_x, N_y ... number of pixels in X- or Y-direction

– Arithmetic mean deviation of all surface height values RS_a

$$- RS_a = \frac{1}{N_x \cdot N_y} \cdot \sum_{i=1}^{N_x} \sum_{j=1}^{N_y} \cdot \left[z(x_i, y_j) - RS_c \right]$$

– Quadratic mean deviation of all surface height values RS_q

$$- RS_q = \sqrt{\frac{1}{N_x \cdot N_y} \cdot \sum_{i=1}^{N_x} \sum_{j=1}^{N_y} \cdot \left[z(x_i, y_j) - RS_c \right]^2}$$

– Skewness of the distribution of all surface height values RS_{SK}

$$RS_{SK} = \frac{1}{N_x \cdot N_y \cdot RS_q^3} \cdot \sum_{i=1}^{N_x} \sum_{j=1}^{N_y} \cdot z^3(x_i, y_j)$$

– Kurtosis of the distribution of all surface height values S_{KU}

$$RS_{KU} = \frac{1}{N_x \cdot N_y \cdot RS_q^4} \cdot \sum_{i=1}^{N_x} \sum_{j=1}^{N_y} \cdot z^4(x_i, y_j)$$

– Maximum peak height RS_p

$$RS_P = z_{max} - RS_c$$

– Maximum valley depth S_V

$$RS_V = RS_c - z_{min}$$

– Maximum roughness depth RS_t (= Peak to Valley / PV)

$$- RS_t = z_{max} - z_{min}$$

maximum height difference of the overall topography.

Classification of topography in 25 equal area elements (rectangles in the 2D mode)

– average roughness depth S_z:

$$- RS_z = \frac{z_{max1} - z_{min1} + z_{max2} - z_{min2} + \cdots + z_{max25} - z_{min25}}{25}$$

Averaging of R_t-values of all the 25 single area elements. When combined, both parameters provide information about the homogeneity of the surface. Big differences are indicative of pronounced inclination of the overall area or of spikes.

– maximum roughness depth RS_{max}:

$$- RS_{max} = Max \ (z_{max1} - z_{min1}, \ z_{max2} - z_{min2}, \ \cdots, \ z_{max25} - z_{min25})$$

maximum of R_t-values of all the 25 single area elements.

☞   Both the roughness parameters and the Z-histogram will be influenced by the use of filters!

(5)   Volume measurement in 3D

- Tick the checkbox ☑ Volume within the Measure line:
  - The volume parameters are calculated and displayed to the right of the image.
  - To copy / save the content displayed, click with the right mouse button into the shown tables and decide to copy or save. The values can be imported to other programs (e.g.: MS Word or MS Excel) via the **Paste** function.
- Setting the **Z-level** slider enables you to change the height level of the topography. The portion of the topography lying below the set height level is filled with "water" (blue color) and the volume parameters are calculated online only for the projecting part of the topography.

☞   To use the **Z-level** function, load the **Profiles** 3D display mode containing the **Glowscale** or **Grey** channel color to obtain optimum display.

- If the **Measure** function **Bearing area ration** is also activated, a red marker line shows the position of the height level in the percentage of contact area curve.

Within the Volume measurement also the ratio of **valid** data points (means signal intensities within a given intensity threshold) is displayed.

Parameters

The following parameters are calculated:

Z: height level (selectable with the **Z-Threshold** and **Fill Level** sliders). The setting of this value influences the following parameters.

Vm (z):   material volume above chosen height level

Vv (z):   void volume below chosen height level

Smr (z):   material volume ratio

$$S_{mr}(z) = \frac{V_m(z)}{V_m(z_{min})}$$

Svr (z):   void volume ratio

$$S_{vr}(z) = \frac{V_v(z)}{V_v(z_{max})}$$

Au: surface bearing area of the topography at Z (= projection area of those parts which are situated above chosen height level)

Smr: surface bearing area ratio of the topography at Z

                percentage of contact area (= Au / (x * y) * 100 %)

Sda: true surface = sum of all triangles formed by adjacent data points of the surface reconstruction

Sdr: developed surface area ratio:

                Σ (surface areaij) - Σ (projected areaij) / Σ (projected areaij) * 100 %

                projected area = x * y

                The percentage of the 3D surface area (sum of all triangles formed by adjacent data points of the surface reconstruction) to the 2D surface area produced by projecting the 3D surface onto the threshold plane.

                absolute flat surface $\Rightarrow$ is equal to base plane (Sdr = 0 %)

                The increase by which the 3D surface is larger than the basic plane (e. g. 625 % is a 3D surface which is about 6.25 times larger than the projected basic plane)

4.6.12.4   Control Block: 3D Measurement

Fig. 4-223   Image view control block: 3D Measurement
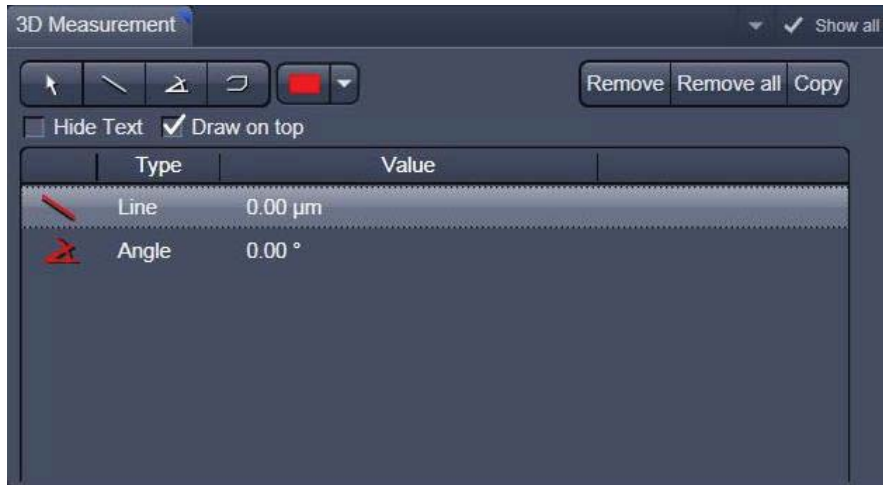
The symbols shown are described in section Profile measurement. These can be used to measure directly on the 3D model. The dimension data shown below can be copied using the "Copy" symbol.

After the open polyline measurement is finished, use a right mouse click to leave the image window.

4.6.12.5   Control Block: Series

See section 4.6.8.10 on 3D (VisArt*plus*).

4.6.13   Mean of ROI: Additional View Type for Time Series

The **Mean of ROI** View Type allows to

- display the intensity - time diagram (mean intensity in user defined ROIs over time),
- use frame - time series and frame - Z-Stack - time series as input,
- show the intensity values in table form and copy table to clipboard or save as text file.

The **Mean of ROI** function permits interactive analysis and monitoring intensity over time.

The settings of the **Dimensions, Display**, **Player** and **Overlay** View Options control block apply. The additional view-specific **Mean of ROI View Option** control block is shown in Fig. 4-224.

Any changes done with this View Option control block are effective immediately.

The Image Display in the Mean of ROI View shows 3 panels: the intensity-over-time diagram, the data table with the intensity values for each ROI over time and the image display with ROIs in overlay (see Fig. 4-225 and Fig. 4-226).

Fig. 4-224   **Mean of ROI View Option control block**

To access the **Mean of ROI** View Type, load or acquire a time series data set and click on the **Mean ROI** View tab.

The mean intensity of one or several regions of interest is displayed over time. Time flags from bleaching experiments of trigger events are marked with little symbols on the time-axis of the graph (see Fig. 4-225.

The **Mean of ROI** analysis always refers to the currently displayed z-section of a Z-Stack (see **Dimension** View Option control block for which section it is).
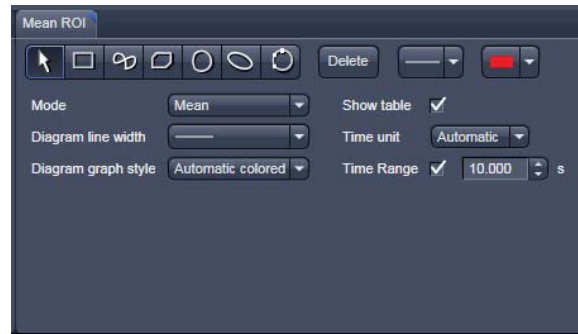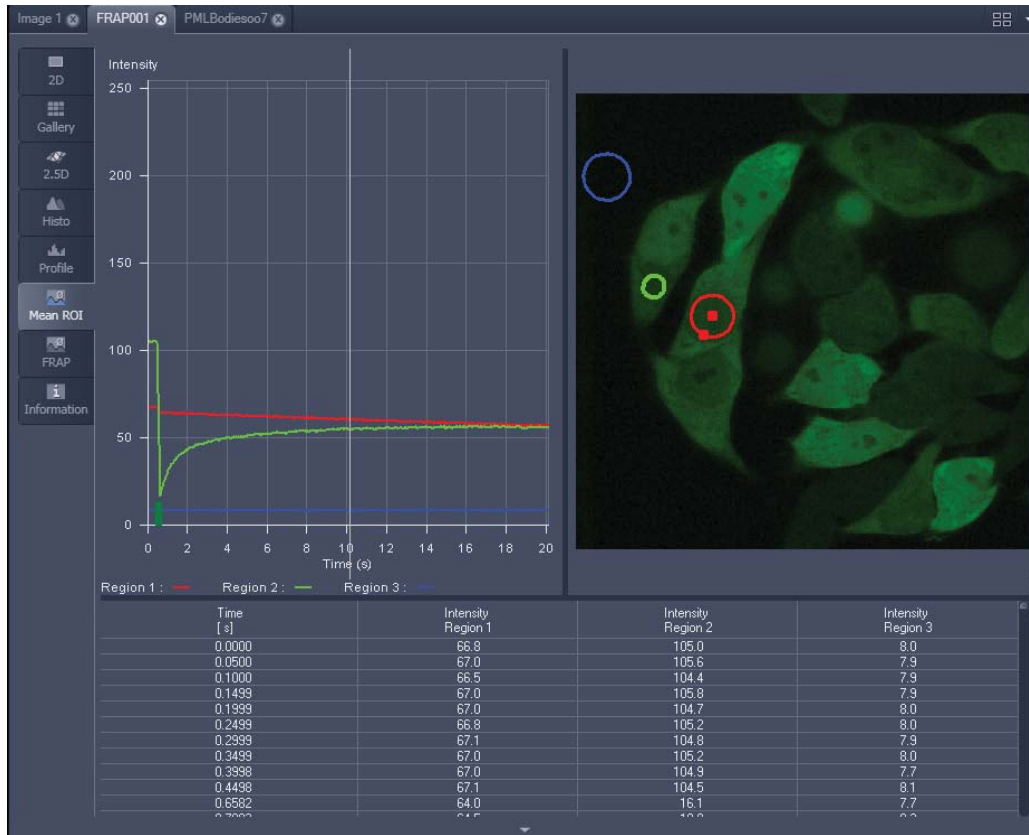
Fig. 4-225   Image Display window, Mean ROI display for time series in single plane
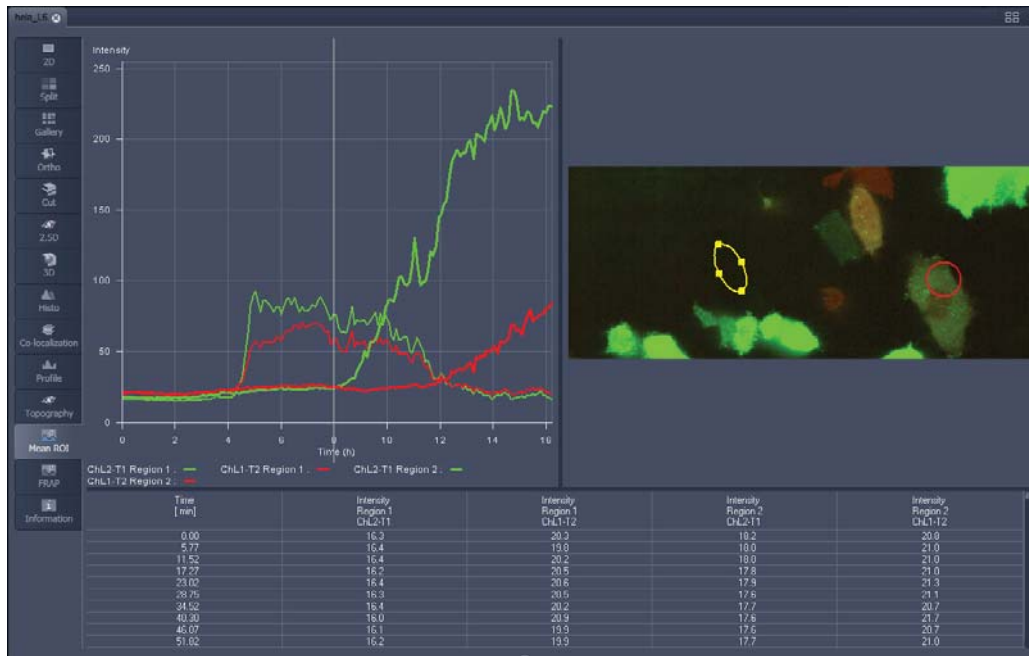
Fig. 4-226   Image Display window, Mean ROI display for time series of Z-Stack

The **Mean of ROI** view options control block contains the following function elements:

ROIs can be managed in the Overlay View Option control block.

Arrow button: Activation of the mouse button for resizing or movement of the ROI in the **Image Display** window.

Bezier button: Activates the Bezier figure drawing mode. The first click sets the starting point, each additional click adds a further line, a double-click on the starting point closes the figure and ends the procedure.

Circle button: Activates the circle drawing mode. Clicking and holding down the mouse button sets the center point; drag the diameter and release the mouse button to end the procedure.
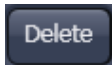
Recycle bin button: All the ROIs to the image are deleted.

Rectangle and 3D Rectangle button: Activates the rectangle drawing mode. Click and hold down the mouse button, drag the rectangle in any direction, release the mouse button to end the procedure.

Ellipse and **3D Elipse** button: Activates the ellipse drawing mode. The first click sets the center point, the displayed line permits the determination of the first dimension, the second click sets the first dimension, the second dimension and the rotation direction can then be determined; the third click sets the second dimension and the direction and ends the procedure.

Polyline and **3D Polyline** button: Activates polyline drawing mode. The first click sets the starting point, each additional click adds a further line, a double-click on the starting point closes the figure and ends the procedure.

Line button: This button allows you to determine the line thickness of the ROI outline.

Buttons for diagram display options:

- **Mode** pull down: choose between area and mean mode
- **Area**: Display of the area of the ROI in the intensity time diagram, depending on the set threshold values. Area measurements of very small areas (< 10 pixels) give only approximate values.
- **Mean**: Display of the mean values of the relevant ROI in the intensity time diagram.
- **Diagram line width** pull down: sets the line with of the curves in the diagram.
- **Diagram graph style** pull down: choose from a set of graph styles
- **Show Table** check box: The table of intensity values is displayed on the bottom of the Image Display window.
- **Time unit** pull down: choose between seconds, minutes, hours or Automatic
- **Time Range** tick box and spin box: reduce the displayed time range to the selected value

In Area Mode: Ch1 / Ch3 / Ch4 pull down: Selection of the channel to be used.
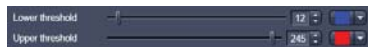
In Area Mode: Threshold low and high slider: The intensity values below /above threshold are not displayed for the **Area** function.

4.6.14   Kinetic / FRAP View: Additional View Type for Time Series

The **FRAP** function permits interactive analysis of bleaching experiments, including

– Correction of FRAP data for bleaching and background,

– Fitting of FRAP data to a mono exponential or double exponential model for intensity.

The settings of the **Dimensions, Display**, **Player** and **Overlay** View Options control blocks apply. The additional view-specific **FRAP View Option** control block is shown in Fig. 4-227.

Any changes done with this View Option control block are effective immediately.

The Image Display in the **FRAP** View shows 4 panels: the intensity-over-time diagram with the fitted curve, the table with the fit parameters, the intensity value data table and the image display (see Fig. 4-228, Fig. 4-230 and Fig. 4-231).

To access the Kinetic / FRAP analysis view type, load or acquire a time series data set and click on the **FRAP** View tab.

Fig. 4-227   FRAP View Option control block
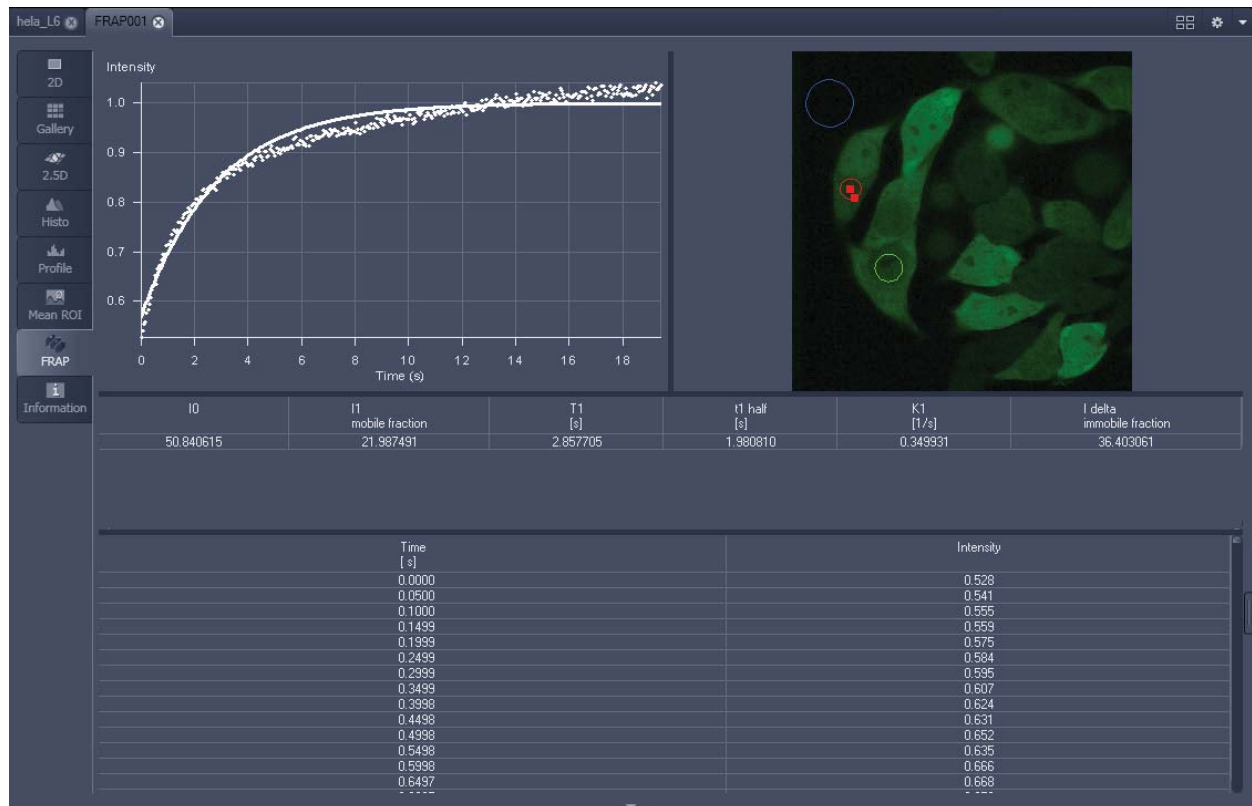
Fig. 4-228   Image display of a time series in the FRAP View tab

Available tools in the FRAP View:

Channels

Select single channels or all channels for analysis in the general **Dimensions** view options control box.

Kinetic Model / Fit formula

Pull down menu to select the mathematical model (mono or double exponential model) for fitting the data.

Analyis Areas / Regions of Interest (ROIs)

The **FRAP** View Options control block includes drawing tools that work identically to the overlay view options control block. ROIs from both control blocks can be combined.

Background Region

Checkbox in the list of ROIs: Mark the region of interest which represents the mean background intensity to be used for data correction.

Reference Region

Checkbox in the list of ROIs: Choose and mark the ROI which represents the fluorescence intensity of a reference cell that has not been bleached. The mean intensity within that region is used to correct the data at each time point for any bleaching artifact that occurred during the imaging process.

Combine Regions

Group 1,2,3

Checkboxes in the list of ROIs which allow to choose more than one ROI for analysis and to group them according to the experimental set up.

Example: FRAP Performed in a Nucleus Expressing GFP Labeled Proteins

Display of the image series in the **Mean ROI** display mode: The drawing tools are used to define the ROI to be analyzed (ROI 1), the background ROI (ROI 2), and the reference ROI (ROI 3) (see Fig. 4-229). The reference ROI must be a neighboring cell which has been imaged with the same laser intensity over time identical to the cell, which has been bleached to induce FRAP. Make sure the whole cell or cell compartment of interest is imaged and therefore illuminated.

☞   Use the **time** slider in the **Dimensions** view options control block of the general view options group to display the first image after the bleach event. This makes it easy to choose the ROI for analysis. The analysis region should be slightly smaller than the region that has been bleached. The latter is listed in the Mean of ROIs list in the overlay view options control block.

- Define your ROIs for analysis in the **Mean of ROI** View tab.

Fig. 4-229   FRAP data displaying the first bleached image of an images series in the Mean of ROI View Type

- Switch to the **FRAP** View tab.

- Mark the checkboxes for background and reference region.

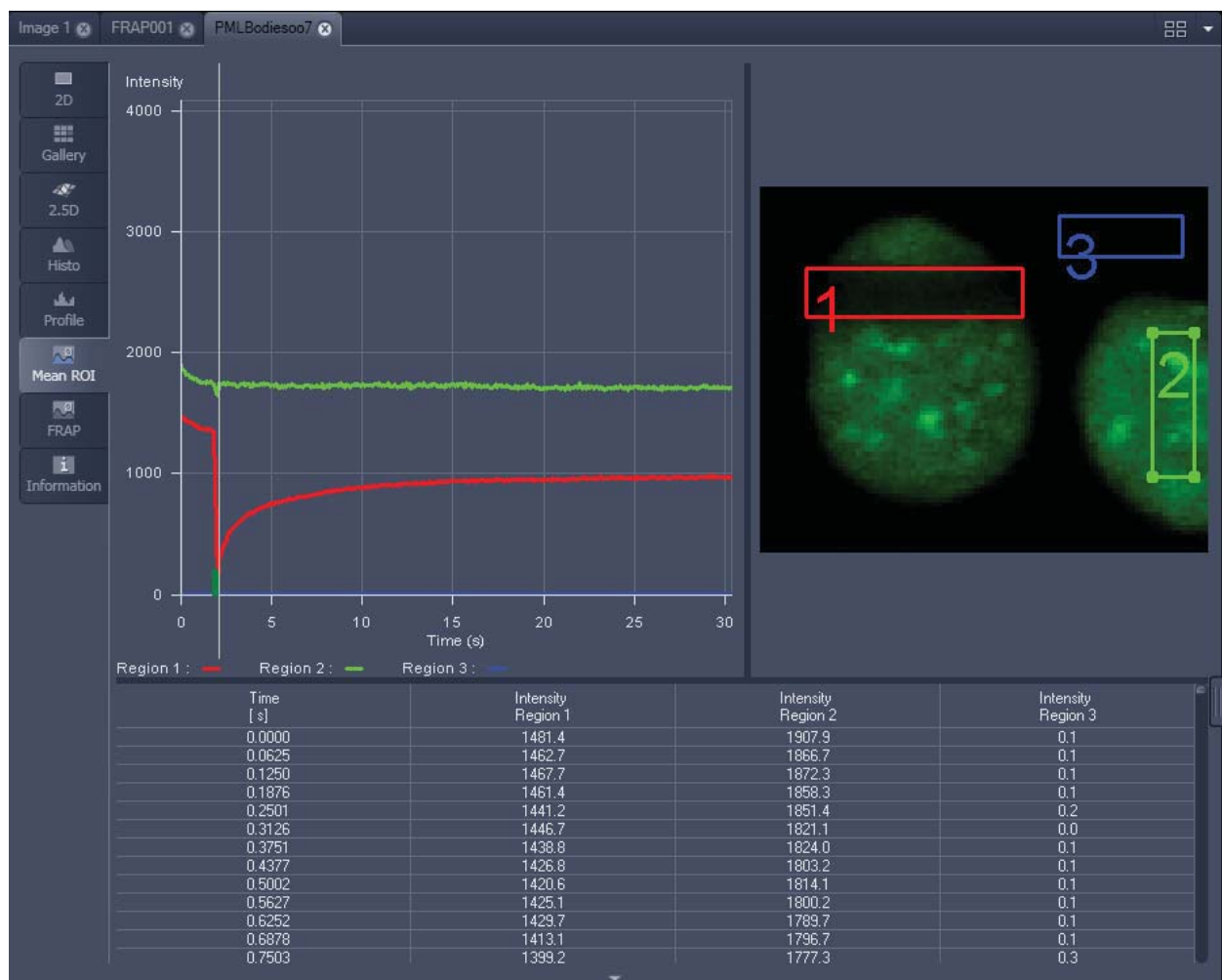- The intensity values of the ROI to be analyzed will be corrected for background intensity and changes in intensity calculated for the reference region. The correction is done for each time point taking the actual intensity difference in the reference region into account.

  The remaining ROI or ROIs are used for analysis when checked active for either group 1, 2 or 3. ROIs assigned to the same group are analyzed added for analysis.

- Chose the **Kinetic Model** in the pull down list.

  – The result of the fit is displayed in the table. The result can be copied to the clipboard (Copy **Results**) or saved as a text file (**Save Results**) (right-mouse click). The following values are calculated and shown:

  – The final signal intensity in the analyzed ROIs following recovery **I0** (of the fitted curve).

  – The amplitude of the fitted curve (which equals the mobile fraction) **I1 mobile fraction**.

  – The fitted parameter **T1** (seconds).

  – The rate constant for the exchange of molecules between the bleached region and the surrounding area **K** (per second).

  – The part of the immobile fraction of the protein **I delta immobile fraction**.

Checking the **Table** tick box opens a further table in the image display area. It shows all intensity values for each channel and ROI analysis group over time. These values are corrected for background intensity and intensity loss of the reference region.

The values can be saved as a text file (**Save Table**) or copied into Excel via right-mouse click (clipboard) (**Copy Table**).

The data can be normalized optionally when marking the checkbox **Normalize** in the FRAP View Options control block.

☞   The calculation of the parameters is based on the same ROIs unless other ROIs or moved ROIs are selected again. The Kinetic Display is always available once the analysis has been performed.

☞   The analysis is not stored with the image.

☞   Note that this modeling is a very basic approach to your experiment. It offers a first hint on the possible presence of only one or, in case of a bad fit, more than one mobile fractions of the labeled protein within the cell or cell compartment examined. For a more advanced analysis refer to the scientific literature.

☞   The half time of the recovery can be calculated using the following formula: $t_{half} = \dfrac{\ln 0.5}{-T1}$
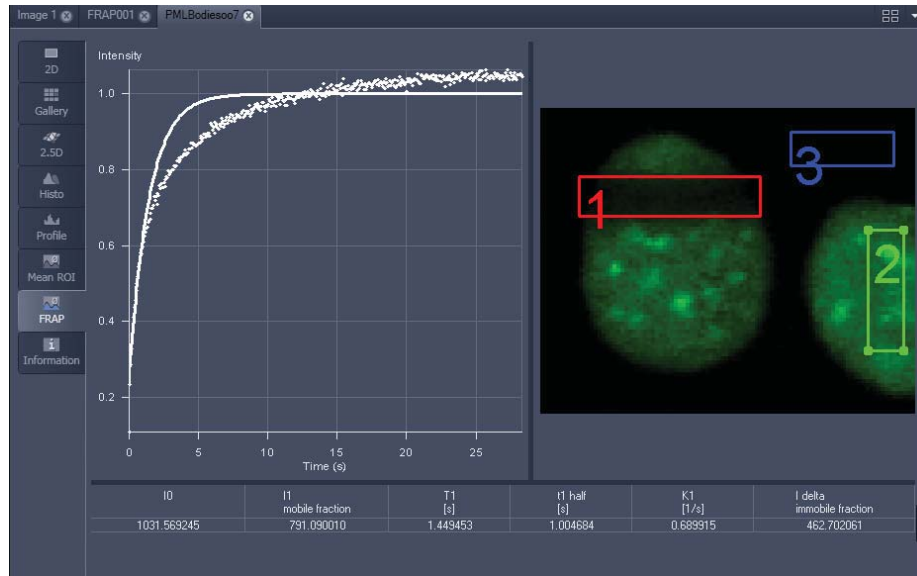
Fig. 4-230   Image window displaying the analysis of FRAP data using a mono exponential fit
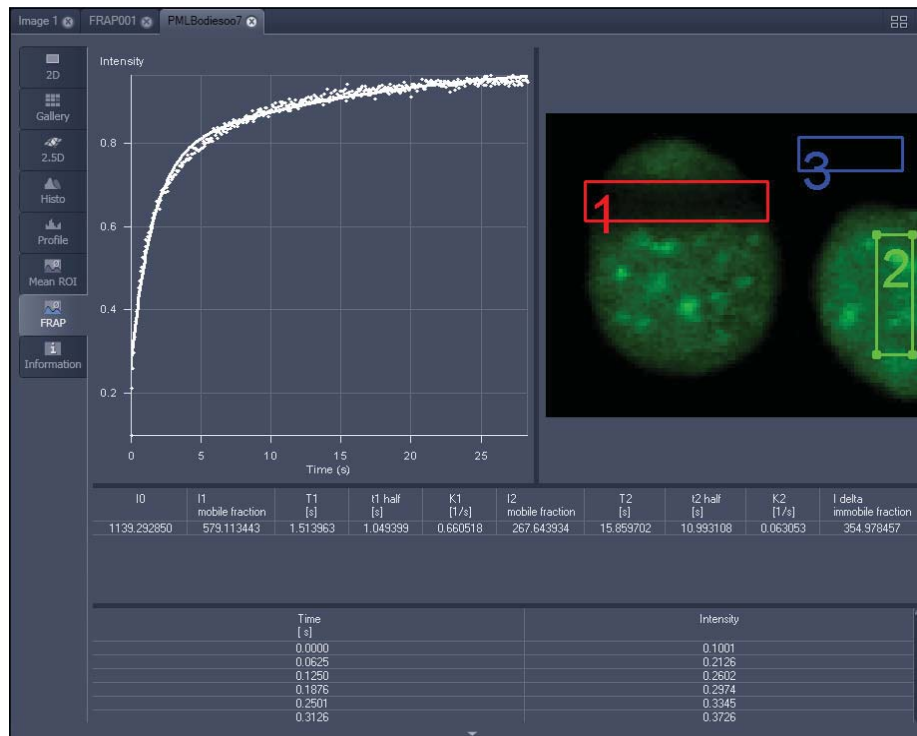
Fig. 4-231   Image window displaying the analysis of FRAP data using a double exponential fit

If the analysis is done using the double exponential fit the fitted curve displays the mean of the fitted values for the two different mobile fractions. The table shows the following additional parameters:

- The amplitude of the two curves, displayed as one (which corresponds to each part of the mobile fractions **I1** and **I2**).

- The fitted parameters **T1** and **T2** (seconds) for each mobile fraction.

- The rate constant for the exchange of molecules between the bleached region and the surrounding area **K1** and **K2** (per second) for each mobile fraction.

☞   The raw data of the experiment can be exported for further analysis using the Mean ROI display mode and, within this dialogue, the table display of the results.

☞   Note that this modeling is also a very basic approach to your experiment. It offers a first hint on the possible presence of two mobile fractions of the labeled protein within the cell or cell compartment examined.

Please refer to relevant scientific literature or the website of the EAMNET (http://www.embl.de/eamnet) for further information on how to set up and perform FRAP experiments. A schematic curve marking the data points that are calculated performing the Kinetic Analysis is shown in Fig. 4-232. Please note, that the naming of the data points is not consistent with the information on the website.

A more general introduction to FRAP experiments can be found in the "Methods" brochure "Photomanipulation with Laser Scanning Microscopes" by Carl Zeiss Microimaging GmbH (order no. 45-0060 e/09.06) and the references cited therein.
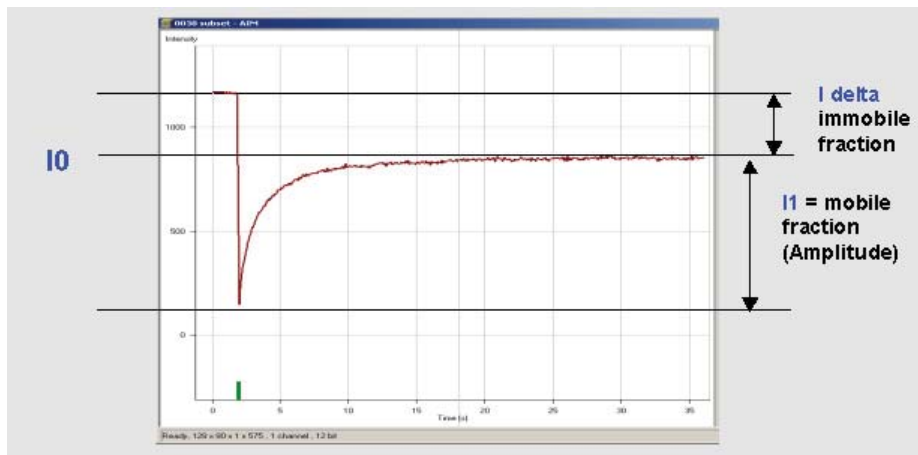
Fig. 4-232   **Schematic FRAP curve with marks at the relevant data points. I0, I1 and I delta which are calculated performing the Kinetic Analysis**

4.6.15 Lambda Coded: Additional View Types for Lambda Mode

The **Lambda Coded** View displays a Lambda Stack in a wavelength-coded color view. A color palette is automatically assigned to the individual images which are then displayed in a merge-type display. As a result, each pixel is represented similarly to a view through the oculars without emission filter (see Fig. 4-234).

The settings of the **Overlay** View Options control block apply as in 2D view. In the **Display** View Options control block, the channel-specific settings of brightness, contrast and gamma don't apply since there are no channels. The **Dimensions** View Options control block is reduced since the wavelength is not a dimension any more when it is color-coded. The modified view-specific **Dimensions View Option** control block is shown in Fig. 4-233.

Any changes done with this View Option control block are effective immediately.
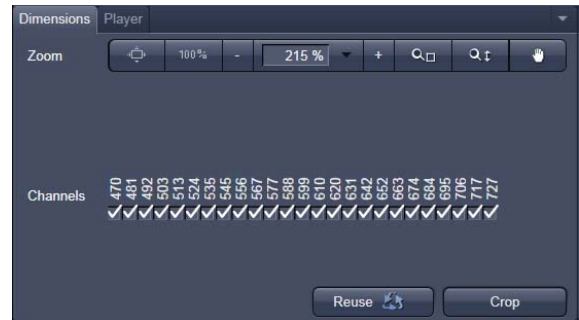
Fig. 4-233   Lambda Coded - specific Dimensions View Option control block
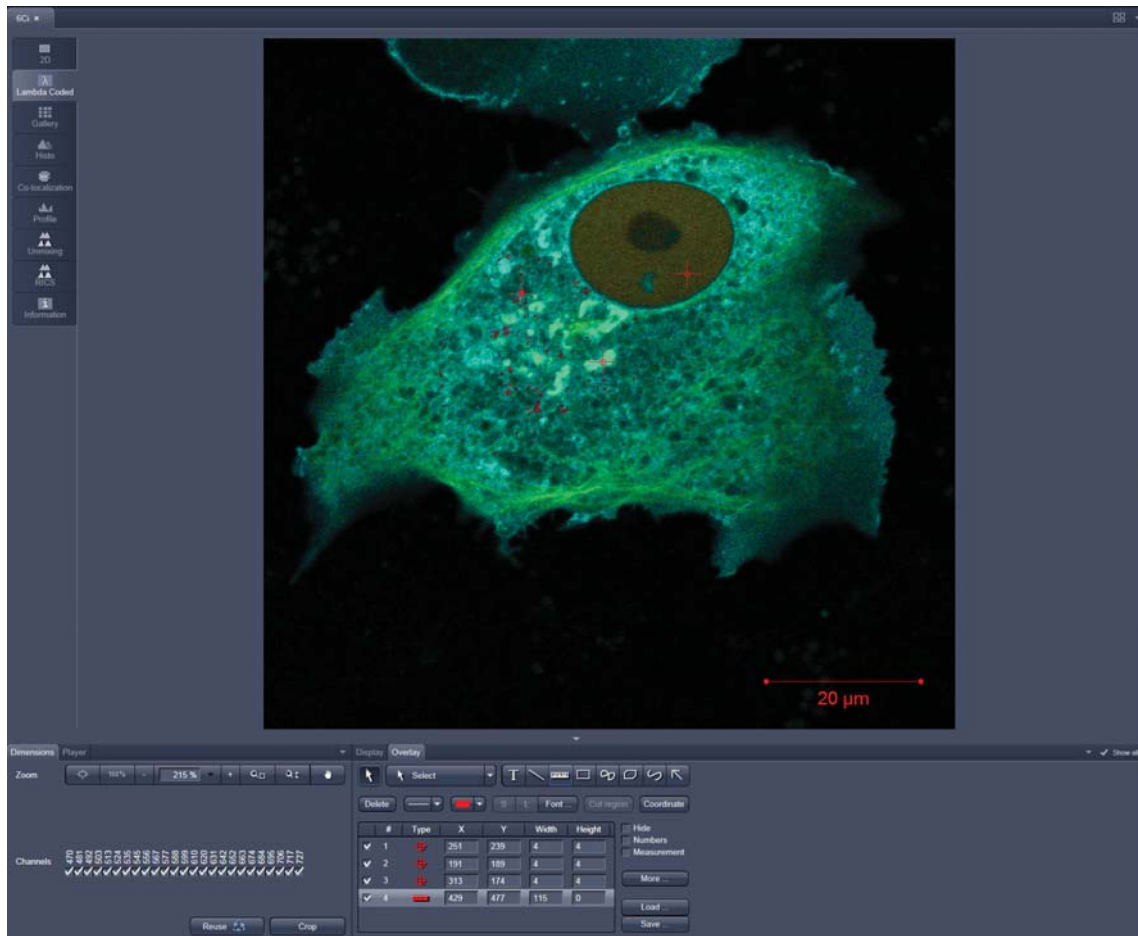
Fig. 4-234   Dimensions view option control block for Lambda Coded View

4.6.16   FRET View

In the **FRET** View type (Fig. 4-235), FRET data sets can be analyzed:

- Data gained performing Acceptor Photobleaching
- Data gained performing Sensitized Emission

The data sets can either be acquired setting up the imaging procedure manually with the system or using the FRETplus Macro.

The **FRET** View will only be present if either a time series with a bleach event or a three channel image is present in the image container.

Any changes done with this View Option control block are effective immediately.

Depending on the type of data the Image Display in the **FRET** View shows 3 panels: the FRET image, the original data and the table with the analyzed data according to the method chosen for analysis.

To access the **FRET** View, load or acquire either a multichannel data set (minimum 3 channels) or a time series with bleach event and click on the **FRET** tab.

Tables can always be saved or copied to the clipboard by right-mouse clicking on the table display!
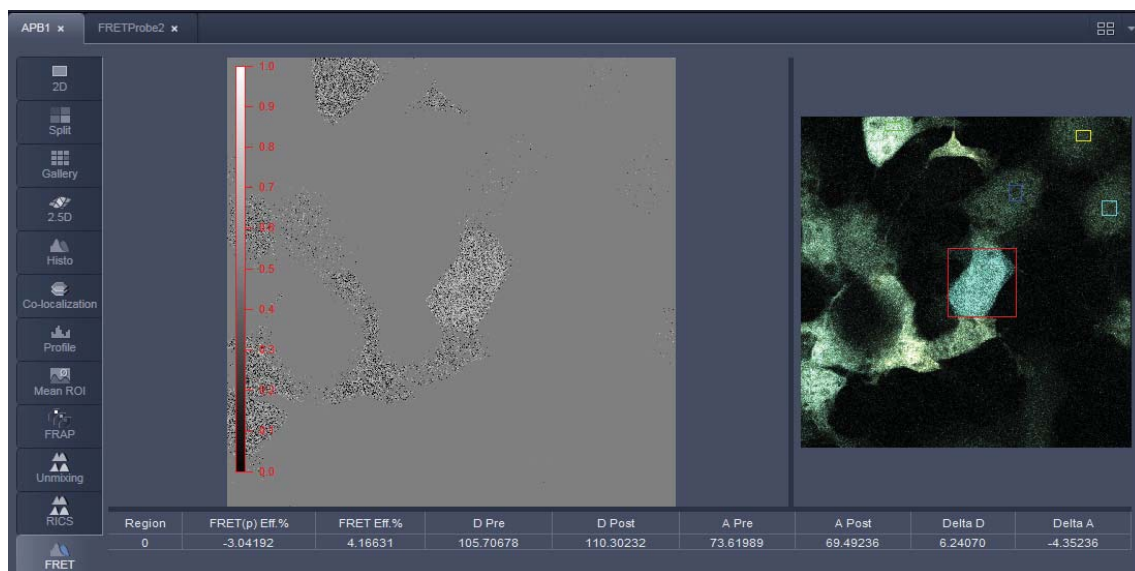
Fig. 4-235   FRET Display of Time series image having performed Acceptor Photobleaching

Independent of the FRET method, the FRET View Options control block offers four tabs:

The available functions within each tab can vary according to the image loaded.

It is assumed that a time series with bleaching represents an experiment with Acceptor Photobleaching and a three channel image represents an experiment performing Sensitized Emission.
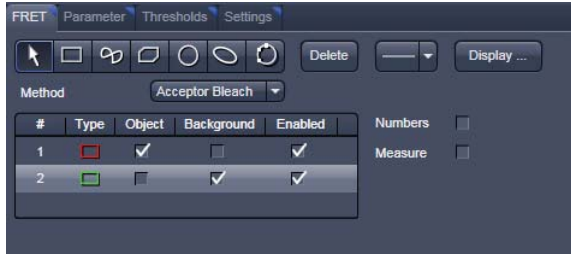
Fig. 4-236   FRET   View   Options   control   block, FRET tab

4.6.16.1   Tools in the FRET View Options Control Block for Acceptor Photobleaching

FRET tab (Fig. 4-236):

Within this tab the overlay regions present in the image (used for bleaching and defined as background or control region) can be defined (The drawing tools correspond to the drawing tools described in section 4.6.2.4).

As **Method** for FRET Analysis, only **Acceptor Photobleach** is available in the drop down list.

The check boxes **Numbers** and **Measure** refer to the overlay regions and annotate the number of the region and its area to the overlay in the image.

Export allows the resulting FRET image to be saved as a separate image file.

The regions defined can be set as **Object** (Region where FRET should be calculated) or **Background** (from which the threshold for the image analysis can be deduced from).

Regions and the assigned status as Object or Background can be selected or deselected for the individual Analysis using the corresponding check box under **Enabled**.
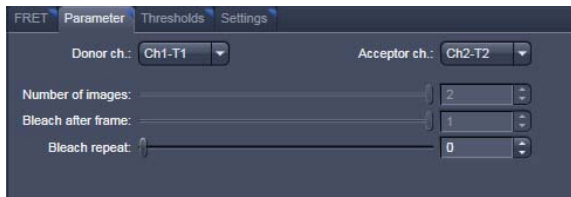
Fig. 4-237   FRET   View   Options   control   block, Parameter tab

Parameter tab (Fig. 4-237):

In case the image series for Acceptor Photobleaching has not been acquired with the FRETplus Macro, various Parameters of the image series can be set in this tab.

The image of the Donor and the Acceptor are assigned to the appropriate channel using the drop down menu next to **Donor ch.:** and **Acceptor ch.:**.

The total **Number of images**, the time for the bleaching event (**Bleach after frame**), and the action of **Bleach repeat** is set using the slider next to each action or by editing the field next to the slider.
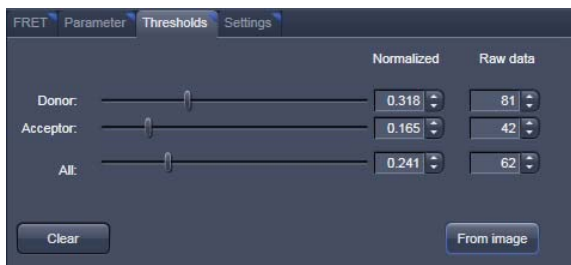
Fig. 4-238   FRET   View   Options   control   block, Thresholds tab

Thresholds tab (Fig. 4-238):

The threshold for the image analysis can be set manually using the slider or editing text box next to **Donor**, **Acceptor** or **All**, where **All** moves both sliders to the same value.

Alternatively, the threshold can be set from the image when clicking **From image** provided a background region and an object region have been defined and enabled in the FRET tab.

The values for the thresholds are either displayed as grey value levels (**Raw data**) or **Normalized** to the value 1.

Settings tab (Fig. 4-239):

This tab provides access to the definition of the parameters used for the image analysis.

The **General** tab applies to both methods, Acceptor Photobleaching and Sensitized Emission.

If checked the listed options will be taken into account for the analysis of FRET.

The **Acceptor Photobleach** tab (Fig. 4-240) sets additional parameters to analyze the Acceptor Photobleaching experiment such as the choice for **Truncation** (as Remove Max or No Truncation) and the option not to display negative values in the image (**Don't show negative values in the FRET image**).
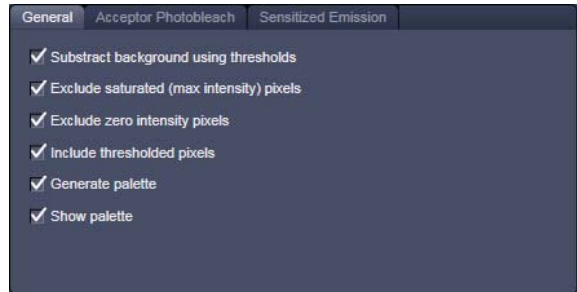
Fig. 4-239   FRET View Options control block, Settings tab, General
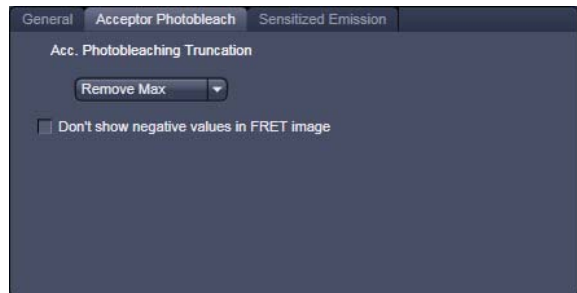
Fig. 4-240   FRET View Options control block, Settings tab, Acceptor Photobleach

4.6.16.2   Tools in the FRET View Options Control Block for Sensitized Emission

FRET tab (Fig. 4-241):

Within this tab overlay regions can be defined. (The drawing tools correspond to the drawing tools described in section 4.6.2.4).

The check boxes **Numbers** and **Measure** refer to the overlay regions and annotate the number of the region and its area to the overlay in the image.

Fig. 4-241   FRET View Options control block, FRET tab

Export allows the resulting FRET image to be saved as a separate image file including the table with the results.

The regions defined can be set as **Object** (Region where FRET should be calculated) or **Background** (from which the threshold for the image analysis can be deduced from).

Regions and the assigned status as Object or Background can be selected or deselected for the individual Analysis using the corresponding check box under Enabled.

As Method for FRET Analysis now three different options are available in the drop down list:

- **Fc (Youvan)**,
- **FRETN (Gordon)** and
- **N-FRET (Xi)**.

Fc or **Youvan** method:

Displays the Fc image with intensities converted from the FRET index calculated for each pixel using the Youvan method. This method assumes that the signal recorded in the FRET channel is the sum of real FRET signal overlaid by donor crosstalk and acceptor signal induced by direct (donor) excitation. There is no correction for donor and acceptor concentration levels and as a result the FRET values tend to be higher for cells that have higher FRET molecular concentrations.

FRETN or **Gordon** method:

Displays the FRET image with intensities converted from the FRET index calculated for each pixel using the Gordon method. This method calculates a corrected FRET value and divides by concentration values for donor and acceptor. This method attempts to compensate for variances in fluorochrome concentrations by overdoes it. As a result cells with higher molecular concentrations report lower FRET values.

N-FRET or **Xi** method:

Displays an N-FRET image with intensities converted from the FRET index calculated for each pixel using the Xi method. This method is similar to the Gordon method with the difference that for concentration compensation the square root of donor and acceptor concentration is used. The resulting image is properly corrected for variances in the fluorochrome concentration.
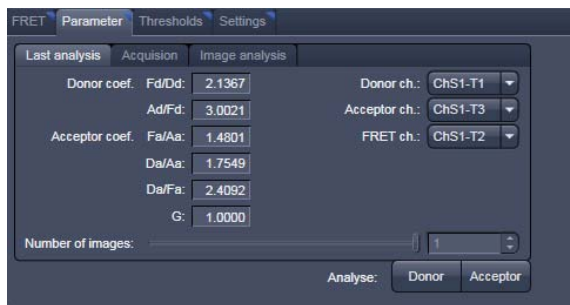
Fig. 4-242   FRET View Options control block, Parameter tab, Last Analysis

Parameter tab (Fig. 4-242):

In case the image series for **Sensitized Emission** has not been acquired with the FRETplus Macro, various Parameters of the image series can be set in this tab for the **Last analysis**, the **Acquisition** and the **Image analysis**.

The values for the different Analysis methods to be set (from the images) for the **Donor coefficient** include the FRET signal detected in the donor channel/Donor signal detected in the Donor channel (**Fd/Dd**) and the Acceptor Signal detected in the Donor channel/FRET signal detected in the Donor channel (**Ad/Fd**).

The values for the **Acceptor coefficient** include the FRET signal detected in the Acceptor channel / Acceptor signal detected in the Acceptor channel (**Fa/Aa**), the Donor signal detected in the Acceptor channel / Acceptor signal detected in the Acceptor channel (**Da/Aa**), the Donor signal detected in the Acceptor channel/FRET signal detected in the Acceptor channel (**Da/Fa**), and the Gordon factor **G**.

The image of the Donor the Acceptor and the FRET are assigned to the appropriate channel using the drop down menu next to **Donor ch.:**, **Acceptor ch.:** and **FRET ch.:**.

The total **Number of images** is set using the slider or by editing the field next to the slider if necessary.

By clicking the button **Donor** or **Acceptor** the analysis is performed with the new/edited parameters and a new image view is added to the image container (if not already present).

Use the buttons for Donor, Acceptor and FRET in the lower left corner of the image view to alternatively display the three different images.

Thresholds tab (Fig. 4-243):

The threshold for the image analysis can be set manually using the slider or editing text box next to **Donor**, **Acceptor**, **FRET** or **All**, where **All** moves all sliders to the same value. Alternatively, the threshold can be set from the image when clicking **From image** provided a background region and an object region have been defined and enabled in the FRET tab. The values for the thresholds are either displayed as grey value levels (**Raw data**) or **Normalized** to the value 1.
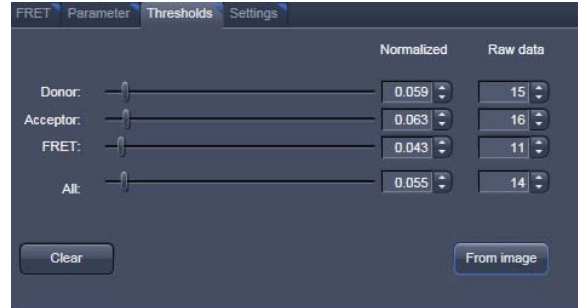
Fig. 4-243   FRET View Options control block, Thresholds tab

Settings tab (Fig. 4-239 and Fig. 4-244):

This tab provides access to the definition of the parameters used for the image analysis.

The tab **General** (Fig. 4-239) applies to both methods, Acceptor Photobleaching and Sensitized Emission.

If checked the listed options will be taken into account for the analysis of FRET.

The tab **Sensitized Emission** (Fig. 4-244) sets additional Truncation parameters for each method to analyze the Sensitized Emission experiment such as the choice for **No truncation**, **Remove maximum** or the percentage of truncation to choose between 100, 200 and 300 %.

For the Fc analysis method, the option "**Don't show negative values in the Fc image**" can be selected using the check box. Also included is a slider to set the value for normalization of the results of the FRETN-method.
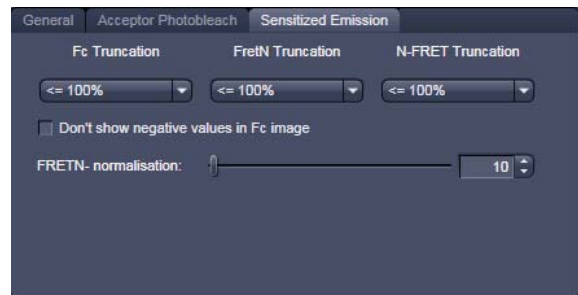
Fig. 4-244   FRET View Options control block, Settings tab, Sensitized Emission

Fig. 4-245 **Unmixing View Option control block**
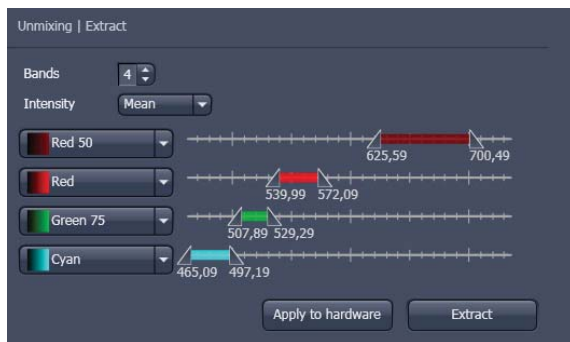
Fig. 4-246 **Unmixing - Extract - View Option control block**

4.6.17 Unmixing View

In the **Unmixing View** type, Lambda data sets can be analyzed and spectrally unmixed. In particular it allows to:

- display the spectra corresponding to user-defined ROIs (mean ROI intensity over Lambda),

- use Lambda Stacks as input

- show the intensity values in table form and copy table to clipboard or save as text file,

- generate unmixed multi channel images.

The available tools and the functionality of the four general View Option control blocks **Dimensions**, **Display**, **and Overlay** is the same as in the **Lambda Coded** View (see the previous section on this view type). The two additional view-specific **Unmixing View Option** control blocks are shown in Fig. 4-245 and Fig. 4-246.

Any changes done with this View Option control blocks are effective immediately.

The Image Display in the **Unmixing View** shows 3 panels: the intensity-over-lambda diagram with the extraction bands (32-channel-LSM 710 only), the table with the intensities in each ROI over lambda, and the image display (see Fig. 4-247). The spectra from images which are acquired on an LSM 700 are shown as integrated spectra. This reflects the way, how Lambda stacks are acquired with the LSM 700 (see the respective description in the LSM 700 Manual).

To access the Unmixing analysis view type, load or acquire a Lambda stack data set and click on the **Unmixing** tab.

☞ The Unmixing View is available (→ displayed) for all Lambda stacks.
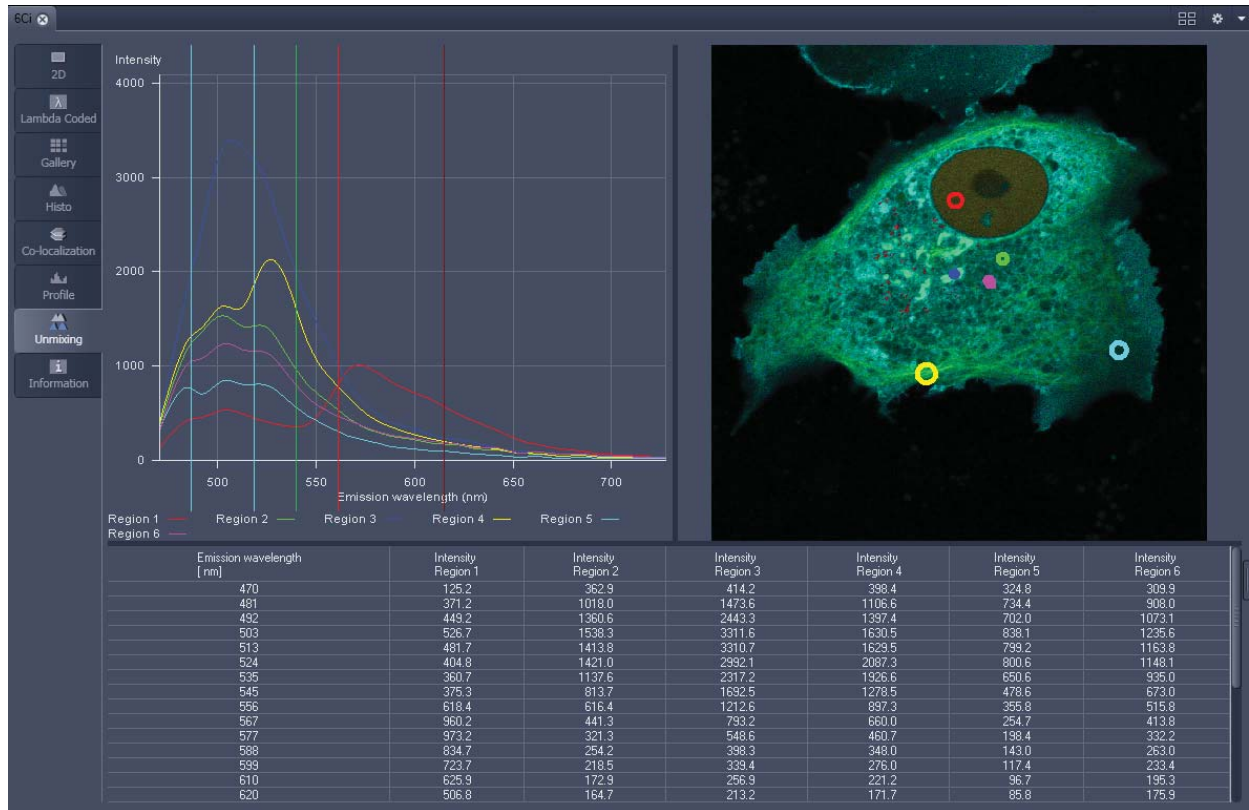
Fig. 4-247   Unmixing Display of a Lambda stack

Tools in the Unmixing View Options control block

– Tools for display customization and drawing:

| **Drawing tools** | Drawing tools work the same as in the Overlay View Option control block |

☑ Lambda coded

Check box for image display: show the image in greyscale or lambda coded
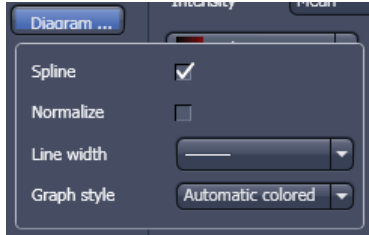
Dialog for diagram display:

- – Spline on/off
- – Normalize the graphs of the spectra to 1
- – Set the line width of the spectra in the diagram
- – Selection of graph style
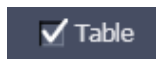
☑ Table

Show Table button: The table of intensity values over Lambda is displayed in the Image Display window.

Tables can always be saved or copied to the clipboard by right-mouse clicking on the table display!

– Tools for unmixing:

Background region   Region 5

Select ROI for background subtraction

Auto scale   ☑

Automatically balances the intensity of unmixed channels to equal levels.

Display channel with residuals   ☑
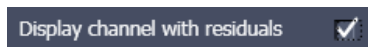
Displays the difference between fit and original data (for the channel of the Lambda stack that shows the greatest deviation) in a separate channel in the unmixed image. (See also section 4.5.10.7).

Save to spectra database...
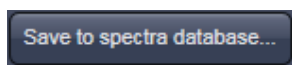
The values of the intensity Lambda diagram are saved to the spectra database in the ZEN\DyeDatabase directory. Make sure to have displayed only data of single dyes in the intensity Lambda diagram when executing this function. (The database interface is shown in Fig. 4-248).

Auto find / ACE...

Auto Find / ACE ("Automatic Component Extraction")
Automatically searches the image for regions with pure signals (Fluorescence originating from one single dye).

Linear Unmixing

Performs the unmixing with the selected spectra. Note: Those channels of the Lambda stack which are de-selected in the dimensions control tab are not included in the unmixing calculation.
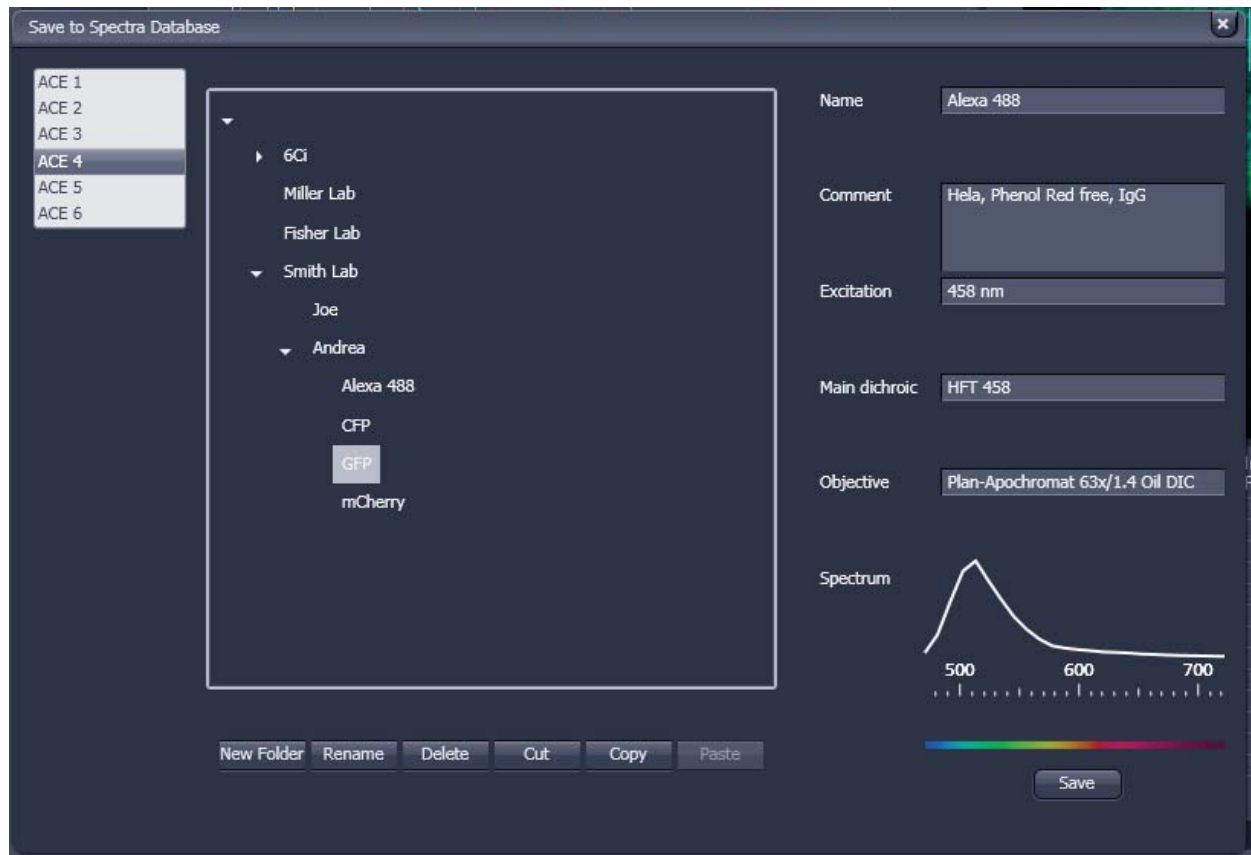
Fig. 4-248   Save to Spectra Database: database interface with subfolder structure
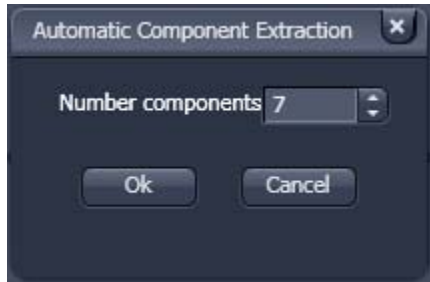
Fig. 4-249   ACE, number of components selection window
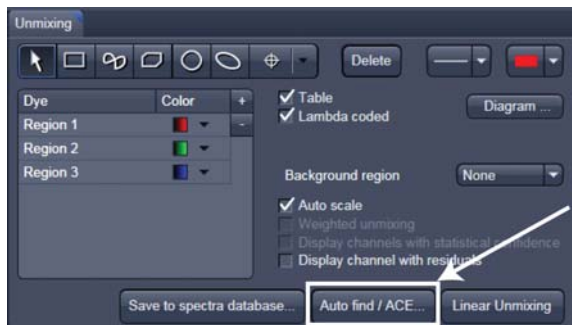
Fig. 4-250   Automatic   Component   Extraction function

4.6.17.1   Automatic Component Extraction

The use of this function permits the automatic search for the individual reference spectra in a Lambda stack.

- Load or acquire a Lambda stack data set.

- Click on the **Unmixing View** tab.

- Click on the Auto find / ACE... button in the Unmixing View Option control tab (Fig. 4-250).

- Select the number of components in the sample and click **Ok** (Fig. 4-249).

- Review the results in graph.

- When obtained spectra appear reasonable, unmix   the   data   set   by   clicking   the Linear Unmixing button (Fig. 4-250).

  – A new and spectrally unmixed multichannel image document is created (as many channels as previously defined components).

Benefit and limitations of the ACE method:

Little to moderate spatial overlap of emission signals is required to obtain good results.

Because it relies on reference spectra extracted from the multi-channel / Lambda stack image itself, ACE could unmix on wrong assumptions and produce poor data.

However, it provides a solution for Emission Fingerprinting in those cases where reference spectra are not accessible via single-labeled controls.

If the resulting data is carefully checked for plausibility and quantification of the results is avoided carried out with the appropriate caution, this often is a helpful tool when working with spectrally overlapping dyes.

Tools in the Unmixing - Extract View Options control block:

Button:
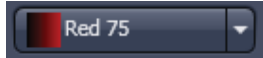
Selection of a wavelength range (use the sliders) that will be assigned to a channel. This assignment can be used for scanning (when **Apply to Hardware** is pressed, see below) and /or for extracting a single or multichannel image from the Lambda stack (when **Extract Channels** is pressed). Clicking on the color box in the channel button opens the color selection list and a LUT can be assigned to this channel.

Slider:

Add (**+**) or reduce (**-**) the number of channels displayed and available for assignment. Up to 8 channels labeled after the assigned LUT are available.

Sum signals from selected detector elements

Mean signals from selected detector elements

Autoscaling of output channels (individually)

channels button

Generates a new image or multicolor images based on the settings made in the channels. Two or more Lambda channels are binned to form the channels 1 ... 8. The generated image is displayed in a new **Image Display** window and is no longer a Lambda stack.
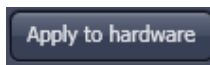
button

Sets the QUASAR detector parameters to match the previously defined spectral ranges / channels. For the used in binning mode as specified in the settings ChS1, ChS2, ChS3 ... ChS8. See **Single Track** and **Multi Track** in **Config Control** for the effect of the action. The **Lambda stack** setting is not affected by this action.

4.6.18   Information View

The **Information View** is a view type that shows a summary information sheet of all relevant image acquisition parameters. Parts of it can be edited – like, e.g., the **Notes** field.

The Information View displays:

– File Name of the data set

– A brief description of the data set

– A Notes field in which the user can note down essential information on the experiment

– The user name of the person who acquired the image

– The pixel scalings in X, Y, Z

– The image size (pixels in x-y-z, channels, bit depth)

– Calibrated Dimensions x-y-z in µm

– The scan mode (e.g., stack)

– The zoom factor

– The objective lens

– The pixel dwell time of the scan

– The average mode used

– The Gain (Master) settings for all channels

– The Digital Gain settings for all channels

– The Digital Offset settings for all channels

– The pinhole sizes

– The filters and beam splitters in the beam path

– The used lasers with the transmission of the respective AOTF

Fig. 4-251   Information View of a 2 channel Z-Stack

4.7 Right Tool Area: Data Management and Storage

4.7.1 General

(1) Document Type

Images are stored as files (*.lsm format) in folders just like in any other Windows application.

Further Information about the .lsm file format for developers and programmers are available in a .pdf document in the ZEN installation folder.

In ZEN there are three ways to access your data:

– The File Menu

– The ZEN File Browser

– Open Images are accessible in the Right Tool Area

The traditional LSM databases have been replaced by

– the ZEN file browser and

– the Information View tab (see section 4.6.18) in the image.

(2) ZEN File Browser

The File Browser allows access to image data of variable image formats and permits displaying them in three views:

– Gallery View,

– Table View and

– Form View.

Images can be loaded, copied and deleted within the ZEN File Browser.

The **Re-use** (see section 4.6.2.1) function is available in the ZEN Image Browser: Hardware parameters can be loaded without opening the files by clicking the **Re-use** button.

(3) Open Images

In the Right Tool Area the **Open Images** section is displaying the images which are loaded in the system memory. Double clicking brings the image to the front. Files can be saved and deleted/closed on this section.

(4) Export

With the **Export** dialog in the **File** menu, the acquired data can be directly exported to various file types.

☞ In the **Save As** dialog in the **File** menu these formats can also be chosen as file type.

4.7.2   File Menu

- In the **Main** menu bar, click on **File**.
  - This displays the menu shown in Fig. 4-252.
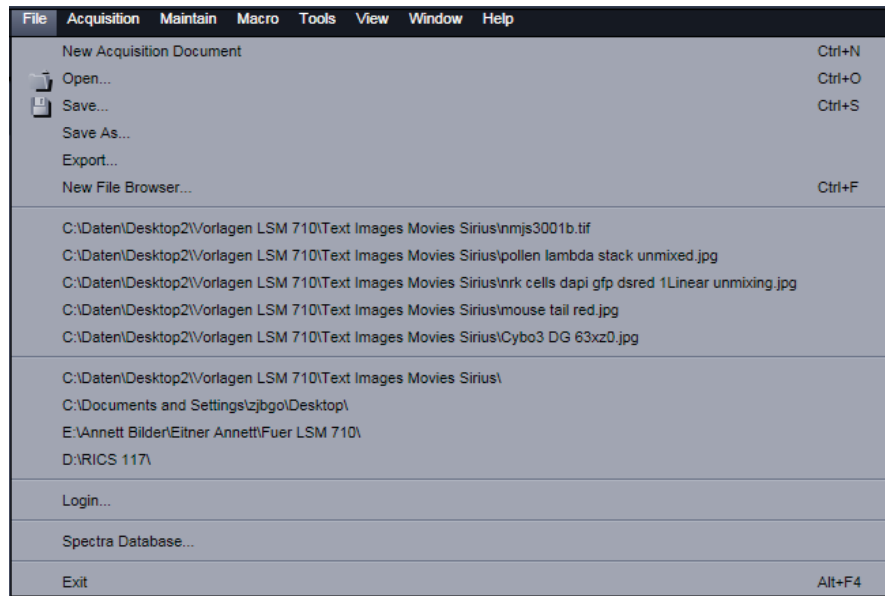
Fig. 4-252   File menu

The items in this list reflect the standard windows files menus with the following exceptions:

- **New File Browser** will open a ZEN File Browser in the currently active container (see section 4.7.3).

- In the second group of entries in the list, the five last accessed files are listed. Selecting a list entry will open the respective file.

- In the third group of entries, the four last accessed folders are listed. Selecting a list entry will open the ZEN File Browser and display the image files which are present in the folder.

- **Login** displays the **Login screen** and permits to change the operation mode of ZEN, e.g. from **Online** (with hardware started) to **Offline** (Image Processing or Demo mode), without quitting ZEN.

- **Spectra database** is starting the dialogue used to handle previously recorded reference spectra.

4.7.3 ZEN File Browser

The ZEN File Browser is a versatile tool to access your image data. It displays the hard drives and connected network drives available on the computer. Clicking on a folder opens it and lists the image files therein.

The ZEN File Browser can be operated in three views:

- Gallery View,

- Table View and

- Form View.
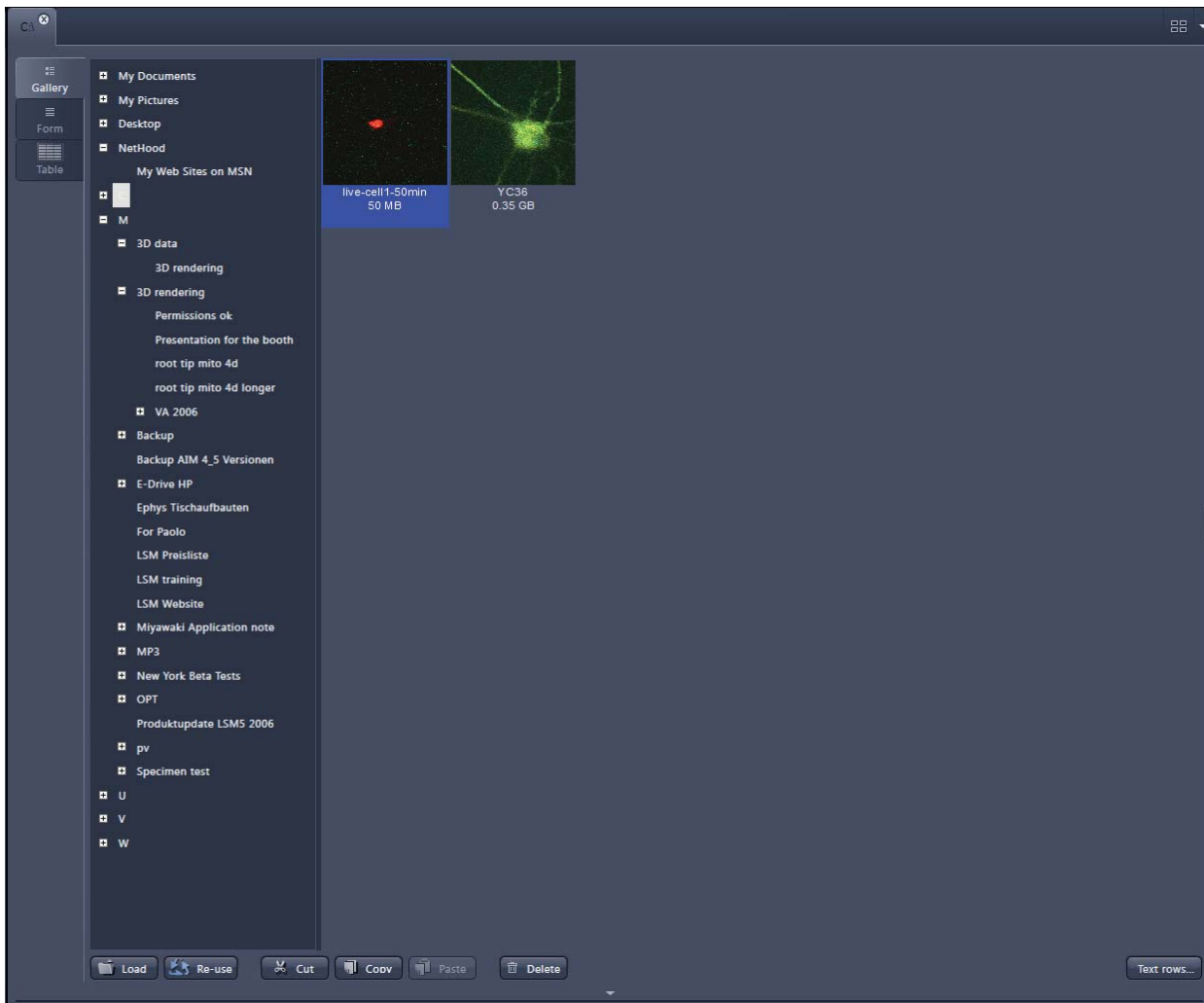
Fig. 4-253   ZEN File Browser

With the use of the button bar underneath the files can be loaded, cut, pasted and deleted.

Pressing the **Re-use** button loads the hardware settings of the .lsm image highlighted (blue) in the browser window. You do not have to open the image to apply the **Re-use** function.

☞ Eventual error messages (Fig. 4-254) fading in the right hand corner inform about missing hardware if applicable. The message box disappears after ~ 10 seconds.

Fig. 4-254   Error message

4.7.3.1   Gallery View of the ZEN File Browser

In the **Gallery View** of the browser the images are displayed as thumbnails. One click selects a single image, press **Shift** and click to select multiple images. If the **CTRL** key is pressed, all clicked images are selected.
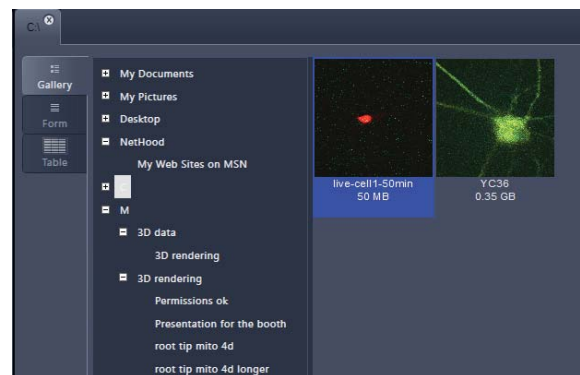
Fig. 4-255   Gallery view
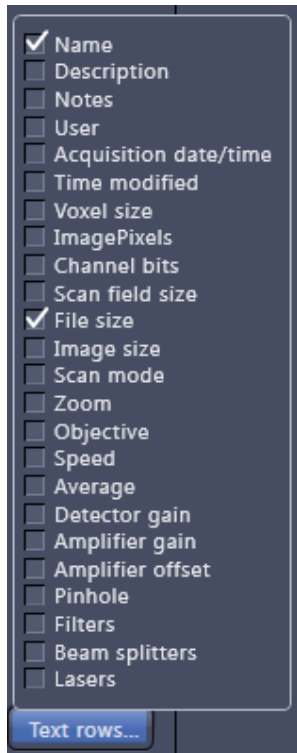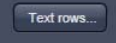
Fig. 4-256   **Image information selection**

Opening Images:

- To open an image, double click on it.

- For opening multiple images at a time, select the desired images and press **Load**.

The information stored in the .lsm file format can be displayed underneath the thumbnail. By pressing the [Text rows...] button a choice of information is available (see Fig. 4-256).

The example below (Fig. 4-257) shows the use of the display of:

- file name,

- acquisition date,

- file size and

- scan mode.
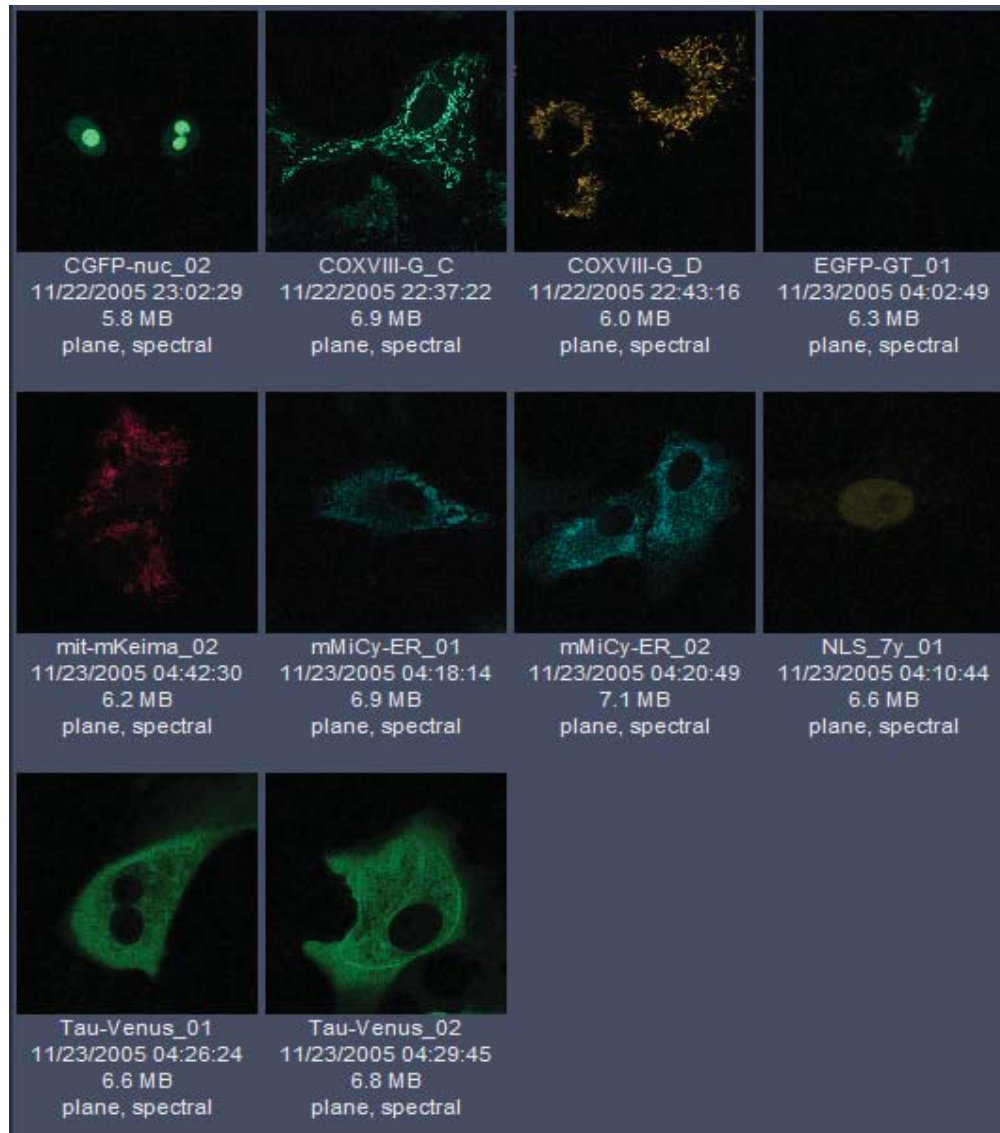
Fig. 4-257   Images with information

4.7.3.2 Form View of the ZEN File Browser

All images in a given folder can be accessed by using the slider below the Info-Panel or using the stepper on the right side of the slider. The total number of images in a folder is indicated on the right side of the slider ![stepper 13 1].

In the **Form View** of the browser all information available (or entered) in the **Info** tab of the image is displayed.

Note that the upper four fields (**Name**, **Descriptions**, **Notes** and **User**) are currently not saved. To edit these fields please load the image and edit the form in the info-view of the image. The information in those fields are – however – accessible by copy and paste.

The buttons for copying and loading images underneath are available in the Form view as well.
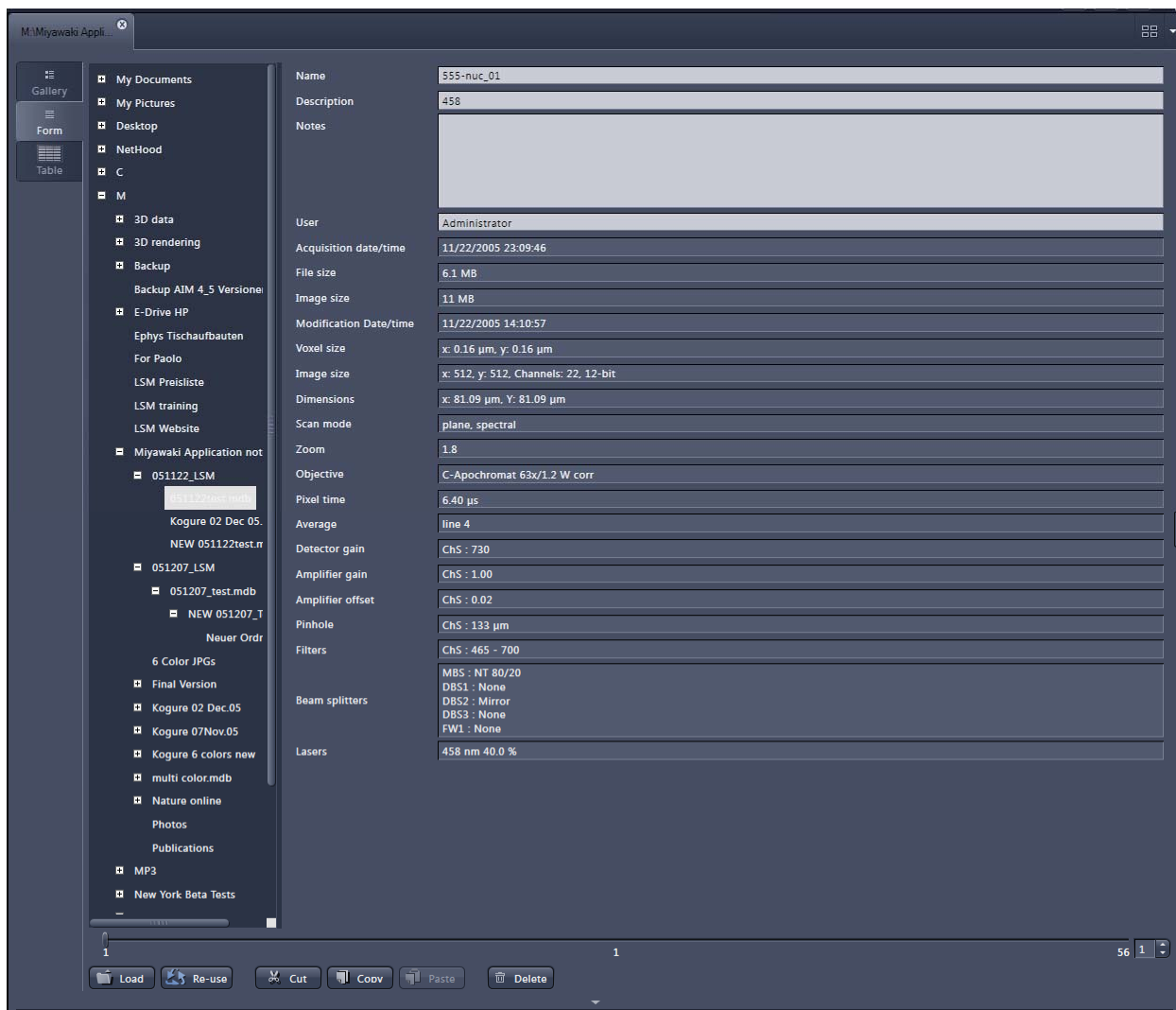
Fig. 4-258   Form view

4.7.3.3 Table View of the ZEN File Browser

The **Table View** is especially useful in case a lot of files are found in one folder.

Fig. 4-259 Table view

Fig. 4-260   Table view options

The columns displayed in the table are selectable from the **Table columns** button. The options shown in Fig. 4-260 are available.

The width of the columns is editable and the order of the columns can be freely shifted by dragging the header of the columns.

4.7.4 Open Images

The Open Images section is located in the Right Tool Area of ZEN. It displays all images and data which are open in ZEN. Its purpose is multi-fold:

– All opened images can be brought to the front by double clicking on them which makes the navigation through lots of images easy and fast.

– Basic file handling functions are implemented for fast operation with the acquired images. The images can be deleted and saved using the following buttons:

☞ On already saved images the button is closing the image, not deleting it.
   Multiple images can be selected by holding down the **CTRL** key on the keyboard and clicking on the list entries.

– The whole section can be hidden by clicking on the white triangle above the section.
   Once hidden, a click on the right boarder of ZEN lets the **Open Images** section slide back into the application.

– All files can be displayed in three ways by using the buttons underneath the section.
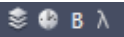
   – List

   – Small Thumbnails

   – Large Thumbnails

   The default setting is **Small Thumbnails** as indcated on the screengrab on the left.

Regardless of which display type is chosen, the individual images contain the following information:

– File name

– Data type indicated by icons: like e.g. **Stack**, **Time**, **Bleach**, **Lambda** or **C** for multichannel images. These icons are the same as underneath the start button when starting multidimensional experiments (section 4.5.2.1).

The file size is indicated in MB or GB

– Example of a 2 Ch. Z-Stack displayed with a small thumbnail in the Open Images section (Fig. 4-261).

Fig. 4-261   2 Ch. Z-Stack image
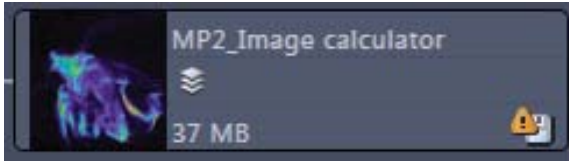
Fig. 4-262   Image with warning symbol

– In case the image is not saved or has been processed, a warning symbol is diplayed on the right side (Fig. 4-262).
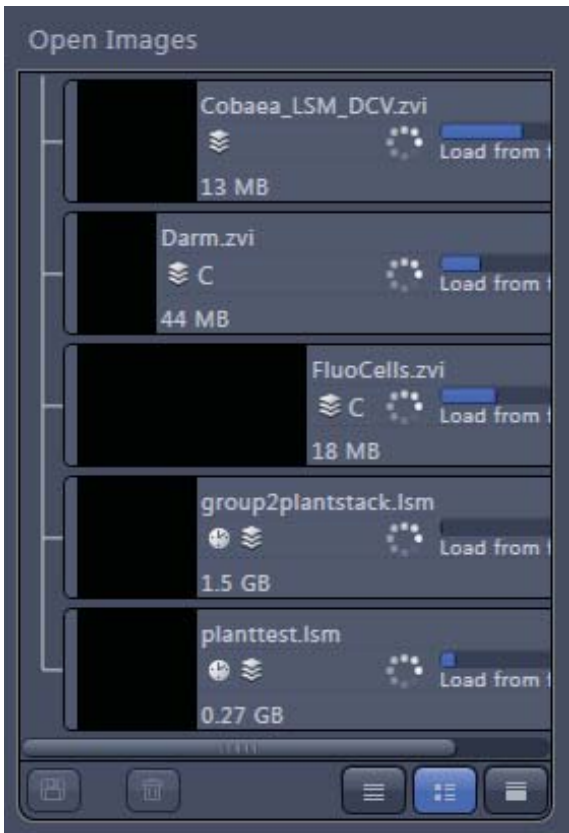
Fig. 4-263   Image progress bar

– A progress bar (Fig. 4-263) is shown for each image facilitating the overview if e.g. many processing function are running at the same time.

Fig. 4-264   Enlarging the image view

– In case long filenames are used the whole section can be dragged to the left and thereby enlarged (Fig. 4-264).

4.7.5   Opening of Files via the "Open" Command in the File Menu

- Click on the File Menu and select **Open**.

    – The standard windows open dialog appears.

- Select the desired file and click **Open**.

4.7.6   Save

The **Save** function allows to store an image together with the acquisition parameters (and processing information) to be stored in a .lsm file.

Proceed as follows to save an acquired or an edited / processed image:

- Click on the **Save** or **Save As** button in the **File** Menu of the **Main** menu.

    – The **Save Image and Parameter As** window appears on the screen.

Save:
Stores a newly created or changed image. Newly created images must be given a name

Save As:
Stores a previously stored and called up image under a different name. If images are called up and stored again, the original data and time display will be retained.
Clicking on either of these Menu Items opens the **Save As** window to create and open an image database.
Click on the **OK** button in the **Save Image** window.
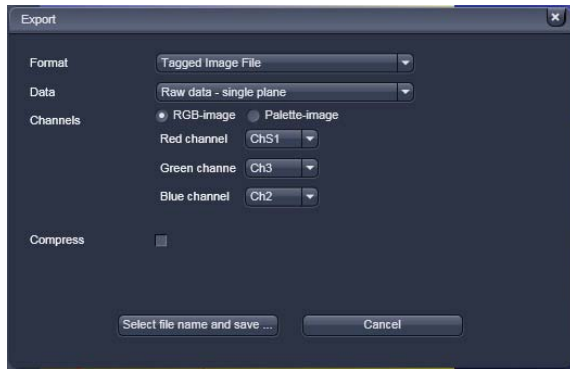
Fig. 4-265   Export window
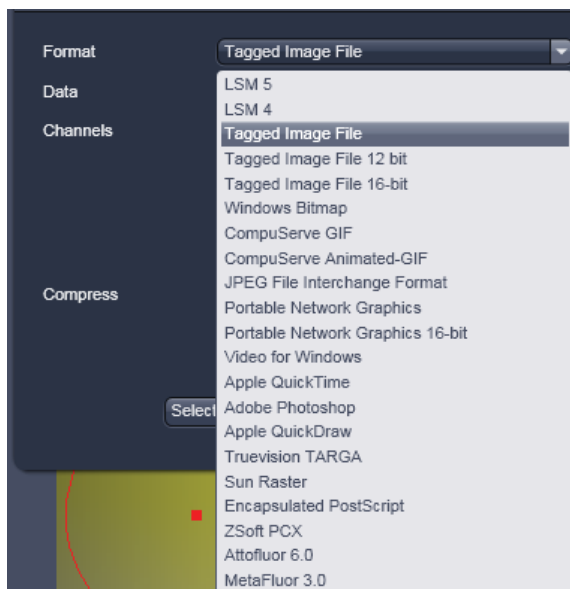
Fig. 4-266   Format selection

Fig. 4-267   Data type selection

4.7.7   Export of Images

The **Export** function allows the export of acquired images and images loaded

- Select the image to be exported.

- Click on the **Export** Menu Item in the **File** Menu of the **Main** menu bar

  – This opens the **Export** window (Fig. 4-265).

- Under **Format**, select the format to which the image is to be exported to (Fig. 4-266).

- Select the data type which the image is to be exported under **Data** (Fig. 4-267).

- Chose a compression level. For some file formats lossless compression or various other compression levels are available. The degree of losses for the image quality is listed according to the type of compression.

- Click on the **Select file name and save ...** button. The standard Windows File saving dialogue appears. Click **Save** to complete the export process.

☞   When stacks or time series are exported, each frame is stored as an individual image.

4.8   Help – About

The **About** window can be accessed via the Help item in the menu bar clicking **About** opens the following dialog:
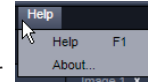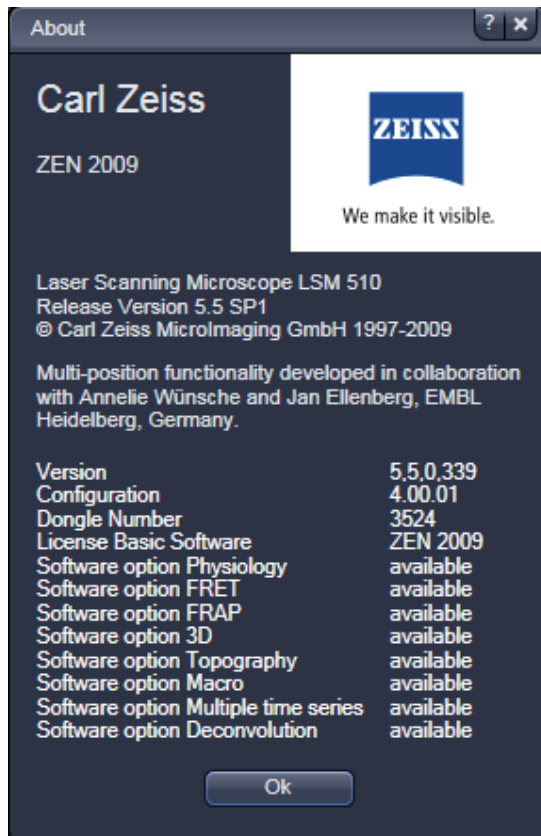
Fig. 4-268   About window

The panel hosts important information about the software version number, the license number and the available software modules.

4.9   Index

CHAPTER 5 MACROS AND VISUAL BASIC

CONTENTS

Page

5   MACROS AND VISUAL BASIC

5.1   Macros

The macro function permits the recording, running and editing of command sequences and their allocation to buttons in the **Macro** menu. Macros can be set up in two different ways: The Visual Macro Editor or alternatively they can be programmed in Visual Basic directly.

- In the **Menu** bar (see Fig. Fig. 5-1), click on **Macro.**
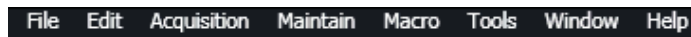
Fig. 5-1   Menu bar
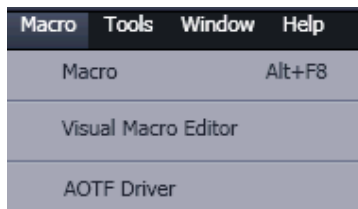
Fig. 5-2   Macro menu

– This displays the **Macro** menu (see Fig. Fig. 5-2).

- The Macro list is tripartite:**Macro** – editing and recording of macros; assign macros to list

- **Visual Macro Edito**r – edit automatic workflows with pre-defined modules

- List assigned **Macros** – start a specific Macro

Selecting **Macro** opens a dialogue for editing and recording Macros based on VBA programming in the **Macro Control** window (see Fig. 5-3). VBA programming is described in Section 5.2 of the printed manual.

Using the **Visual** Button (optional) allows to create automated work flows using the arrangement of symbols which depict the single steps within a work flow. See **Visual Macro Editor**, page 5-32 for further details.

5.1.1   Macro Language

"Visual Basic for Applications", called VBA in the following, is used as the Macro language. This language is well known and widely used as Macro language in the "Microsoft Word for Windows" and "Microsoft Excel for Windows" products. Experience with "Microsoft Visual Basic" would also be beneficial for macro-programming ZEN.

An Integrated Development Environment (IDE), the "Microsoft Visual Basic Editor", is available for editing and debugging of macros. The IDE includes an "online help" with a detailed description of the VBA language.

Macros are stored in project files. One project file can include several macros.

☞   **The VBA functionality described in this paragraph refers to VBA as implemented in the Carl Zeiss LSM software 4.2. With the introduction of ZEN, the range of functions is and will be extended, while VBA can still be used with the functions described below. The extension of functionality applies mostly to display and access of image data. If you need assistance or further information please contact your local Carl Zeiss LSM specialist**:

5.1.2   Macro Control

To open the Macro Control, choose **Macro** from the **Macro** List in the main **Menu Bar**.

This opens the **Macro Control** window.

5.1.2.1   Edit Macro Function

This function allows you to manage project data. Macros can be recorded, stored, performed, edited and, if required, deleted.

- Press the **Edit Macro** tab to switch to the **Macro** and **Recording** panels (see Fig. 5-3).
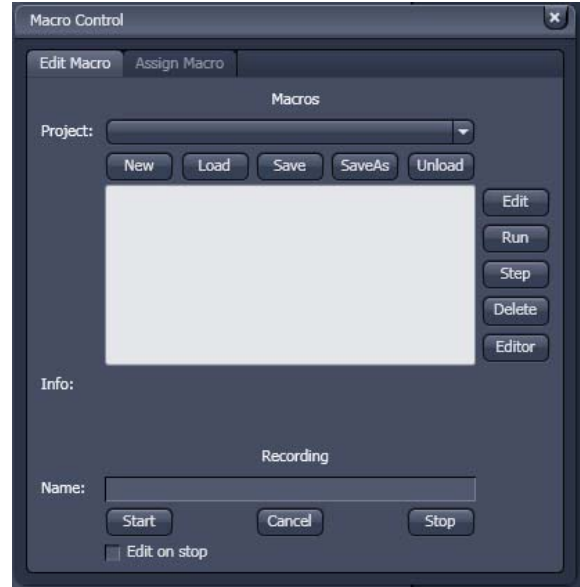
Fig. 5-3   Macro Control window

(1)   Macro panel

New button: Creates a new project.

Load button: Opens an existing project.

Save button: Stores the project on the hard disk.

Save As button: Stores an existing project under a new name.

Unload button: Removes the selected macro from the **Macros** list.

Edit button: Allows macros to be edited and debugged. The editor (**Microsoft Visual Basic**) is automatically located at the beginning of the relevant macro.

Run button: Runs a macro.

Step button: Opens the editor for line-by-line editing / debugging.

Delete button: Deletes the selected macro.

Editor button: Opens the editor. Displays the processed area of the macro edited last.
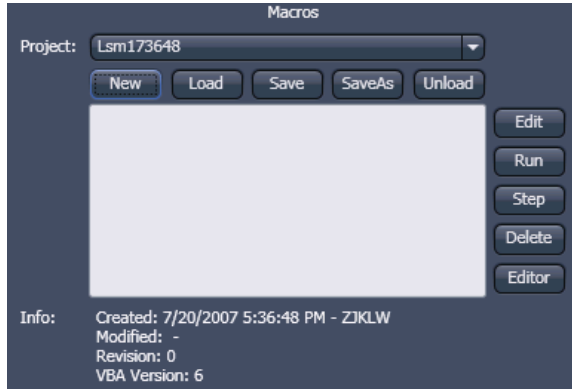
Fig. 5-4   **Macro panel with new Macro assigned**
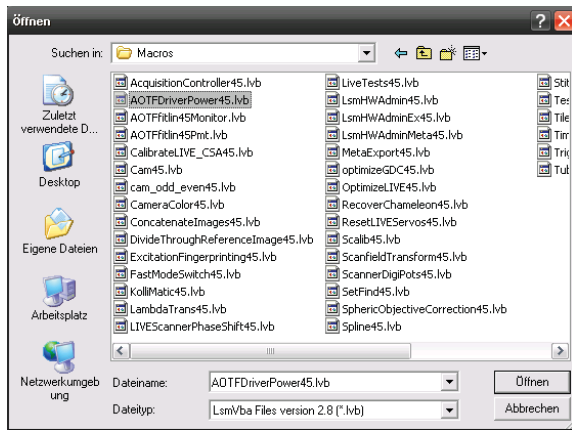
Fig. 5-5   **Open window**

Fig. 5-6   **Macro panel with existing Macro assigned**

Macros are stored and managed in project files (*.lvb). Before you can record or edit a macro, you have to create a project as follows:

- Press the **New** button to create a project file.

  – A new project is created and displayed in the **Project** selection box. The project name is automatically default (LSM followed by a number), but can be edited afterwards.

To activate an existing project, proceed as follows:

- Press the **Load** button.

  – The **Open** window will be opened (see Fig. 5-5).

- Select the relevant project file (data extension: ***.lvb**) from the **Macros** list box. Click on the **Open** button.

  – The project file will be opened and the macros contained in it are displayed in the **Project** selection box of the **Macro Control** window. You can use the pull down menu to load any listed Macro.

Recorded macros are stored in main memory first. Before the macros can be assigned to the list in the **Macro** submenu, the project must be stored on the hard disk.

- Press the **Save** button under the project name in the **Macro Control** window and determine the file name in the **Project** selection box, if required.

(2)   Recording panel

Before recording a command sequence, you can enter the name for the macro to be created in the **Name** input box of the **Recording** panel.

Start button: Starts recording.

Cancel button: Cancels the recording procedure.

Stop button: Stops recording.

☑ **Edit On Stop**:   On stopping the recording procedure, the macro editor is automatically opened at the relevant position.

Proceed as follows to record a macro:

- Enter a name for the macro to be created under **Name** in the **Recording** panel.

- Click on the **Start** button to start recording the macro.

Fig. 5-7   Recording section

- Then perform the operations to be stored, e.g.:

    – Click on the LSM **Find** action button. A Find scan will be performed.

    – Click on the LSM **New** action button. A new **Image Display** window will be opened.

    – Click on the LSM **Single** action button. A **Single** scan will be performed.

- Then click on the **Stop** button to end the recording. (**Cancel** enables you to cancel recording)

    – If recording was successful, the entered **Name** will then also be available in the **Macros** list box of the **Macro** panel. The new macro is automatically assigned to the current project. It is possible to assign as many macros as required to a project.

- Click on the **Save** button to store the new macro.

Proceed as follows to run a macro:

- Select the required macro from the **Project** list box of the **Macros** panel.

- Click on the **Run** button to start performing the macro.

☞   Provided that a macro is linked to a list in the **Macro** section of the **Menu Bar**, you only need to click on its name to perform it.

Proceed as follows to delete a macro:

- Select the required macro from the **Project** list box of the **Macros** panel.

- Click on the **Delete** button. The macro will be removed from the list.

Proceed as follows to edit a macro:

- Select the required macro from the **Project** list box of the **Macros** panel.

- Click on the **Edit** button. The Microsoft Visual Basic editing window will be opened.

- Make the required changes.

Fig. 5-8   **Macro Control window – Assign macro to button**

5.1.2.2   Assign Macro to the Macro list

This function permits stored macros to be linked to the assigned Macros list in **Macro** of the **Menu Bar**.

- Press the **Assign Macro** tab to switch to the **Assign Macro** panel (see Fig. 5-8).

Define Buttons panel

Proceed as follows to link a macro to the **Macro** list:

- Select the button number corresponding to the position in the list from the **Button** selection box.

- Enter the button labelling in the **Text** editing box.

- Select the name of the project file from the **Project** box using the **...** button.

- Select the macro name from the **Macros** box.

- Press the **Apply** button to assign the relevant macro to the specified list position in the **Macro** toolbar.

Delete a macro from the Macro list

Proceed as follows to delete the linking between a **Macro** list position:

- Select the button number from the **Button** selection box.

- Press the **Delete** button to delete the list entry.

5.1.2.3 Editing and Debugging of Macros

The **Edit** in the **Edit Macro** panel button activates IDE (Integrated Development Environment), which allows macros to be edited and debugged. Under the **Help - Macrosoft Visual Basics Help** menu item, IDE contains detailed "online" help on its operation and on the VBA macro language. Therefore, only a few hints are provided in the following:

You should activate the required toolbars. We would recommend you to activate the **Debug** toolbar via the **View - Toolbars -Debug** menu item.

The following buttons in the toolbar can help you when debugging macros:

▶	Starts running the command lines.
■	Stops running the command lines.
‖	Interrupts processing of the command lines (pause).
✋	Sets a breakpoint in the line with the text cursor.
⤵	Processes a command line and steps into subprocedures.
⤷	Processes a command line and steps over subprocedures.
⤴	Exits the subprocedure (step out).
👓	Displays the value of the marked expression (Watch). If nothing is marked, the value of the variable above the text cursor is displayed.
▦	Activates the Watch window in which values of variables and expressions can be displayed. For this, text is marked in the code window and dragged into the Watch window. Variables can be modified in the Watch window.

In the left-hand edge of the code window you will find an arrow beside the current command line. A new current command line can be determined by moving the arrow via the mouse. This makes it possible to skip command lines or to process command lines several times.

5.1.3 Overview of Available Macros

Documentation files (*.rtf, *.doc) of advanced macros will be located in the macro directory.

Name	Description
AOTFfitlin.lvb	Linearize laser attenuation (AOTF or mechanical)
AOTFDriverPower.lvb	Attenuate laser light by the AOTF by additional dampening factors
Autofocus.lvb	Automatic focusing according to a set configuration
CameraColor.lvb (also Button in Maintain)	Color balance of Axiocam HRc
Centerv.lvb	Centers the field of view around the position marked with the cross tool
ConcatenateImages.lvb	Enables the combination of all kinds of images irregardless of the size and data depth to be presented in a time series. Imported images can also be included. Type of concatenation (fitted to the smallest frame or ROI, fixed size or fitted to the largest frame) can be defined by the user.
ExcitationFingerprinting.lvb	Automated generation of excitation lambda stacks for generating excitation spectra which can be used for linear unmixing to separate overlapping dyes. Any detector can be used, also NDDs.Description as guide tour on the installation CD-ROM or DVD.
FRET plus.lvb	(Option) Enables the user to perform FRET experiments based on sensitized emission or acceptor photo bleaching (depending on the type of LSM) including commonly accepted analysis algorithms.
Kollimatic.lvb	Automatically generates the calibration matrix of a LSM 5 *LIVE* system for optimal confocal aperture and collimator settings
Lambdatrans.lvb	Time series alternating between lambda and transmission mode
LsmHWAdmin.lvb (also Button in Maintain)	Direct service hardware access (password protected)
LsmHWAdminEx.lvb	Calibration service macro (password protected)
ModifySeries.lvb	Modifies Z Stacks and Time Stacks like Rotation of the stacks, being mirrored, Conversion of time stacks into z-stacks and vice versa;
StitchArtPlus.lvb	StitchArt Plus macro (**Option**)
MultiTime.lvb	Set up of time series experiments including repeated imaging, bleaching and autofocusing with defined configurations at multiple locations and for various views at each location (**Software option**)

OptimizeLIVE.lvb	Temporarily adjusts the confocal aperture position of a LSM 5 *LIVE* without changing the general calibration values
RecoveryChameleon.lvb	Imitates recovery of chameleon NLO laser in case the laser is falling into CW lasing and does not get to mode lock anymore.
ScanfieldTransform.lvb	Adjusts LSM 510 or LSM 5 *LIVE* image match relative to each other or to LSM *DuoScan* ROIs
SphericObjectiveCorrection.lvb	Calibrates the spheric component of objective error by automatic acquisition of a Z stack on a flat surface and a spherical fit of the topographic data.
TestGrid.lvb	Projects a testgrid into the image window (password protected).
TileScanRotation.lvb	Defines rotation angle of scanners during tile scan

☞ During installation, default macros will be installed according to their type either inZEN\AZEN\HWT or ZEN\Macros\. Self generated Macros will be in ZEN\Macros.
In case of a new installation, old macros will be stored in ZEN\Macros\BackupMacros or ZEN\Backup\, to avoid problems with identical names of existing and new macros.

5.1.4   Sample Macros

The ZEN software package includes a set of basic macros free of charge

Other macros from the list (previous page) are part of the product range of Carl Zeiss MicroImagniing GmbH. Please contact your sales representative for a quote.

In the following paragraphs you find a short despcription of some representative and common macros. Please contact your local LSM specialist for more detailed and extensive information.

☞ **Please check the C:\ZEN\Macros folder on your system for separate documentation in the more extensive macros in .pdf format**.

5.1.5   Kollimatic

This VBA Macro is used to calibrate the LSM 5 *LIVE* on the current system configuration (e.g. objectives, filters, etc.)

Prerequisites

- System Warm-Up (at least 2 hours; for procedure please read the User's Manual) and environmental conditions according to setup requirements.

- "OptimizeLIVE.lvb" Macro: ensure both channel sliders set to 0

- Proper adjustment of collimators and Confocal Slit Aperture (CSA) 1 + 2 (this has to be done by the local Service Representative)

- AOTF RGB + V linearized properly with Macro AOTFFitlin.lvb

- All used objectives are stored in the database according their exact order in the nose piece.

- Necessary calibration slides are available
  1x Bead Slide 1 µm for dry objectives, **# 000000-1371-460**,
  1x Bead Slide 200 nm for immersion objectives, **# 000000-1371-461**);
  to be ordered from Carl Zeiss Jena.

Basic Functions of the Macro

The KolliMatic macro features two main parts, Basic Calibration and Calibration Matrix:

A - Basic Calibration

- Needs to be done only once with one of the recommended objectives (should be part of the shipment in order to be able to repeat the Basic Calibration without calibrating all objectives again (see objective list down below)

- Is for calibrating both collimators RGB + V

- Is for calibrating the Y-position of the Beam Shift Compensator (BSC) for all present NFT positions

☞   On currently delivered LSM 5 *LIVE* Systems, the Basic Calibration is done in the factory (This can be confirmed by checking that **BasicCalibrationLog.txt** is present in C:\ZEN\Bin).

Objective list (objectives are listed in order of most to least recommended for this calibration task)

- Plan Apochromat 20x / 0.8

- Plan Neofluar 40x / 1.3 Oil

- C-Apochromat 10x / 0.45W

- C-Apochromat 40x / 1.2W corr

- Plan Apochromat 5x / 0.16

- Plan Neofluar 10x / 0.3

- Plan Apochromat 63x / 1.4 Oil

- C-Apochromat 63x / 1.2W corr

- Plan Neofluar 20x / 0.5

B - Calibration Matrix

- Is based on the calibration results of the Basic Calibration

- Needs to be performed for each objective separately

- Is for calibrating the collimator position of both collimators RGB + V only for the selected Objective depending on:
  Laser Line, Zoom + EF Filter Position

- Is for calibrating the BSC Y-position depending on:
  Laser Line, Zoom + EF Filter Position

☞ On currently delivered LSM 5 *LIVE* Systems, the Calibration Matrix is set up in the factory for every objective belonging to the system (This is done together with the Basic Calibration).

Without running Calibration Matrix, the positions of the collimators and BSC Y should be ok. This is due to the Basic Calibration, but the results are not fully optimized.

Installation of the Macro:

If not already done, copy the required files to the following destinations:

File Name	Destination
KolliMatic.lvb	C:\ZEN\Macros
PotentialKollimaticConfig.ini	C:\ZEN\Bin
KolliMatic_Instructions.pdf	C:\ZEN\Macros

Starting the Macro:

- Start the LSM software and check first, if all prerequisites have been fulfilled
  (see section Prerequisites)

- Put the appropriate calibration slide (depending on current objective: immersion or non immersion) onto the microscope stage

- Activate all lasers, and using the and focus with the 488 nm line start scanning and focus onto the beads

- Put the appropriate calibration slide (depending on current objective: immersion or non immersion) on the microscope stage, start single scan

- Use the **Macro** menu of the main LSM software to **Load** and **Run** the Macro **KolliMatic** (see Fig. 5-9)

  Please wait a few seconds, the Macro will set up beam path and all necessary settings automatically.
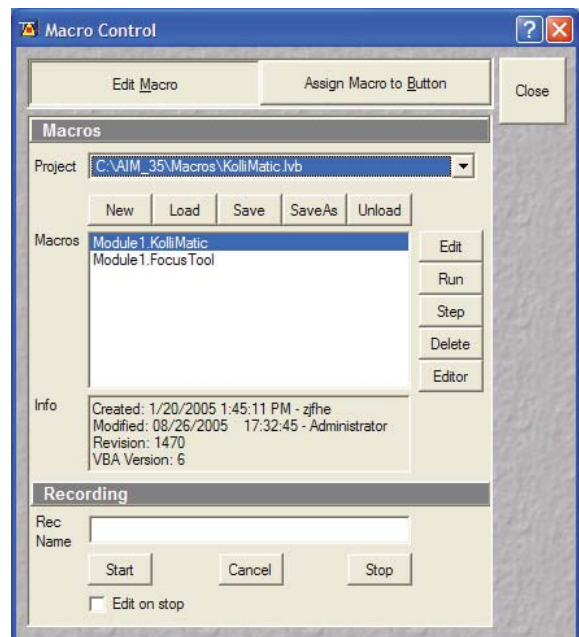
Fig. 5-9   Macro Control Window of the main LSM Software
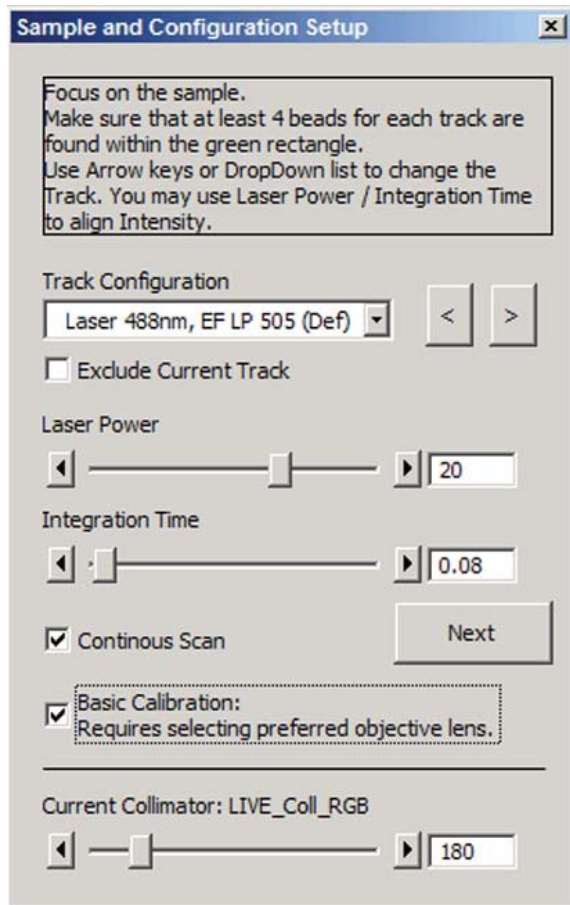
Fig. 5-10   **Figure Sample and Configuration Setup Window of the KolliMatic Macro**

Preparations for using the Calibration Slides:

- Follow the steps shown in the macro **Sample and Configuration Setup** window.

- Use the arrows or the drop down menu in order to select one track for each available laser line (no need to select several tracks for the same laser line), start with a track for the lowest wavelength laser (i.e. typically the 405 nm laser line.).

- Select an area of the bead sample that provides as many beads as possible, at least 4 (fit into the green rectangle)

- In Fig. 5-10, the Basic Calibration has been selected (Check box **Basic Calibration: Requires selecting preferred objective lens.**), therefore some more tracks are available

- Use the **Laser Power** as a first option in order to set the intensity, second option is **Integration Time**.

- In order to start / stop the scan, just select / deselect the check box **Continuous Scan**.

- Focus on the beads.

- Defocus with the **Collimator** slider until the beads become line shaped.

- Focus with the microscope focus drive to get the vertical lines as thin as possible (Fig. 5-11).

- Now use the **Collimator** slider to get a round spot out of the line (Fig. 5-12).

- Repeat these steps for a track with the 488 nm laser line.

- After one track for each laser line has been adjusted, click **Next**.
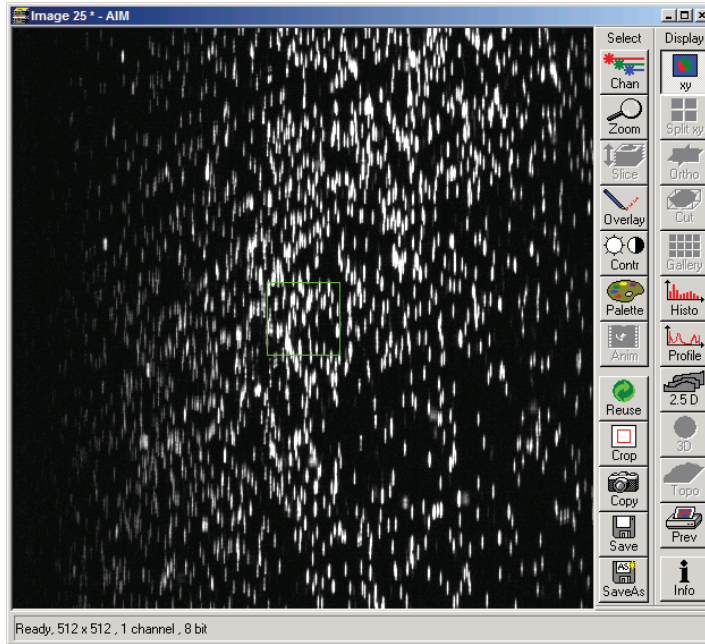
Fig. 5-11   Beads focused for maximal vertical thinness
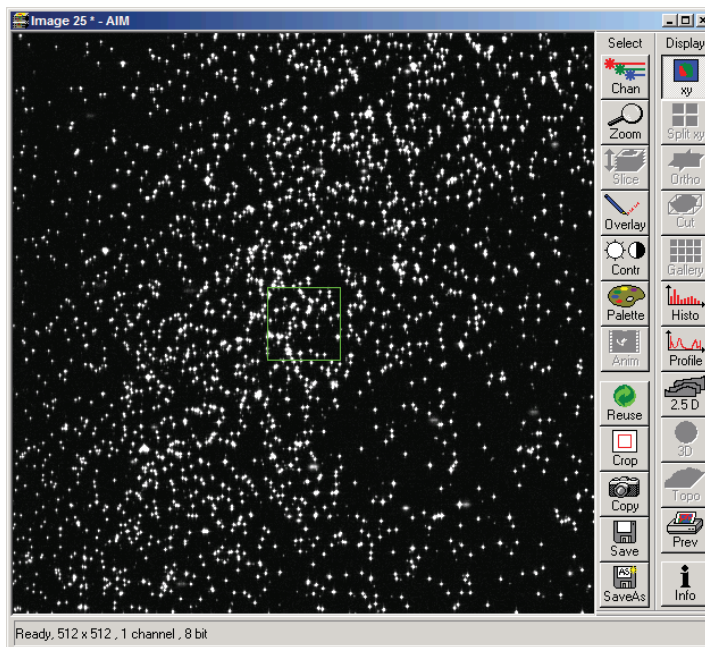
Fig. 5-12   Beads optimally focused

Running the Basic Calibration

☞ If you have selected the check box for **Basic Calibration**, please follow these instructions. In order to run the **Calibration Matrix** only, move on to Running the Calibration Matrix.
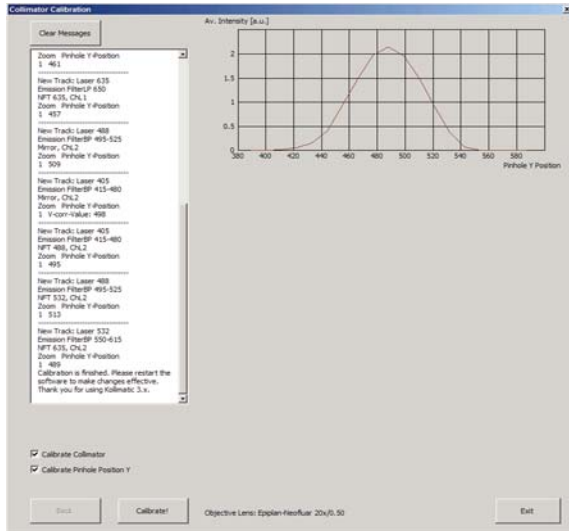
Fig. 5-13   **Collimator Calibration Window**

- Make sure that both check boxes have been selected (Fig. 5-13)

- Double check if the objective settings (shown at the bottom of the macro window) match the currently used objective

- Click the **Calibrate** button

- In order to follow the calibration process, just click into the **Message** window; a scroll bar will appear

- The **Basic Calibration** takes about 15 min

- The displayed curve should resemble a Gaussian profile

- After the macro has finished, a message will pop up with the request to restart the LSM software to make the changes effective.

- Press **Exit** and restart the LSM software

Running the Calibration Matrix

☞ If you haven't selected the check box for Basic Calibration, the **Calibration Matrix** will be selected automatically. In this case, when the Calibration Matrix is running, the Basic Calibration is disabled.

- This window provides three check boxes (Fig. 5-14)

- The recommended setting is to select **Create new calibration file.** All previous files will be deleted.

  Exceptions:

  No check box checked

  – Especially after an unexpected interruption of the program, it is possible to continue from the position where the process stopped (saving time).

  Overwrite Collimator values

  – The values for the chosen objective will be added or updated.

  – After selecting the collimator option, the Pinhole Y option will be selected automatically.

- Double check if the objective settings (shown at the bottom of the macro window) match the currently used objective.

- Click the **Calibrate** button.
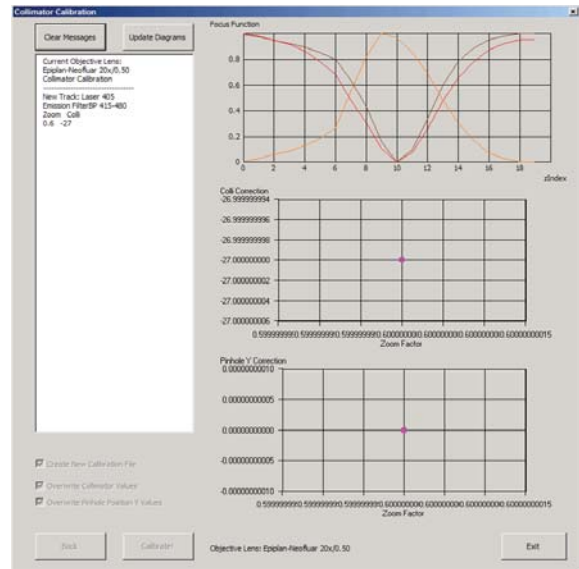
Fig. 5-14   Running the Calibration Matrix

Meaning of the Graph (Figure):

Brown: Focal plane of the CCD Line Detector

Red: Focal plane of the collimator

Orange: Estimated focal plane

The brown and red graphs should be close to this curve.
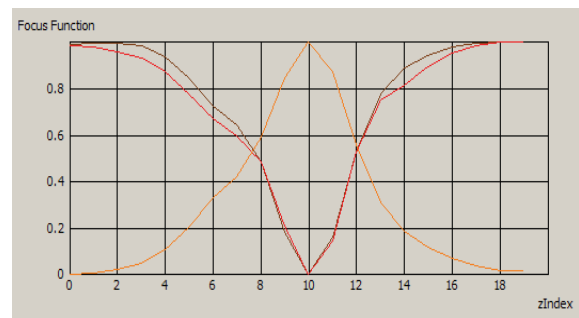
Fig. 5-15   Graph of Focus Function
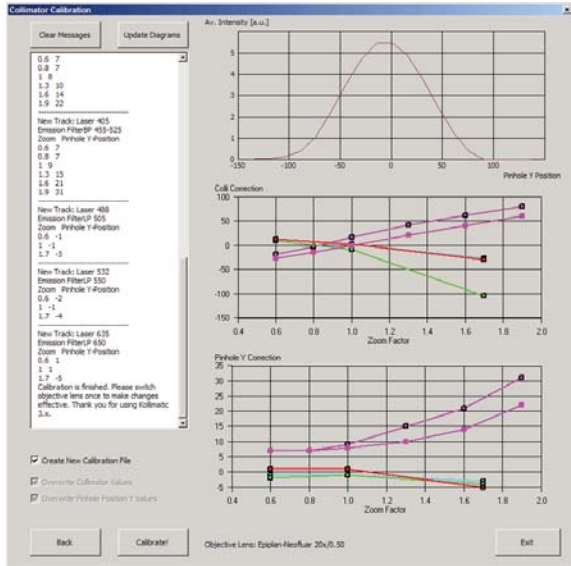
Fig. 5-16 **Collimator Calibration window after calibration**

- In order to follow the calibration process, just simply click into the Message Window and; a scroll bar will appear (Fig. 5-16).

- The Calibration Matrix takes about 25 min for each objective.

- After the macro has finished, a message will pop up with the request to switch the objective lens in order to make the changes effective.

- If necessary, repeat the procedure for the other objectives.

- Use the **Update Diagrams** button in order to check, if an objective has already been calibrated.

   - Select an objective, press the **Update Diagrams** button

   - If no graphs appear, this particular objective has yet needs to be calibrated.

- Click **Exit** to finish.

Meaning of the Graph (Figure):

   - Each coloured line represents an individual laser line.

   - Each coloured calibration point represents an emission filter.

   - Standard graphs are displayed with black points.

Meaning of the Colours:

   - <420 nm: → Pink

   - 421…475 nm: → Blue

   - 476…495 nm: → Turquoise

   - 496…550 nm: → Green

   - 551…590 nm: → Yellow

   - >590 nm: → Red

Important Information!

☞ If the calibration has been completed successfully with the Calibration Matrix, do no longer use the pinhole and collimator functionality of the LSM software in the **Maintain** menu.

Fig. 5-17   Optimize Macro

The use of the pinhole sliders to raise the quality of the images is well known from the other LSM Systems.

On the LSM 5 *LIVE*, only the **Optimize** sliders of the **OptimizeLIVE.lvb** macro have to be used (Fig. 5-17) to compensate for misalignments (e.g. temperature influences).
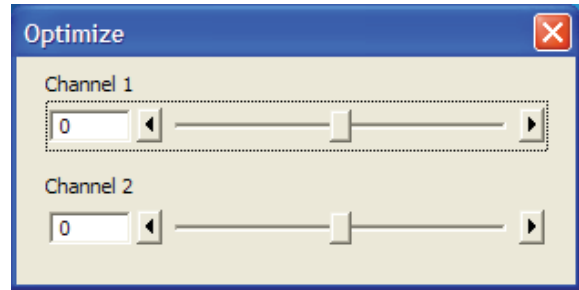
After the Calibration Matrix has optimized a particular objective, the results will be stored automatically in a file as shown below. These files can be found in C:\ZEN\BIN.

☞ Do not change any of the values!
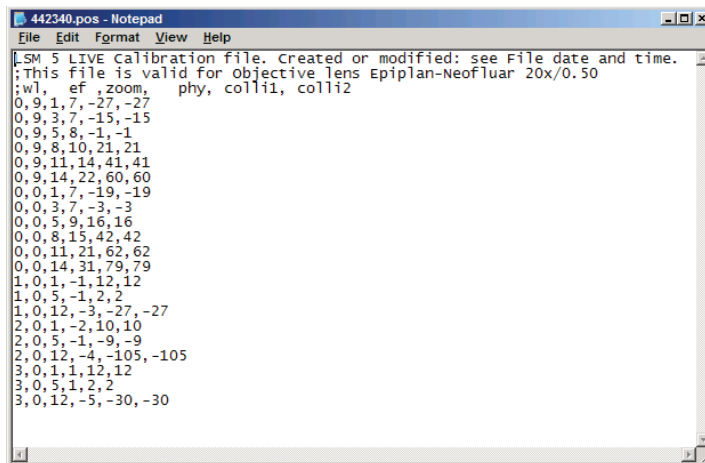
Fig. 5-18   Calibration file

```
BasicCalibrationLog.txt - Notepad
File  Edit  Format  View  Help
Current Objective Lens:
Epiplan-Neofluar 20x/0.50
Collimator Calibration
-----------------------------------
New Track: Laser 488
Emission FilterLP 505
Zoom    Colli
1    184
-----------------------------------
New Track: Laser 405
Emission FilterBP 415-480
Zoom    Colli
1    257
Current Objective Lens:
Epiplan-Neofluar 20x/0.50
Pinhole Y-Position Calibration
-----------------------------------
New Track: Laser 488
Emission FilterLP 505
Plate, ChL1
Zoom    Pinhole Y-Position
1    458
-----------------------------------
New Track: Laser 488
Emission FilterLP 505
NFT 488, ChL1
Zoom    Pinhole Y-Position
1    459
-----------------------------------
New Track: Laser 532
Emission FilterLP 550
NFT 532, ChL1
Zoom    Pinhole Y-Position
1    461
-----------------------------------
New Track: Laser 635
Emission FilterLP 650
NFT 635, ChL1
Zoom    Pinhole Y-Position
1    457
-----------------------------------
New Track: Laser 488
Emission FilterBP 495-525
Mirror, ChL2
Zoom    Pinhole Y-Position
1    509
```

Fig. 5-19   BasicCalibrationLog file

The calibration results of the Basic Calibration will be saved in a file named **BasicCalibrationLog.txt** (to be found in the BIN-Folder as well).

☞   Changes won't effect the stored values.

5.1.6   OptimizeLIVE Macro

For temporary optimization of the LSM 5 *LIVE* beampath, try to avoid a modification of the general adjustment in the pinhole dialogue. Use the **Optimize** slider instead. The general setting of the confocal aperture might still be correct for other scan parameters.

The Optimize slider can be set independently for each channel. The slider is moved to the one or the other side while scanning continuous. The brightest image result is the optimal setting. This setting does not affect the general adjustment of the LSM 5 *LIVE* collimator and confocal aperture, but can enhance image brightness efficiently.
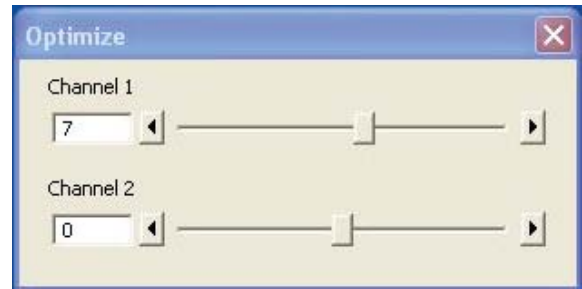
Fig. 5-20   Optimize window

5.1.7   Image Match (Scanfield Transformation) Macro

Adjusts the Image match between two scanning imaging systems or a manipulation and a scanning imaging system.

To adjust the image match, set up a multitracking that creates one channel for each scanning and/or manipulation system. In case of the LSM *DuoScan*, use a T-PMT to create an image with the *DuoScan* system.

- Start the Image Match (Scanfield Transformation) Macro.

- Use the Image match calibration sample delivered with the system. This sample has a fluorescent layer and a reflective grid, useful to create images in fluorescent, reflective or transmission light mode.

- Start continuous scan, and set laser power and gain to appropriate levels. The point scanning system will be the speed limiting system, so choose a fast scanning speed to get a faster image refresh.

- Use unidirectional scan to avoid adjustment errors due to bidirectional image jitters.

- Set the matching zoom factors with one of the **Match DuoScan zoom/offset** or **Match LIVE zoom/offset** buttons relative to each other. It is recommended to adjust the LSM zoom.
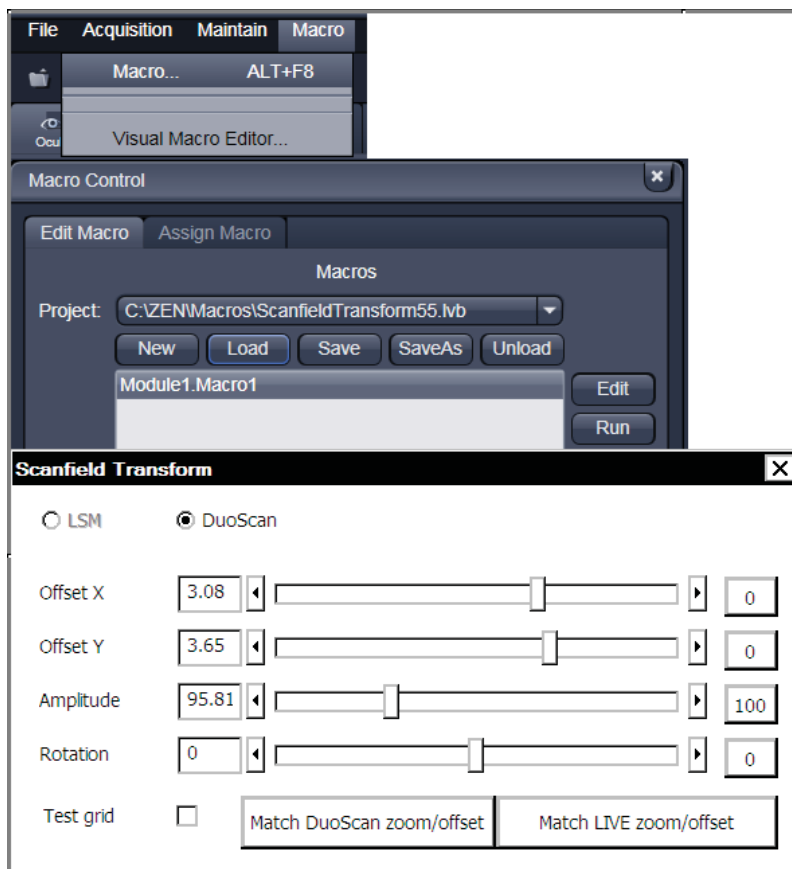
Fig. 5-21   Scanfield Transform window showing after slecting Macro…, loading and running the corresponding macro "ScanfieldTransform55.lvb"

These buttons set the two scanheads at a matching scanfield diameter, according to the following table (LSM 5 *LIVE* = Zoom 1.0):

Microscope stand	LSM 5 *LIVE DuoScan*	LSM 5 *DUO*
Inverted (port config)	(SP/RP) *DuoScan* = Zoom 1.4142	(RP/SP) LSM 510 = Zoom 1.33978
Upright (port config)	(Tube/RP) *DuoScan* = Zoom 1.4142	(RP/Tube) LSM 510 = Zoom 1.25295

- Choose split image for the image window display. You will see the scanned areas of both scanning systems and an overlay.
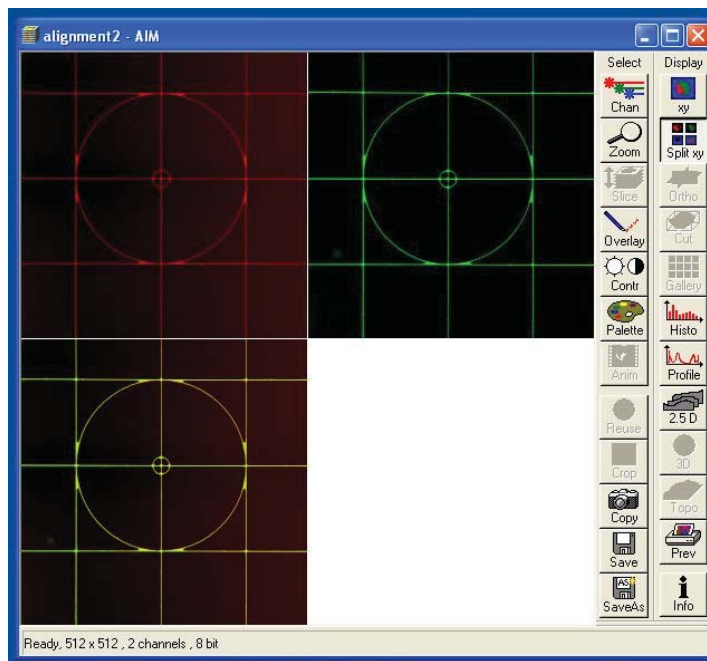
Fig. 5-22   Split image

- Choose LSM (510) or *DuoScan* to adjust the image match.

- While scanning, move the sliders of the Image match macro to get a perfect overlay of the scanning and/or manipulation system relative to each other.

- Offset X/Y adjusts the horizontal and vertical image shift

- Amplitude adjusts a size difference between the images

- Rotation compensates for a tilt in one of the images.

☞   Store the final image, and use the ReUse function for future image matching. The Multitrack setup and the Zoom factors will then be set automatically.

5.1.7.1 StitchArt plus Macro

Purpose of the StitchArt plus macro

The **StitchArt plus** macro is useful for the acquisition of larger profiles and larger fields of view than a single scan field could cover. It is applicable in reflection mode confocal imaging only.

With the StitchArt plus software profile lengths, which are required in standard tactile roughness evaluation, could be performed (for instance: 12.5 mm profile length with 10x objective, 4.0 mm profile length with 20x objective, 1.25 mm with 50x and 100x objective).

9 x 9 stacks   **3.6 mm x 3.6 mm**

Single scan field size   **Epiplan Neofluar 20x/0.5**
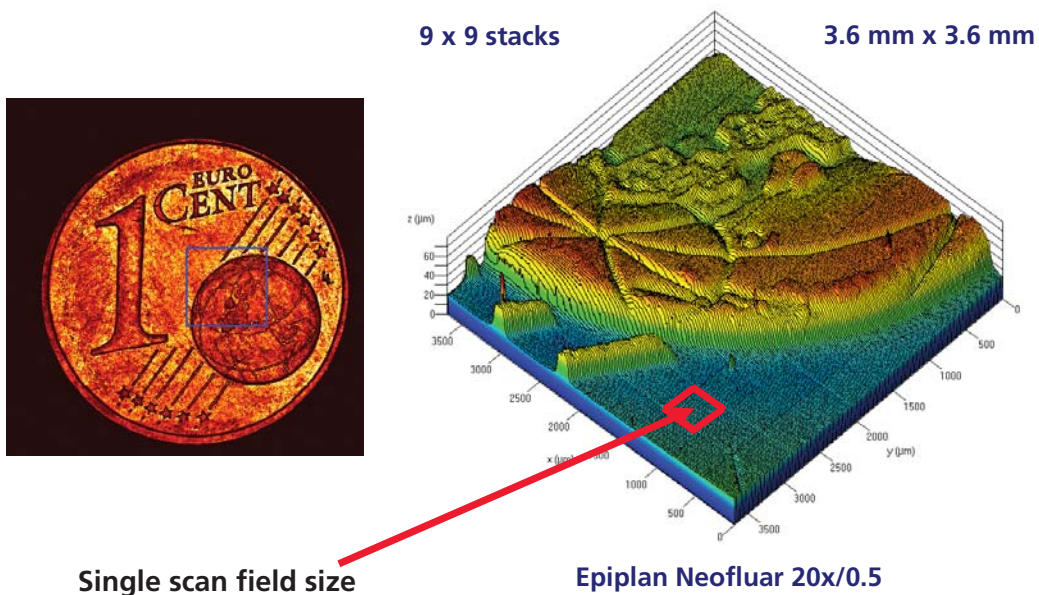
Fig. 5-23   StitchArt plus macro

General requirements

- The system and especially the lasers have to be in operation for at least one hour.

- The motorized XY scanning stage has to be oriented as accurate as possible perpendicular to the direction of the optical axes.

- The parfocality adjustment of all objectives has to be performed and saved before running the macro.

- The pinhole has to be adjusted properly on a plane mirror or on the sample itself.

- The calibration of the objective magnification in X and Y has to be performed (An image of an XY calibration standard or another known length scale has to be taken and measured with the system in X and Y direction. The ratio between the measured and the real length value has to be calculated. The objective magnification has to be corrected with this factor and put in the objective list as a new objective).

- Pinhole diameter and amplifier offset have to be optimized by a prescan. They are automatically taken from the **Channels** tab of the **Scan Control** window.

(1) Loading the StitchArt plus macro

- Click on the **Macro** button 1.

- Click on the **Macro** button 2.

  – The **Macro Control** window opens.

- Click on the **Load** button in the **Macro Control** window.

- Load the macro from **ZEN/Macros/StitchArt-plus.lvb**.

- Click on the **Run** button in the **Macro Control** window.

(2) Starting the StitchArt plus Macro

- Click on the **Macro** button.

- Activate the **StitchArt plus** button.

  – The **Profile & Stack Stitching** window opens.
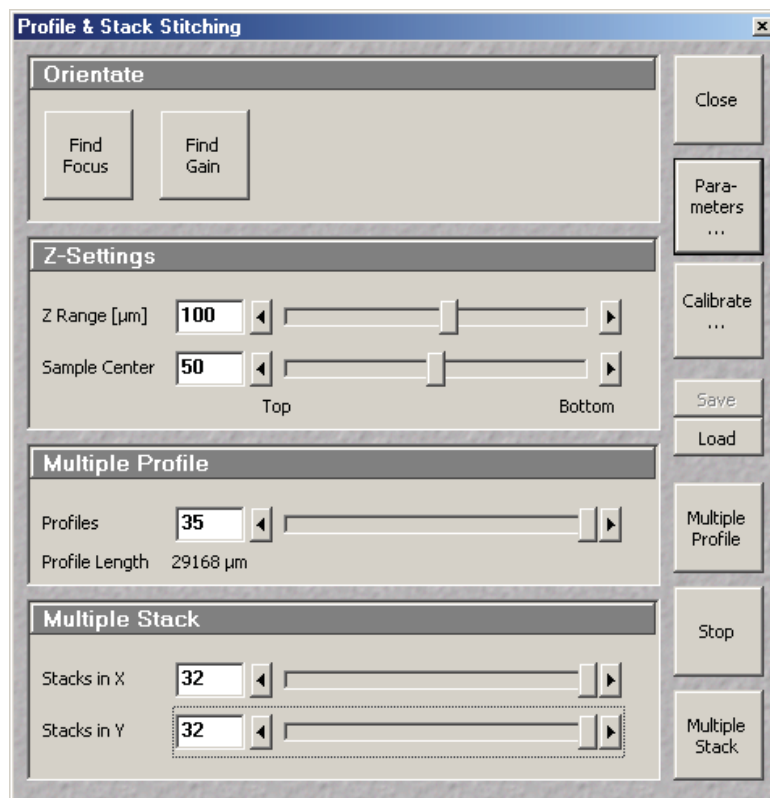
Fig. 5-24   Profile & Stack Stitching window

Orientate panel

Find Focus button   The objective chosen in the **Multiple Profiles Parameters...** window is performed and a fast line scan over Z is acquired. The **Z range** of this line scan is 0.5 mm, the single Z step 300 nm. The stage Z Position is set to the height of the major maximum intensity position.

Find Gain button   When clicking on the **Find Gain** button a **Find** procedure is performed on the current **Z slice**.

Z-Settings panel

Z Range [μm]   This parameter defines the total **Z height** a **Multiple Profile** and/or a **Multiple Stack** is performed. **Z range** values could be changed between 20 microns and 4 mm (if working distance of the objective will permit this, otherwise an error message appears on the screen.

    ☞   Because in **Multiple Profile Scan** the single Z step could only be varied from 100 … 600 nm and the maximum number of Z slices is limited to 2000, the overall Z travel distance of the stage is also limited to 200 … 1200 μm, respectively.

Sample Center [%]   This button defines whether the actual Z position of the stage is rather placed on top or on the button of the materials surface.

Multiple Profile panel

Profiles   The number of single profiles which has to be acquired, can be varied between 1 and 35. (Depending on the overlap total maximum size is restricted to less than 32k).

Total Profile Length   The calculated total profile length is displayed

Multiple Stack panel

Stacks in X   The number of stacks in X dimension can be varied between 1 and 32.

Stacks in Y   The number of stacks in Y dimension can be varied between 1 and 32.

Multiple Profile button

If you click on the **Multiple Profile** button, the stage moves out of the center position of the sample and is moved to the left-hand side to perform a first single profile. After that an interim image is performed before and after the next step of the motorized XY stage. A cross correlation of the two profiles (Z) and the two (not displayed) images (lateral) is performed to assure an accurate profile match online during the experiment.

    ☞   If you acquire the **Multiple Profile Scan** in **Range Indicator** palette the results of the correlation could directly be observed as "blue boxes".

Select subprofiles from Multiple Profile data

After acquisition of a **Multiple Profile** it is displayed as an XZ projection.

By variation of the **Z:XY ratio** slider on the right hand side of the image window the Z:X ratio could be changed.

- Click on the **Overlay** button in the main display window and define a region with **Rectangular Frame** button in the **Overlay** menu.

- Click on the **Extract Region** button in the **Overlay** menu. (For control the masked region is displayed in blue colour in the range indicator mode.)

Mask out Multiple Profile data

After acquisition of a **Multiple Profile** it is displayed as an XZ projection.

- Click on the **Overlay** button and define a region with the rectangular frame.

- Click on the **Cut** button. (For control the masked region is displayed in blue color in the range indicator mode.)

Multiple Stack Button

If you click on the **Multiple Stack** button, the stage moves out of the center position of the mould (lens) and is moved to the upper left corner to perform a first single stack. After that all other stacks are acquired successive neighbors is performed.

☞   If a **Multiple Stack** scan is stopped before the regular end of the experiment, no correlation will be performed.

Stop Button

If you click on the **Stop** button the acquisition is stopped immediately and the stage position returns to the initial XY center position.

Close Button

If you click on the **Close** button the **Profile & Stack Stitching** window is closed.

(3)   Setting Parameters for Profile & Stack Stitching

- Click on the **Parameters ...** Button in the **Profile & Stack Stitching** window.

    – The **Parameters** window opens.

☞   Pinhole diameter and receiver offset are applied as defined in the **Channels** tab of the **Scan Control** window of the LSM 5 software.
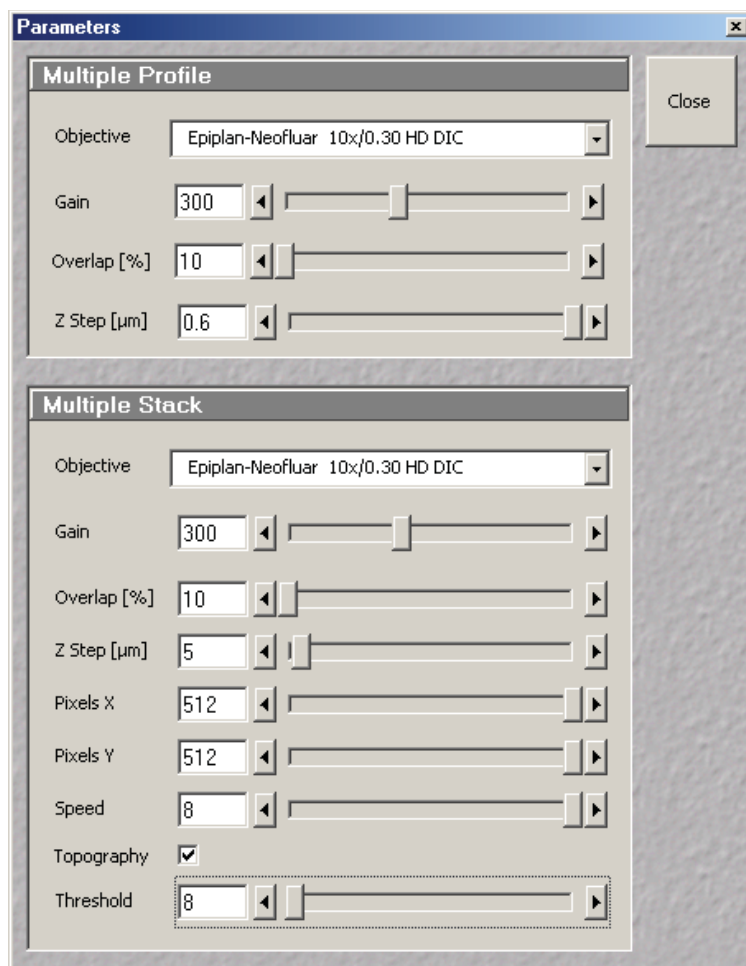
Fig. 5-25   Parameters window

Multiple Profile panel

In the Multiple Profile panel the parameters necessary for the **Multiple Profile** procedure can be defined.

Objective   An objective has to be chosen from the currently used objectives of the stand.

Gain   The detector gain can be modified. If the **Find Gain** procedure has been applied the optimized gain value is displayed. This value can be changed by use of the slider or by clicking on the FORWARD / BACKWARD buttons.

Overlap [%]   The relative overlap between single profiles can be adjusted between 10 % and 50 % of a single profile length. For more pronounced surface features a smaller overlap is sufficient, for rather smooth surfaces larger overlap values are recommended.

Z step [μm]   The single Z step in the **Multiple Profile** Mode can be varied between 0.1 μm and 0.6 μm in 0.1 μm steps.

Profile data:

Maximum format:	16384 pixels x 2048 slices
Variable overlap:	10 ... 50 % of single profile length
Scan Speed:	8 (fixed)
Scan time:	20 ... 210 seconds (depending on Z)
Height difference DZ:	0.1 ... 0.6 μm [in steps of 0.1]
Total height Z:	50 μm ... 1 mm
Auto alignment:	Cross-correlation in X, Y & Z

Multiple Stack panel

In the **Multiple Stack** panel the parameters necessary for the **Multiple Stack** procedure can be defined.

Objective	An objective has to be chosen from the currently used objectives on the stand.
Gain	The detector gain can be modified. If the **Find Gain** procedure has been applied the optimized gain value is displayed. This value can be changed by use of the slider or by clicking on the FORWARD / BACKWARD buttons.
Overlap [%]	The relative overlap between single stacks can be adjusted between 10 % and 50 % of the single image length. For more pronounced surface features a smaller overlap is sufficient, for rather smooth surfaces larger overlap values are recommended.
Z Step [μm]	The Z step between the single slices of a stack can be defined between 0.1 and 100 microns.
Pixels X	The X pixel resolution of a slice of a single stack can be varied between 4 and 512 Pixels.
Pixels Y	The Y pixel resolution of a slice of a single stack can be varied between 4 and 512 Pixels.
Speed	The scan speed of the **Multiple Stack Scan** mode can be varied between scan speed 5 and 8 (see **Scan Speed)**.
Topography	This checkbox determines whether the result of the **Multiple Stack Scan** is treated as Multiple intensity stack array or in an reduced XYZ topography format. If this tickbox is disabled all treatments like for a single confocal image stack can be applied. If the tickbox is enabled, only topography based operations could be performed. This **Multiple Stack** experiment needs much less storage when performing the topography based experiment. But operations like orthogonal projections and intensity profiles are not any more available.
Threshold	This intensity threshold has to be chosen before performing a **Multiple Stack** experiment in a topography based format and could not be changed therafter.

Adjustment functionality:

Find Focus:	Autofocus by fast Z line
Find Gain:	Auto Brightness & Contrast
Adjust scan mirrors:	To XY direction of MOT stage
Adjust spherical objective error:	On a plane mirror

☞ Macro VBA programming is described in Chapter 5.2 of the printed manual.

(4)   Calibrating Routine

- When clicking on the **Calibrate** button in the **Profile & Stack Stitching** window the **Calibrate** window opens.
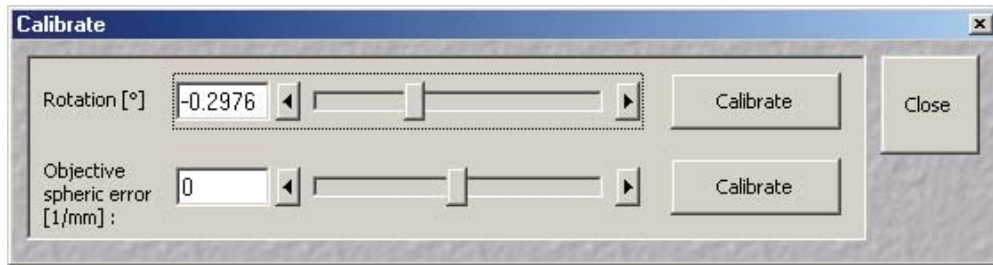
Fig. 5-26   Profile & Stack Stitching window

Calibrate Rotation Routine

With this calibration routine the direction of the scanning mirrors are adjusted to the axes of the motorized XY scanning stage.

For the **Calibrate Rotation** routine a sample with a pronounced structure in the center of the scan field has to be used. This should be a non-periodic structure, not a periodic pattern with a high spatial frequency.

The stage is moved a single step in X direction, an image before and after the step is taken. By correlation of the 2 images the scan field orientation is matched to the stage coordinate system (XY). The rotation angle between both systems is displayed.

☞   This function is also available independently from the **StitchArt plus** option.

- Load the macro from ZEN/Macros/TileScanRotation.lvb

Calibrate Objective Routine

With this calibration routine the objective error of first order (spherical component) is determined and corrected. This routine has to be performed when accurately measuring radii.

For the **Calibrate Objective** routine a sample with a pronounced structure in the center of the scan field has to be used. We recommend the mirror sample provided with your system or a flatness standard. Try to bring the surface of the flatness sample in focus.

- Press the **Calibrate** button.

  – An image stack of 50 slices in 100 nm steps is acquired (centered around current position). After the topography reconstruction a 3D sphere fit is applied. The correction factor 1/R is displayed in 1/mm. This calibration is only valid for the current objective position. With a change of the objective position this calibration has to be applied for the new objective, first.

☞   This function is also available independently from the **StitchArt plus** option.

- Load the macro from ZEN/Macros/SphericObjectiveCorrection.lvb

(5)   Saving the images

The acquired **Multiple Profiles** and intensity based **Multiple Stacks** can either be saved as image stack based data or as topography based data.

☞   If the **Multiple Stack** data are saved in an intensity stack based format, correlation is performed only in X- and Y- direction, **NOT** in Z-direction.

If the **Multiple Stack** data should be saved in the topography based format, the **Topography** checkbox in the **Parameters** window has to be activated and an intensity threshold has to be chosen before the experiment will be started.

☞   Try to perform a single stack first to find the optimal acquisition settings and the appropriate lower intensity threshold for topographic reconstruction.

☞   In the topography based experiment the data are correlated in X-, Y- and Z-direction.

- The **Save** button in the **Profile & Stack Stitching** window saves topography based **Multiple Stacks** experiments in two file formats.

(a)   XYZ-data file without correlation results (*.inf / .dat)

The topography data file (.dat) contains the height data in 32-bit IEEE floating-point format. The file has no additional header. All data of the first tile are stored in the file first then followed by the data of the second tile and so on. The tile resolution and size information is stored in the .inf file.

The height values (32-bit IEEE floating-point format) are stored in the range 0…1 where the value 0 indicates that no height value has been determined for the pixel due to low intensity. All other values can be multiplied with the **RangeZ** entry from the *.inf file to obtain the metric height value.

(b)   Information file with size of Multiple Stack Scan and automated correlation results (*.inf)

The information file (.inf) is formatted like a MS-Windows ini-file with sections and entries.

Section [Image information]:

Entries:

ImageSizeX ImageSizeY	The horizontal and vertical number of pixels for the tiles.
TilesX TilesY	The horizontal and vertical number of tiles that have been acquired
VoxelSizeX VoxelSizeY	The horizontal and vertical pixel to pixel distance in meter.
RangeZ	The factor that has to be applied to the floating-point height data in the data file to obtain metric height data.
Overlap	The relative fraction of the overlap region for adjacent tiles that has been used to acquire the data in the range 0…1. The value 0.5 indicates that half of the tiles overlap.

The other sections contain offsets for the individual tiles.

The section name has the format [Tile(xxx,yyy)] where xxx is the zero based horizontal index of the tile and yyy is the zero based vertical index of the tile.

Entries:

TileOffsetX TileOffsetY	The horizontal and vertical offset of the left-top corner of the tile relative to the left-top corner of the result topography in pixels. These offset values have been calculated with cross correlation from the original acquired stacks.
TileOffsetZ	The height offset for the data in the tile in meters. Note that the tile data already contain height corrected data and the macro writes always the value 0. By a multiple manual correction already manually corrected data could be more easily distinguished from uncorrected data. The value can be modified in the file and the **Load** command will use this value to shift the tiles in z-direction.

(6)   Loading Multiple Stack files

Multiple Stack scan *.inf files can be loaded in the **StitchArt plus** software.

5.1.8   Visual Macro Editor

The Visual Macro Editor is an optional software module which allows the user to program a variety of scanning procedures, image calculations or image processing routines as well as the combination of these functions.

5.1.8.1   Open / Close the Visual Macro Editor Window

- Choose the **Visual Macro Editor** from the **Macro** menu in the Main Menu Bar.

- Click on the [Close] button in the upper right side of the window to quit.
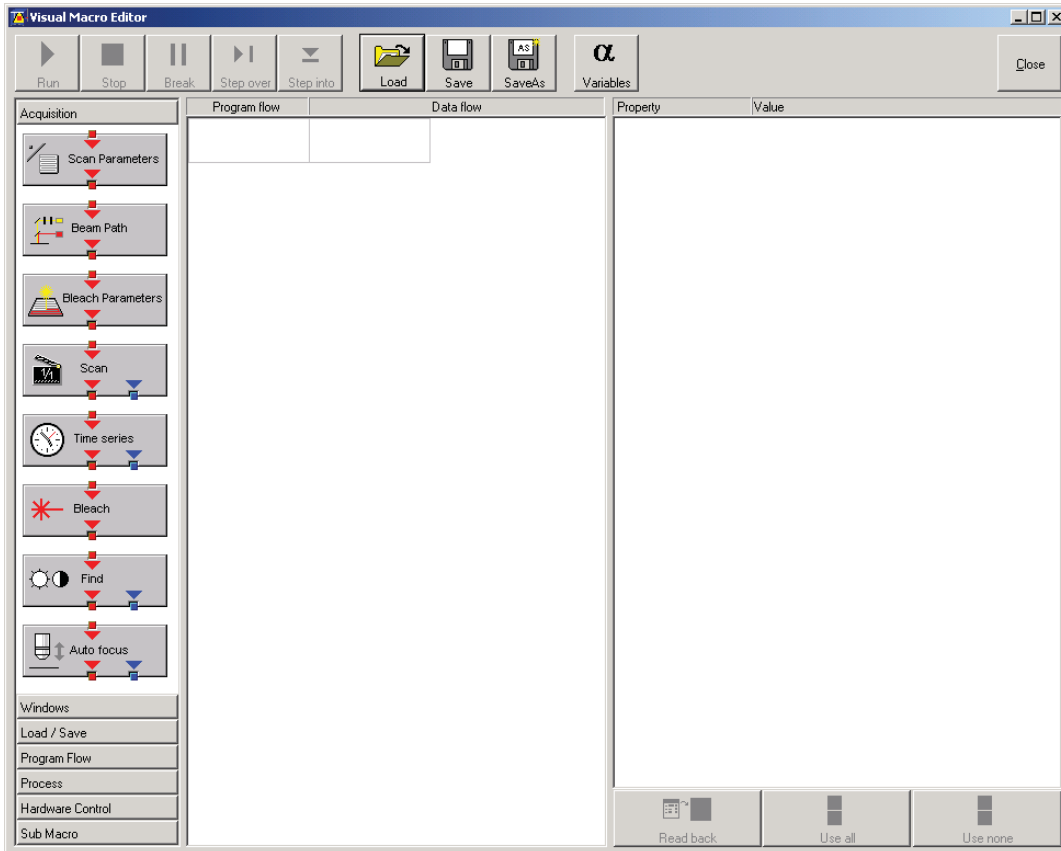
Fig. 5-27   Visual Macro Editor window

5.1.8.2 Function Description

Toolbar buttons

Run	Starts the macro starting with the first block in the program flow column.
Stop	Stops the macro at any time.
Break	Interrupts the macro. Clicking the button again will resume the actual program flow of the Macro.
Step over	Runs a sub-macro and stops at the first action block in the main macro.
Step into	Stops at the first action block in a sub-macro.
Load / Save / Save as	Use **Load** to open an existing macro and **Save** to store the macro**. SaveAs** allows storing the same macro under a different name.
Variables	By clicking the button the list of defined variables is displayed. New ones can be added by typing in name and value in the next free line. Variables are assigned to macros. For each new macro a new list of variables has to be generated.

The editor uses predefined action blocks. The action blocks for setting up the work flow of the macro are categorized according to their properties. The following categories are displayed on the left-hand side of the **Visual Macro Editor** control window.

- Acquisition
- Windows
- Load/Save
- Program Flow
- Process
- Hardware Control
- Sub Macro

- Click on one of the category selection buttons will display the available action blocks.

  The action blocks can be positioned according to their function either in the **Program Flow** column or the **Data Flow** column. The color of the connection arrows of the action block defines the possibility to position the block in the program flow column or the data flow column(s).

- Click at the action block with the left mouse button (hold down the mouse button) and drag the mouse cursor into the flow column. Release the mouse button.

  - Action blocks for the program flow show red connection arrows ▼ as well as blue output arrows ▼ to connect to the data flow.

  - Action blocks for the data flow show blue connection arrows ▼ or green output arrows ▼ to connect back to the program flow.

☞   It is not possible to drag an action block for program flow into the data flow or an action block for data flow into the program flow.
Action blocks can be removed using the **Delete** key of the keyboard.

☞   A block is highlighted by clicking onto the block icon. To highlight more than one block drag the cursor over the block icons to be marked. A click on the right mouse button also allows the use of the functions **cut**, **copy**, **paste**, and **delete** to modify a macro.

☞   Without using the **Image Display** action block in the data flow column, the result of the scan will not be displayed (see also description of **Scan** and **Time Series** action blocks).

The work flow is generated by connecting the action blocks using the mouse cursor.

- Click onto an output arrow with the left mouse button (hold down the mouse button) and drag the mouse cursor away.

    – A connecting line appears.

- Drag the line to the input arrow of another action block. Only arrows of the same color can be connected.

According to the action blocks a set of properties is listed which can be assigned with free or predefined values. These values can also be variables. A set of defined properties is assigned to one block only. If this block is used more than once the properties can be set differently.

Clicking the **Read Back** button will take over the current settings from the LSM main program for the displayed properties list.

- Mark the check boxes next to the parameters individually or, click **Use all** or **Use none** to check or uncheck all parameters with one click.

- For changing value click into the **Value** column.

It is possible to define variables. A variable has a (property) name and a mathematical value. The **Assignment** action block can be used to assign a new value to a variable when inserted into the program flow using mathematical algorithms. The new value is then used for any subsequent calculation in the program flow where the variable is part of the calculation.

- Click the **Variable** button on the toolbar to display the variable list. Click once more to display the parameter list again.

- New variables can be added by typing in name and value in the next free line.

Acquisition Action Blocks

Loads a Beam Path Configuration form the Configuration list or uses the current one that is set up in the main program. Use the pull down menu in the **Property/Value** list to select the Configuration that should be used for imaging. Changing between Single Track and Multi Track allows to choose between the different Configuration lists. Multi Track also lists tracks that are used for Emission Fingerprinting or Online Fingerprinting. Depending on the type of track different settings can be loaded optionally. If they are not loaded and the parameters list of the Scan Parameter block does not offer them as being editable, for example detector gain and offset, default values will be used. Read back will use the actual parameters of the main program.
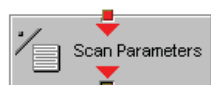
The list of properties lists all parameters that can be adjusted for the scan procedure. The value for each parameter can be typed in individually. Clicking the **Read Back** button will take over all current settings from the main software. The check boxes next to the parameters can be marked individually or, clicking **Use all** or **Use none** they can be all checked or unchecked with one click. Only the parameters with a mark in the check box will be taken into account and adjusted when the Scan Parameter block is set in the program flow column. This allows to change only some of the parameters, for example the zoom, at a certain position within the program flow as for each block the check boxes can be set.
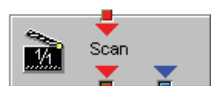
This block performs the scanning of the sample according to the settings chosen with Beam Path and Scan Parameters. It can be linked to the Data flow column. If no connection to the data flow is made (for example: Display Image) the result of the scan will not be displayed.

The parameters for bleaching the sample can be loaded from the list of bleach settings. Bleach settings are assembled and stored in the Bleach control window of the main software.

This block performs the bleaching of the sample according to the bleach settings that have been defined in the Bleach Parameters block.

The list of properties lists all parameters that can be adjusted for the acquisition of the time series.The value for each parameter can be typed in individually. Clicking the **Read Back** button will take over all current settings from the main software. The check boxes next to the parameters can be marked individually or, clicking **Use all** or **Use none** they can be all checked or unchecked with one click. Only the parameters with a mark in the check box will be taken into account and adjusted when the Time series block is used in the program flow column. This allows to change only some of the parameters at a certain position within the program flow, for example the trigger used, as for each Time series action block the check boxes can be set individually. Activating the Bleach parameter will bleach the sample according to the settings in the Bleach Parameters action block during the time series. The Time series action block can be connected to the data flow column.

This block performs the **Find** function according to the main software.

This action block sets the Z-position according to the focusing procedure set with the parameters available. The image generated displays the image or image series aquired to set the focus position. The following properties are available to define the auto focus function:

– Mode/Precise: Uses fast XYZ-scan(s); the scan parameters can be defined via Scan Parameters Action Block

– Mode/Fast: Uses fast XZ-scan(s)

– Method/Intensity: auto focus mode which searches for maximum intensity.

– Method/Contrast: auto focus mode which searches for maximum contrast.

– Range (µm): The system will perform the auto focus search within that range.

– Offset (µm): Defines the distance between the focus plane set by the auto focus function (e.g. coverslip reflex) and the focal plane for imaging.

– Assign position to: Chose a variable to which the new position of the focus plane set by the auto focus function (e.g. coverslip reflex) should be assigned.

– Assign offset to: Chose a variable to which the offset value should be assigned.

Windows Action Blocks

This block has to be used if the actually scanned image(s) should be displayed. For each Image Display block a new image window is opened. If not saved, the image will be overwritten when the program flow is passing by again.

Load / Save / Action Blocks

The image(s) are stored to an image database. The database taken is either the last one, that was open or the current open one. Clicking Read back updates the database to be used for storing the image(s). In addition some other parameters can be set according to the list of properties displayed.

Within the program flow already existing images can be loaded.

The database taken is either the last one, that was open or the current open one. Clicking Read back updates the database to be used to load the image(s) from. The images can be loaded by Index or by the name of the image.

It is possible to export images choosing a certain file format from the pull down list that appears when highlighting the line File Format in the properties list. Specific parameters are assigned to each file format which can be set for the image export.

Images can be imported within the program flow. The image to be loaded can be selected by typing in the file path. The import of single images or an image series is possible. The images to be imported as series must have the same name and must be numbered in ascending order.

Program Flow Action Blocks
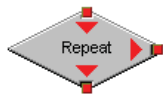
If any action(s) should be repeated a defined number of times, the Repeat block should be set into the loop of the program flow. The number of repeats is set in the properties list. The repeat is only performed using the connection of the right arrow. After finishing the repeats, the program flow connected to the arrow pointing down is performed.

Using this block in the program flow allows to assign new values to a selected variable using mathematical calculations which can be typed into the value line. All predefined variables can also be used for calculations. By passing this block the variable will be transiently changed and the new value can for example be used to assign different numbers to the image name or to increase the time delay during a time series acquisition.
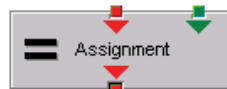
This block assignes a new value to a variable which has to be selected from the drop down list. The value assigned results from a calculation taken from the statistics action block (see below).
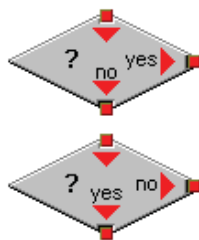
The two blocks are used as decision makers. Depending on the value of a predefined variable, the program flow can be directed to one of the two possible directions. The expression of the variable's value which should be used to decide on the program flow is set in the properties list individually for each used decision making block. It takes the actual transient value of the variable into account.

This block introduces a delay in the program flow. The delay time is started when the block is reached within the program flow.

The block allows to assign a potential error message with a textbox and to decide on the Error where to direct the program flow. If no textbox is assigned the program flow will just continue according to the presence of an error message. Choosing a textbox requires the input of the user to continue with the program flow.

Process Action Blocks
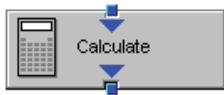
This block allows to do image calculation using the pixel intensities of the image. The operators for the formulas that can be used are described in the Ratio Dialogue within the Process Menu. The formulas set up in the Image calculation window can be copied and pasted into the properties list of the Visual Macro Editor.
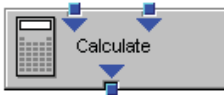
For calculations using two different sources the Calculate block with two possible inputs is used. The calculation starts when both inputs have arrived. Within program loops the next calculation will start when the inputs have arrived again in the same sequence as the first time. The operators for the formulas that can be used are described in the Ratio Dialogue within the Process Menu. The formulas set up in the Image calculation window can be copied and pasted into the properties list of the Visual Macro Editor.

The input image will be copied to a new image format that can be defined by changing the values of the listed properties.

Using this block enables to copy one image into another with the coordinates and settings put into the properties list. The destination image is connected to the left input; the source image is connected to the right input.

The subsequently arriving images will be concatenated according to the coordinate setting and treated as one image. A subsequent image display will be updated each time a new image arrives.

The second concatenate action block allows to concatenate images from two different acquisition blocks.

The block uses an input image (left input) and choses a region within that image according to a mask (right input) that has been defined in the Mask action block (see below). Within that mask region of a selected channel, one of the following result values can be selected which is then assigned to the chosen variable in the corresponding assignment action block:

– Mean intensity: Equals mean intensity within mask region

– Standard deviation: Equals standard deviation of intensity distribution within mask region

– Number Pixels: Number of pixels that constitute the mask region

– Area (µm x µm): Area of mask region

– Minimum: Minimum intensity value found within mask region

– Maximum: Maximum intensity value found within mask region

This action block defines a mask (image region with defined properties) derived from an image window. The image can be a newly acquired or imported image. The properties of the mask based on the image data can be selected according to the following properties:

- Scan regions(s): The mask is based on the scan region(s) defined in the **ScanControl**.

- Bleach region(s): The mask is based on bleached region(s) defined in the **BleachControl**.

- Image overlay: The mask is based on image overlay regions.

- ROI list entry: The mask is based on ROIs listed in Edit ROI control window.

- Interactive overlay: The mask is defined using overlay regions which are defined while the macro is executed.

- Interactive histogram: The mask is defined via histogram settings (min/max) interactively defined while the macro is executed. This works in conjunction with ROI selection (interactive or stored) as described above.

- Region index: Index of ROI (i.e. ROI number) if for example multiple bleach regions are active.

- Channel: Image channel which is used to derive the mask parameters from. This works in conjuction with interactive settings or loaded images.

- Intensity range: Defines the intensity range which is applied for mask definition.

- Lower threshold: Defines lower intensity threshold which is applied for mask definition. This threshold can be an absolute number or a floating point.

- Upper threshold: Defines upper intensity threshold which is applied for mask definition. This threshold can be an absolute number or a floating point.

Hardware Control Action Blocks

If a motorized stage is available the movement of the stage can be set up as part of the program flow. The stage can be moved to an absolute position which can also be taken from the actual stage position using the Read back function or typed in individually. Using a relative position will use the current stage position as relating position. The values have to be typed in.

It is also possible to choose the position from the **Mark and Find** position list which must have been set up in the stage control menu to be available.

The movement of the focus can be set up as part of the program flow. The focus can be moved to an absolute position which can also be taken from the actual focus position using the Read back function or typed in individually. Using a relative position will use the current focus position as relating position. The values have to be typed in. For large travel ranges which exceed half of the objectives working distance a warning can be displayed.

Sub Macro Actions Blocks

This block allows to set a sub macro within a main macro. The sup macro is opened by clicking onto the arrow in the block icon and closed clicking the ⊠ top right of the program flow column. The number of program flow in- and outputs as well as the number of program flow in- and outputs (= number of available connection arrows indicated on the block icon) can be defined for each sub macro. Multiple connections are therefore possible. The maximum of inputs and outputs for each flow is ten.

Using this block allows to integrate macros or macro procedures programmed with visual basic into a VME macro. The number of data flow inputs and outputs can be set to a maximum number of ten. The name of the macro indicates the name within the VME macro. The file name refers to the path and name of the VBA macro to be loaded into the VME macro. If no procedure available within the VBA macro is selected the whole VBA macro will be executed.
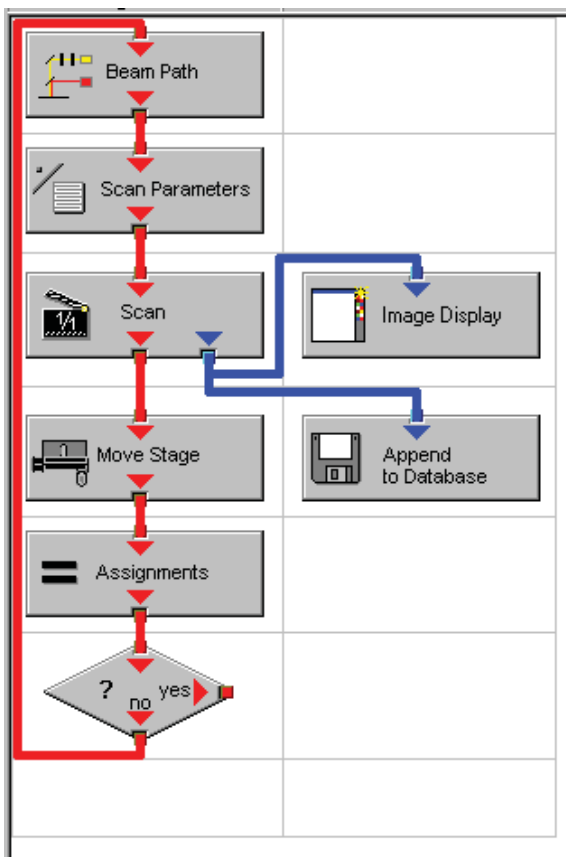
5.1.8.3 Editing a Macro

Following Example shows a macro which acquires five subsequent images and moves the stage 10 microns in X and Y following each image. The images are stored and numbered in ascending order.

- Arrange and connect the action blocks (Fig. 5-28).

Fig. 5-28   **Arrangement of action blocks**

- Assign the value 1 to a variable named Index (Fig. 5-29).

Property	Value
Index	1

Fig. 5-29   **Assignment of the variable**

- Click the single action blocks and assign the values to the listed properties (beam path and scan parameters can be chosen according to the sample).

Property	Value
Configuration Type	Single Track
Configuration	488 FITC
Load Speed	No
Load Bits per Sample	No
Load Pinhole Diameter	No
Load Gain and Offset	No

Fig. 5-30   **Property list of the Beam Path action block**

Use	Property	Value
✔	LSM Pixels X	512
✔	LSM Pixels Y	512
✔	LSM Step Y	1
✔	LSM Zoom	1.600000
✔	LSM Rotation [°]	45.000000
✔	LSM Offset X [µm]	0.000000
✔	LSM Offset Y [µm]	0.000000
✔	LSM Pixel Period [µs]	2.560000
✔	Camera Mode	Mono 1300 x 1030
✔	Camera Pixels X	1300
✔	Camera Pixels Y	1030
✔	Camera Offset X [Pixels]	0.000000
✔	Camera Offset Y [Pixels]	0.000000
✔	Pixels Z	1
✔	Scaling Z [µm]	1.000000
✔	Offset Z [µm]	0.000000
✔	Bits per Sample	8 bit

Fig. 5-31   **Property list of the Scan Parameter action block**

- The database the images will be appended to is automatically displayed when highlighting the **Append to Database** action block. The last one used will be taken. To update this, open the desired database and use the **Read back** function. Type in a name for the images with or without quotation marks. All images will get the same name then. For numbering the images use quotation marks for the name and add the variable Index, which has been assigned with the value 1. This value or the transiently changed value of the variable will be added to the name of the image.

Property	Value
Database	D:\Projekte\Release 3.5 RT\SW\Scripting\test 2.mdb
Name	"Cell culture" + Index
Description	
Notes	
Compress	Yes

Fig. 5-32   **Property list of the Append to Database action block**

Property	Value
Mode	Relative Position
Distance X [µm]	10
Distance Y [µm]	10

Fig. 5-33   **Property list of the Move stage action block.**

Property	Value
Index	Index + 1

Fig. 5-34   **Property list of the Assignments action block**

Property	Value
Expression	Index > 5

Fig. 5-35   **Property list of the Decision action block**

- The movement of the stage is defined highlighting the Move stage action block, chosing the Mode Relative Position in the Property list and typing in the value 10 for X and Y which then will move the stage 10 microns into X and Y direction starting with the current stage position on passing that action block.

- Now the first complete scan has been performed and the image is stored. For the next cycle the variable Index has to get another value to make sure the numbering of the images works. Using the **Assignments** action block, the Variable Index is chosen and the value assigned is **Index + 1**. Such the variable will get the transient value 2 for the next cycle, the value 3 for the over next cycle and so on.

- The number of repeats can be defined using a Decision block and using the value of the variable as base for the decision. By defining that if the Expression of the variable is larger than 5 and not connecting the output arrow marked with 'yes' to any other block, the macro will stop after the acquisition of five images. Alternatively one can use at this part of the program flow the Repeat block and define the number of repeats to be performed, which would be 5 in this case (The first scan is already counted!)

5.2 VBA PROGRAMMING FOR LSM

5.2.1 VBA Programming

5.2.1.1 General Syntax

Object oriented programming

Class Modules

5.2.1.2 Working with Files

I/O operations, file operations are implemented in VBA

Use of the implemented functions

Example:

```
Type CALIBRATION_PARAM
    lambda As Long
    angle As Double
    Translation As Double
End Type

Function DataFromCalibFile(strGetFile As String, calibArray() As CALIBRATION_PARAM) As Integer
    Dim hFile As Long
    Dim strLine As String
    Dim varData(1 To 3) As Variant
    Dim paramCnt As Integer

    paramCnt = -1
    'get the next free file handle
    hFile = FreeFile
    'check if file exist
    If (Len(Dir(strGetFile)) > 0) Then
        Open strGetFile For Input Access Read Shared As hFile
            If (Not EOF(hFile)) Then
                Line Input #hFile, strLine

                paramCnt = 0
                Do Until EOF(hFile)
                    Input #hFile, varData(1), varData(2), varData(3)
                    calibArray(paramCnt).lambda = varData(1)
                    calibArray(paramCnt).angle = varData(2)
                    calibArray(paramCnt).Translation = varData(3)
                    paramCnt = paramCnt + 1
                Loop
            End If
        Close #hFile
    End If

    DataFromCalibFile = paramCnt
End Function Objektstruktur
```

Use of the API function for additional functionality

5.2.1.3 Access to Windows API Function and external DLLs

- with Type Libraries

OLE

- with Declare Statements

API Text Viewer

searches WIN32API.TXT
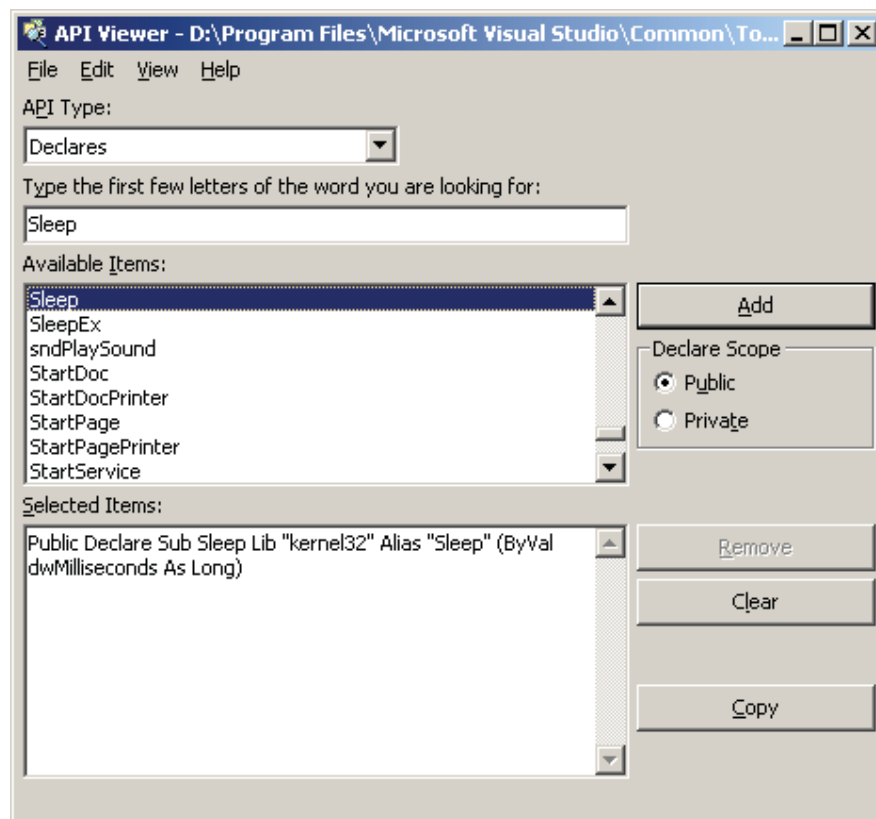
Fig. 5-36 **API Viewer window**

Example:

```
Declare Sub Sleep Lib "kernel32" (ByVal dwMilliseconds As Long)

Declare Function RegOpenKeyEx _
    Lib "advapi32.dll" Alias "RegOpenKeyExA" _
    (ByVal hKey As Long, ByVal lpSubKey As String, _
    ByVal ulOptions As Long, ByVal samDesired As Long, _
    phkResult As Long) As Long


Public Declare Function RegCloseKey _
    Lib "advapi32.dll" (ByVal hKey As Long) As Long


Public Declare Function RegQueryValueEx _
    Lib "advapi32.dll" Alias "RegQueryValueExA" _
    (ByVal hKey As Long, ByVal lpValueName As String, _
    ByVal lpReserved As Long, lpType As Long, _
    lpData As Any, lpcbData As Long) As Long          ' Note that if you declare the lpData  parameter
    as String, you must pass it By Value.


Public Const HKEY_CLASSES_ROOT = &H80000000

Public Const SYNCHRONIZE = &H100000
Public Const READ_CONTROL = &H20000
Public Const STANDARD_RIGHTS_READ = (READ_CONTROL)
Public Const KEY_QUERY_VALUE = &H1
Public Const KEY_ENUMERATE_SUB_KEYS = &H8
Public Const KEY_NOTIFY = &H10
Public    Const    KEY_READ    =    ((STANDARD_RIGHTS_READ    Or    KEY_QUERY_VALUE    Or
KEY_ENUMERATE_SUB_KEYS Or KEY_NOTIFY) And (Not SYNCHRONIZE))

Public Const REG_SZ = 1                    ' Unicode null terminated string
Public Const ERROR_SUCCESS = 0&
```

```
Function FServerFromDescription(strName As String, strPath As String) As Boolean
    Dim lngResult As Long
    Dim strTmp As String
    Dim hKeyServer As Long
    Dim strBuffer As String
    Dim cb As Long
    Dim i As Integer

    FServerFromDescription = False

    strTmp = VBA.Space(255)
    strTmp = strName + "\CLSID"
    lngResult = RegOpenKeyEx(HKEY_CLASSES_ROOT, strTmp, 0&, KEY_READ, hKeyServer)

    If (Not lngResult = ERROR_SUCCESS) Then GoTo error_exit
    strBuffer = VBA.Space(255)
    cb = Len(strBuffer)

    lngResult = RegQueryValueEx(hKeyServer, "", 0&, REG_SZ, ByVal strBuffer, cb)
    If (Not lngResult = ERROR_SUCCESS) Then GoTo error_exit

    lngResult = RegCloseKey(hKeyServer)
    strTmp = VBA.Space(255)
    strTmp = "CLSID\" + Left(strBuffer, cb - 1) + "\LocalServer32"
    strBuffer = VBA.Space(255)
    cb = Len(strBuffer)
    lngResult = RegOpenKeyEx(HKEY_CLASSES_ROOT, strTmp, 0&, KEY_READ, hKeyServer)
    If (Not lngResult = ERROR_SUCCESS) Then GoTo error_exit

    lngResult = RegQueryValueEx(hKeyServer, "", 0&, REG_SZ, ByVal strBuffer, cb)
    If (Not lngResult = ERROR_SUCCESS) Then GoTo error_exit
    strPath = Left(strBuffer, cb - 1)
```

```
lngResult = RegCloseKey(hKeyServer)
hKeyServer = 0

i = Len(strPath)

Do Until (i = 0)
    If (VBA.Mid(strPath, i, 1) = "\") Then
        strPath = Left(strPath, i - 1)
        FServerFromDescription = True
        Exit Do
    End If
    i = i - 1
Loop

error_exit:
    If (Not hKeyServer = 0) Then lngResult = RegCloseKey(hKeyServer)
    hKeyServer = 0

End Function
```

5.2.2   Programming for LSM

5.2.2.1   Object structure
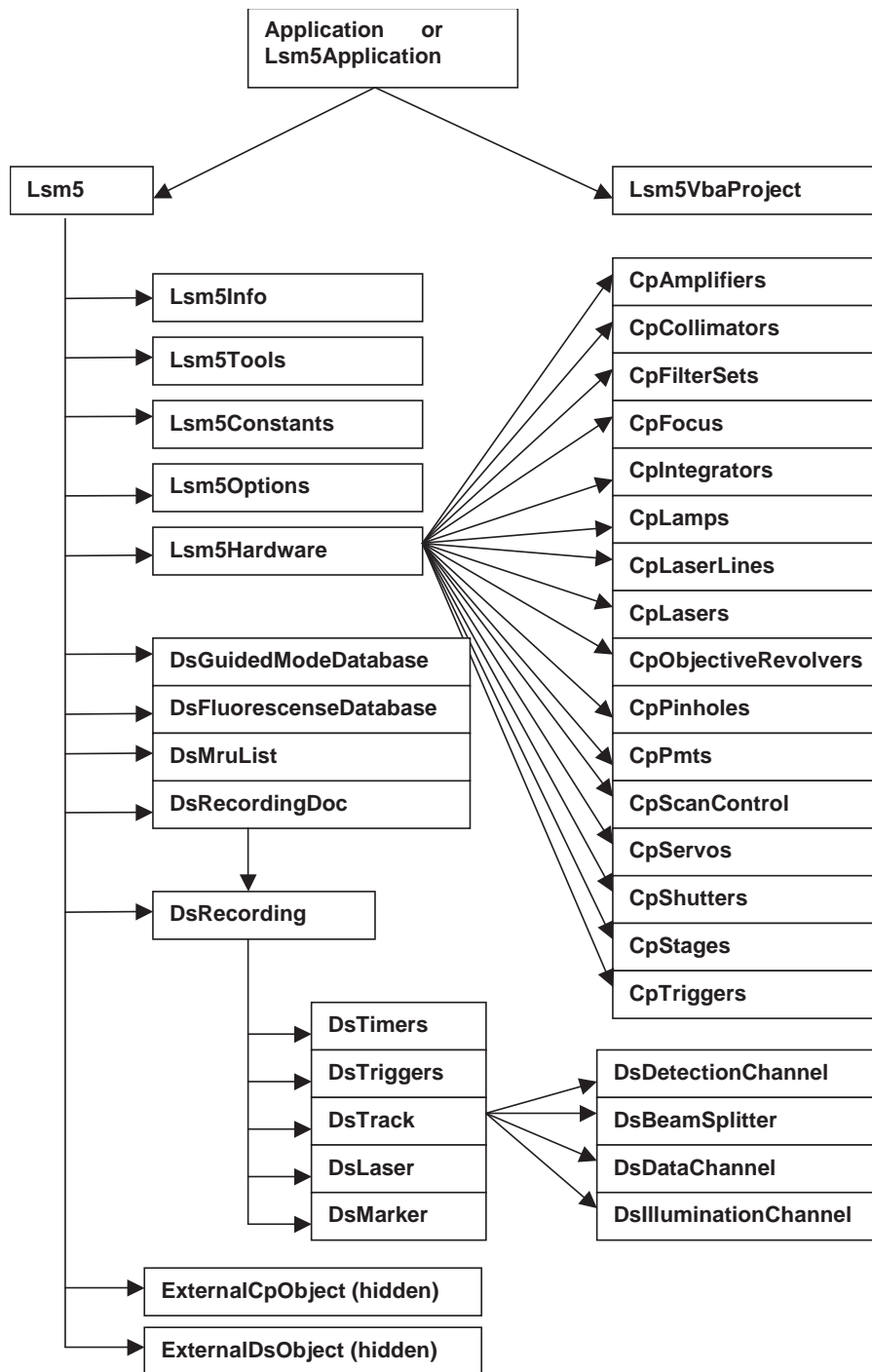
Fig. 5-37   Object structure

To search the object structure, use the object browser.
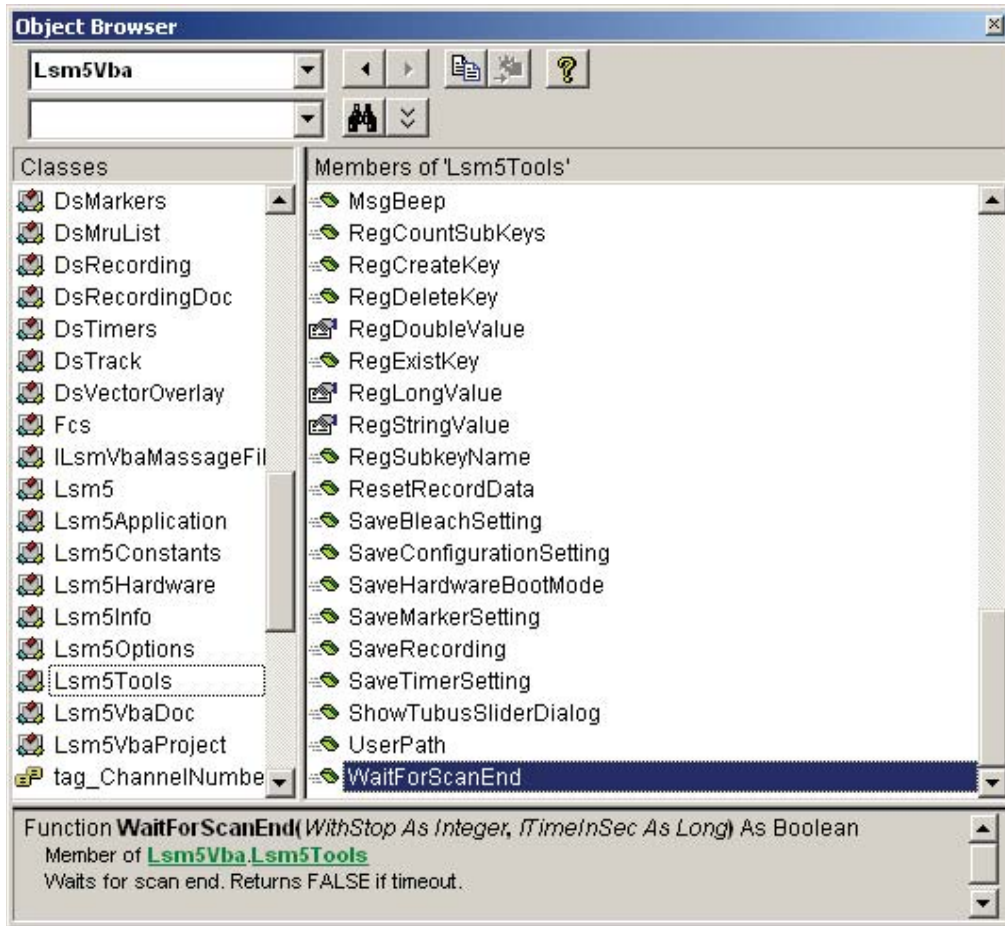
Fig. 5-38   Object Browser window

5.2.2.2 Differences in access to LSM Hardware with LSM 5 Hardware Object / DsRecording Object

Object Browser Project / Library LsmVba auswählen und Objekt Struktur untersuchen

- Access with DsRecording Object

Status maintains at Scan Start

```
Public Sub CopyRecording(Destination As DsRecording, Source As DsRecording)
    Dim TS As DsTrack
    Dim TD As DsTrack
    Dim DataS As DsDataChannel
    Dim DataD As DsDataChannel
    Dim DetS As DsDetectionChannel
    Dim DetD As DsDetectionChannel
    Dim IlS As DsIlluminationChannel
    Dim IlD As DsIlluminationChannel
    Dim BS As DsBeamSplitter
    Dim BD As DsBeamSplitter
    Dim lT As Long
    Dim ll As Long
    Dim success As Integer

    Destination.Copy Source
    Destination.Objective = Source.Objective
    For lT = 0 To Destination.TrackCount - 1

        Set TS = Source.TrackObjectByIndex(lT, success)
        Set TD = Destination.TrackObjectByIndex(lT, success)

        TD.Collimator1Position = TS.Collimator1Position
        TD.Collimator2Position = TS.Collimator2Position

        For ll = 0 To TD.DataChannelCount - 1
            Set DataS = TS.DataChannelObjectByIndex(ll, success)
            Set DataD = TD.DataChannelObjectByIndex(ll, success)
            DataD.ColorRef = DataS.ColorRef
        Next ll
```

```
For ll = 0 To TD.DetectionChannelCount - 1
    Set DetS = TS.DetectionChannelObjectByIndex(ll, success)
    Set DetD = TD.DetectionChannelObjectByIndex(ll, success)
    DetD.Filter1 = DetS.Filter1
    DetD.Filter2 = DetS.Filter2
    DetD.DetectorGain = DetS.DetectorGain
    DetD.AmplifierGain = DetS.AmplifierGain
    DetD.AmplifierOffset = DetS.AmplifierOffset
    DetD.PinholeDiameter = DetS.PinholeDiameter
Next ll

For ll = 0 To TD.IlluminationChannelCount - 1
    Set IlS = TS.IlluminationObjectByIndex(ll, success)
    Set IlD = TD.IlluminationObjectByIndex(ll, success)
    IlD.Acquire = IlS.Acquire
    lD.Power = IlS.Power
    IlD.DetectionChannelName = IlS.DetectionChannelName
Next ll

For ll = 0 To TD.BeamSplitterCount - 1
    Set BS = TS.BeamSplitterObjectByIndex(ll, success)
    Set BD = TD.BeamSplitterObjectByIndex(ll, success)
    BD.Filter = BS.Filter
Next ll

    Next lT
End Sub
```

- Access with Lsm5Hardware Object

    Status will be updated at Scan Start to current recording parameters

```
Private Sub FillPinholeList()
    Dim count As Long
    Dim i As Long
    Dim ObjPinholes As CpPinholes
    Dim Servos As Object
    Dim ServoX As Object
    Dim ServoY As Object
    Dim success As Boolean

    CmbPinhole.Clear

    Set Servos = Lsm5.ExternalCpObject.pHardwareObjects.pServos
    Set ObjPinholes = Lsm5.Hardware.CpPinholes
    count = ObjPinholes.count
    For i = 0 To count - 1
        success = ObjPinholes.Select(i)
        If (success) Then
            Set ServoX = Servos.pItem(ObjPinholes.Name + "X")
            Set ServoY = Servos.pItem(ObjPinholes.Name + "Y")
            If ((Not ServoX Is Nothing) And (Not ServoY Is Nothing)) Then
                CmbPinhole.AddItem ObjPinholes.Name
            End If
        End If
    Next i
    If (count) Then CmbPinhole.ListIndex = 0      ' select first pinhole

    Set ObjPinholes = Nothing
    Set Servos = Nothing
    Set ServoX = Nothing
    Set ServoY = Nothing
End Sub
```

- Access with external Lsm5Hardware Object

5.2.2.3   Access to hidden Interface

- When do I need to use the hidden Interface?
- How do I find the desired Object / Method?

      Open Tool / References
      Browse CP.dll search, open
      Choose Object Browser Project / Library DS or CP
      Examine Object Structure

Example:

```
Function GetLaserKind(WaveLength As String, kind As Integer)
    Dim count As Long
    Dim CpObject As Object
    Dim Lines As Object
    Dim i As Long
    Dim Success As Integer
    Dim WaveLenghtOfIndex As Long
    Dim Attenuation As Double
    Dim Enable As Integer
    Dim Name As String

    GetLaserValueMax = False

    Set CpObject = Lsm5.ExternalCpObject()
    Set Lines = CpObject.pHardwareObjects.pLaserLines

    count = Lines.lCount
    For i = 0 To (count - 1)
        Success = Lines.bLineInfo(i, WaveLenghtOfIndex, Attenuation, Enable, Name)
        If (Success And (WaveLenghtOfIndex = WaveLenght)) Then
            kind = Lines.AttenuatorType(WaveLenght)

            Set Lines = Nothing
            Set CpObject = Nothing
            Exit Function
        End If
    Next i

    Set Lines = Nothing
    Set CpObject = Nothing

End Function
```

5.2.2.4 Access to scanned pictures

Example:

```
Function ChannelIndexFromChannelName(ChannelName As String, ChannelIndex As Long)
    Dim num As Long, num1 As Long, num2 As Long
    Dim i As Long
    Dim Success As Integer
    Dim DetectionChannel As DsDetectionChannel
    Dim LsmInfo As Lsm5Info
    Dim Recording As DsRecording

    ChannelIndexFromChannelName = False

    Set Recording = Lsm5.DsRecording
    Set LsmInfo = Lsm5.Info
    If (LsmInfo.NumberOfPmtsInSystem(num, num1, num2) = True) Then
        For i = 0 To (num + num1 - 1)
            Set DetectionChannel = Recording.DetectionChannelOfActiveOrder(i, Success)
            If Success Then
                If (StrComp(DetectionChannel.Name, ChannelName) = 0) Then
                    ChannelIndex = i
                    ChannelIndexFromChannelName = True
                    GoTo exit_function
                End If
            End If
        Next i
    End If

exit_function:
    Set LsmInfo = Nothing
    Set Recording = Nothing
End Function
```

```
Function GetAveragePixel(ScanDoc As DsRecordingDoc, ChannelName As String, mean As Double)
    Dim line As Variant
    Dim x As Long, xmax As Long
    Dim y As Long, ymax As Long
    Dim spl As Long
    Dim bpp As Long
    Dim Sum As Double
    Dim Channel As Long

    GetAverageScanLineParams = False

    If (ChannelIndexFromChannelName(ChannelName, Channel) = False) Then
        Exit Function
    End If

    'check for valid doc
    If (ScanDoc Is Nothing) Then Exit Function
    Sum = 0
    xmax = ScanDoc.Recording.SamplesPerLine
    ymax = ScanDoc.Recording.LinesPerFrame
    For y = 0 To ymax - 1
        line = ScanDoc.ScanLine(Channel, 0, 0, 0, spl, bpp)

        For x = 0 To spl - 1
            Sum = Sum + line(x)
        Next x
    Next y
    mean = Sum / (xmax*ymax)

    GetAverageScanLineParams = True
End Function
```

5.2.2.5 Backup Recording

```
        .
        .
        .
Dim Recording As DsRecording
Dim BackupRecording As DsRecording
Dim ScanDoc As DsRecordingDoc

Set Recording = Lsm5.DsRecording
'create a backup recording
Set BackupRecording = Lsm5.CreateBackupRecording
Success = BackupRecording.Copy(Recording)

Recording.ScanMode = "Line"
Recording.FramesPerStack = 1
Recording.StacksPerRecord = 1
Recording.ScanDirection = eSingleForeward
Recording.SpecialScanMode = "NoSpecialScanMode"
Recording.TimeSeries = False
Recording.ZoomX = ZOOM_X
Recording.ROTATION = ROTATION
Recording.Sample0X = OFFSET_X
Recording.Sample0Y = OFFSET_Y
Recording.SamplesPerLine = IMAGE_SIZE
Recording.StartScanTime = 8

Success = SetChannelDetection(ChannelName, 1, AmpOffset, 100)
Set ScanDoc = Lsm5.StartContinuousScan


        .
        .
        .


Lsm5.StopScan
'restore the backup recording
Recording.Copy BackupRecording
Set Recording = Nothing
Set BackupRecording = Nothing
```

5.2.2.6 Events

- What are Events good for?

- Mouse Events

- Scan state events

- Hardware Events


```
Private Sub Lsm5VbaDoc_SystemEvent(ByVal EventNr As Long, ByVal Param As Variant)
    Dim x As Long
    Dim y As Long
    Dim z As Long
    Dim t As Long
    Dim c As Long
    Dim tmp As Long
    Dim pt As POINT
    Dim dsDoc As DsRecordingDoc

    'get the active Recording Document
    Set dsDoc = Lsm5.DsRecordingActiveDocObject
    If (dsDoc Is Nothing) Then Exit Sub
    If (Not dsDoc.IsValid) Then Exit Sub

    If (EventNr = eImageWindowLeftButtonDownEvent) Then
        If dsDoc.GetCurrentMousePosition(c, t, z, y, x) <> 0 Then
            'do something
            .
            .
            .


        End If
    End If

    End Sub
```

CHAPTER 6　TOOLS, ADDITIONAL SOFTWARE

CONTENTS

Page

6 TOOLS, ADDITIONAL SOFTWARE

6.1 3D for LSM

6.1.1 Overview and Explanations

Fig. 6-1

6.1.1.1 The Image Sequence

The "3D for LSM" handles image sequences generated by the Zeiss LSM software. This can be three-dimensional image data or a time sequence of two-dimensional images (slices). Each slice (as well as the sequence) can consist of up to eight channels. An image sequence consists of a series of individual (2D) images and has a name that designates the entire sequence. In general an image sequence is handled as a single object in the system. Individual channels or slices can be addressed.

The following terms and definitions apply for the "3D for LSM" software.

– An image sequence is a number of individual sequential images (usually called slices in the dialog boxes), the spacing between which is equal.

– Image sequences can contain up to 12 bit of image data (per channel).

– A sequence (slice) can consist of up to eight channels.

– The maximum size of an image sequence is limited by the provided memory of the operating system.

– A voxel is the smallest element of an image sequence (the equivalent of a pixel in a 2D image). All voxels in a given image sequence are the same size.

– The coordinate system originates in the left upper front corner of the image sequence. This point has the coordinates 0, 0, 0.

– All angles are positive for rotations to the right in the direction of the positive coordinate axis (right-handed coordinate system).

– A slice is an individual image in a sequence of images. The numbering of the slices starts with "1".

Image sequences can consist of several channels. Most functions and the **Display window** are providing buttons to select all or a subset of channels stored in the selected image sequence. The **Output** image sequence will only get those channels which are selected on the input side. The button selects all channels in the image sequence to be used clicking with the left mouse button on it.

Clicking with the left mouse button on any of the number buttons toggles the state of this single channel.

Clicking with the right mouse button on any of the number buttons selects this single channel exclusively. All other channels are deselected.

6.1.1.2 The Image Properties

Every image sequence has its own set of properties. They contain the scaling and the scaling units. The scaling and its units are required for 3D reconstruction and measurement. If a sequence of LSM-TIFF images is read in, the image properties are loaded automatically from the file header and allocated to the image properties of the new image sequence.

6.1.1.3 Memory Usage

All images shown in the **Gallery** are currently loaded in the system memory of the operating system. Some functions need additional temporarily used memory during their execution.

If the memory is running low delete some images from the **Gallery**. If the images are needed afterwards they must be saved to disk first. Normally all functions produce a new result (output) image sequence. In order to save some memory, other image sequences currently presented in the **Gallery** can be selected as result position. The output image is overwritten by entry execution of a function.

6.1.2   User Interface

6.1.2.1 Introduction

This section describes the following main components of the system:

Main window   **Main window** with the **Menu**, the **Tool bar** and **Gallery**. All general system functions are located here.

Gallery   Normally several images are required in order to accomplish a particular task. These images are displayed in reduced size to provide an overview and facilitate selection. This area is located just below the **Tool bar**.
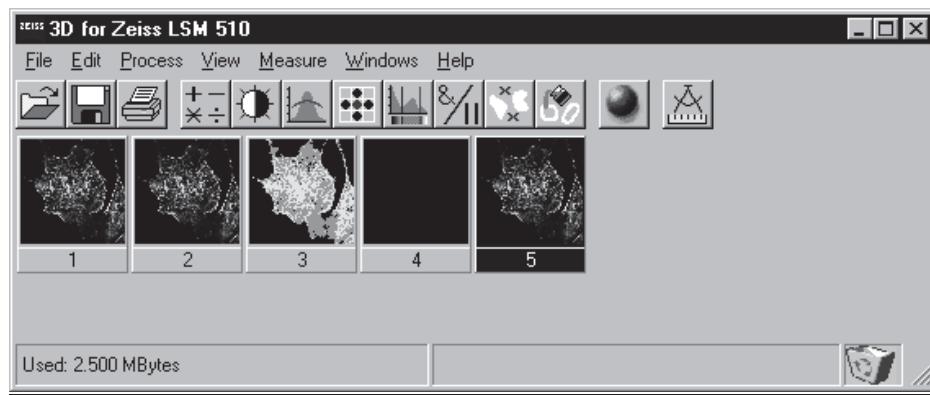
Fig. 6-2

Tool bar   This menu shows all image processing functions.

Display window   This window is used to display image sequences.
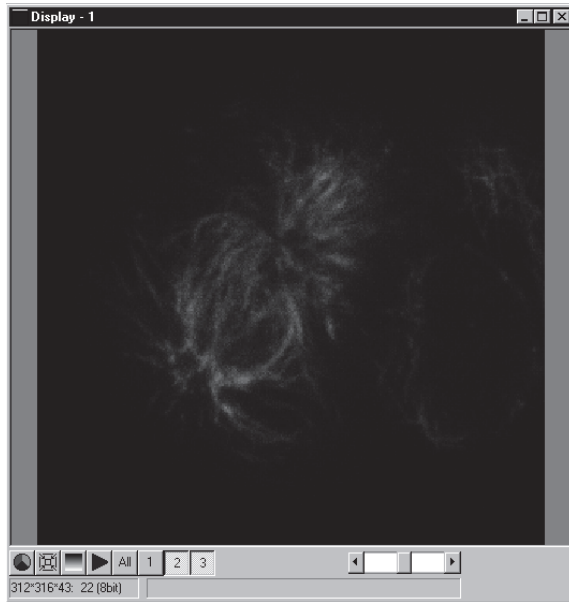
Fig. 6-3   Display window

Dialog boxes   All dialog boxes provide three buttons. Pressing the **OK** button executes the function with the defined parameters and closes the dialog window. Selecting the **Cancel** button does not execute the function, restores the parameters, and closes the dialog window. Pressing the **Apply** button executes the function with the defined parameters; the dialog window will stay opened.

6.1.2.2 Main Window

The **Main window** includes:

the **Menu**

the **Tool bar**

and the **Gallery**
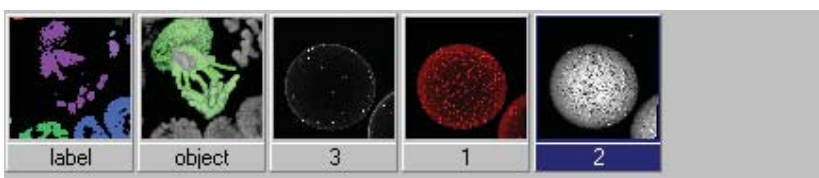
File Menu

Open Image
Opens a file selector dialog to load an image sequence.

Save Image As
Opens a file selector to save an image or image sequence.

Save Display As
Saves the currently shown contents of the Display window as a single colour image.

Print
The printer parameters can be set with this tool. The standard Windows printer dialog is opened.

Exit
Terminates the application.

Edit Menu

Copy
Copies the contents of the **Display window** to the clipboard.

Edit Channels
Allows to add or to remove channels to a single or multichannel image.

Delete All Images
Deletes all images and image sequences from the memory.

Process Menu

Arithmetics
Adds or subtracts the grey values of two image sequences
(**Add**, **Subtract**).

Contrast
Enhances the contrast and brightness of an image sequence
(**Interactive**, **Automatic**, **Linearize**).

Smooth
Smoothes an image sequence.

Morphology
Performs morphological operations on image sequences
(**Erode**, **Dilate**, **Open**, **Close**).

Segment
Segmentates an image sequence to propose measurement
(**Interactive**, **Automatic**).

Boolean
Combines two image sequences by Boolean operations
(**And**, **Or**, **Not**, **Xor**, **Mask**).

	Scrap	Selects or deletes objects of a defined size.
	Fill Holes	Fills holes in objects.

View Menu

Set Channel Colour	The colour and the weight of the single channels can be defined.
Properties	The properties of the image (e.g. scaling, use laser etc.) are displayed.

    Render   Calculates 3D reconstructions of an image sequence (Surface, Alpha).

Measurement Menu

    Automatic Object   Measures geometrical and densitometrical features (**General**, **Object Features**, **Volume Features**, **Condition**).

Windows Menu

Arrange All	Arranges the windows automatically.
Display	The current image is displayed in this window.

Help Menu

Content	Opens the help for the software.
About 3D for LSM	Displays status and release message of the software.

Tool Bar

This bar provides buttons with iconized images of nearly all functions. Clicking on one of the buttons will open a dialog window to define the function parameters. Selecting an entry from the menu alternatively can activate the same functions. Placing the cursor on a tool bar button will show a short description, if the window is activated.

Gallery

The **Gallery** is used as an overview of the images available in memory and their contents. It is located just below the **Tool bar**. Each small image represents a sequence. The middle slice of each image sequence is shown. The status bar of each image shows the name. The name might be a number or a string.

Every image sequence has its own channel colour assignment (see **Display window**). When an image is copied the channel colour assignment is copied too. Drag and drop techniques can be applied to copy images or define the function parameters **Input** and **Output** using the **Gallery** thumbnails.

- Position the cursor on an image in the **Gallery**.

- Press the left mouse button.

- Hold the mouse button down and move the mouse to the destination position.

- At the destination release the left mouse button, the destination image will be overwritten.

To delete an image, drag it, move it to the wastebasket, and drop it.

6.1.2.3   Display Window

This window is used to display an image sequence, regardless of size or type. To show multiple channel sequences each channel could have its own base colour. The user can set these colours and the weighting for each channel by pressing the corresponding button at the bottom of the window. To display a different image or image sequence, it can be dragged from the **Gallery** and dropped to the **Display window**.

The image can be displayed in full size (one pixel on the screen represents one pixel of the image) or in a zoomed size. To zoom the display view click and hold down the right mouse button on the window border and resize the window. The aspect ratio of the image will not be changed. Clicking on the button resets the **Display window** to a full size view of the image (see above).

The title bar shows the currently displayed sequence name. The status bar displays the size of the current sequence and the selected slice on the left. On the right the cursor position within the window and the corresponding intensity (grey) value of each channel is shown.

The **Display window** can be closed without any effect to the image processing functions. If no **Display window** is opened select the entry **Display** in the **Window menu**.

The scroll bar at the lower right of the window enables to show the images in a sequence. The range reaches from one to the maximum slice provided by the current sequence.

To start the automatic animation of an image sequence start the Player tool by clicking on the button . The colour selection for the channels can be activated by clicking on the button . A colour image can be displayed as a grey value image by clicking on the button .

Player

This function plays back the sequential images of an image sequence.
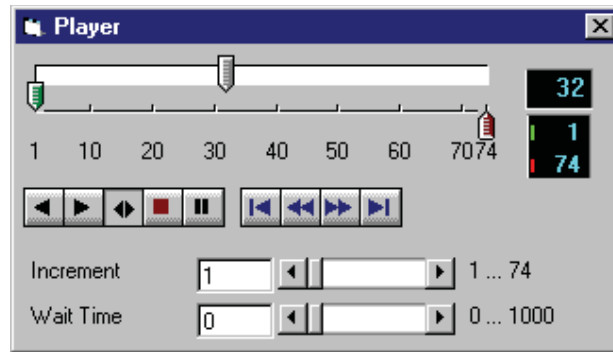
Fig. 6-4

The image sequence is displayed in the **Display window**. The display process is working as a background task; other functions can be executed while the player is running. There are several ways to stop the player:

by closing the player window

by pushing the red Stop button of the player window (the window remains open)

by closing the image window.

The **Increment** parameter specifies whether each sequential image (1) should be displayed or whether some sequential images should be skipped during display. The value 2 skips one image for every sequential image displayed, in other words, it displays only every second image.

The parameter **Wait Time** states the delay in milliseconds between two successive sequential images. The maximum display speed depends mainly on the hardware. The sequential images are always displayed in their entirety, regardless of the set delay.

Control Element of the Player

The three arrow shaped controls on the scale show the start slice and the currently displayed sequential image. The values (positions) can be changed using the mouse. Press and hold the left mouse button and move the pointer to the desired position. The set values are shown in the numerical windows at right.

Start slice

Currently displayed sequential image

End slice

The buttons in the left group start and stop playback of an image sequence.

Reverse playback

Forward playback

Play forward and then backward again (jojo)

Stop playback

Pause playback

The buttons in the middle group control the settings of the current sequential image.

Reset to start slice.

Single step backward (1 sequential image each regardless of Increment).

Single step forward (1 sequential image each regardless of Increment).

Set to end slice.

Increment   Image increment.

Wait Time   Displays delay between two images (in milliseconds).

Set Channel Colour

This function sets the colour and weight for the channels.
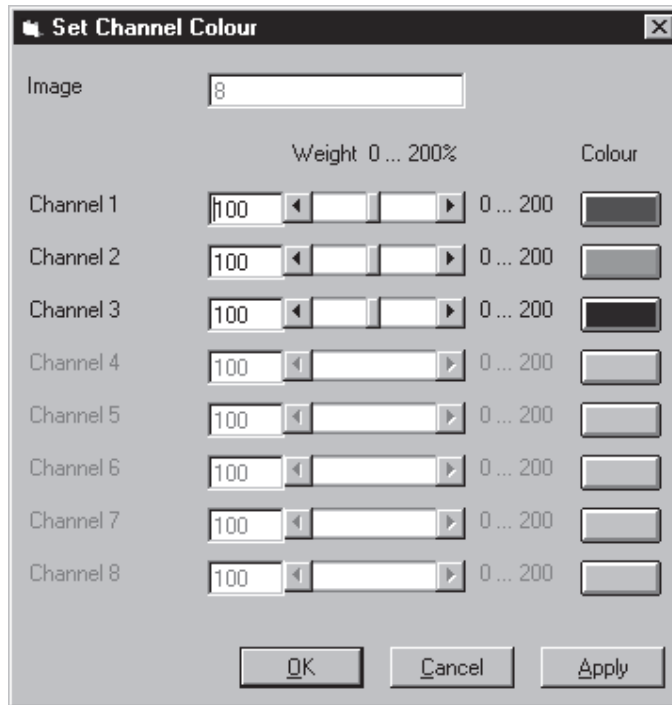
Fig. 6-5

Each image sequence can get its own colour definitions. All functions will inherit the colour definition from the **Input** sequence to the **Output** sequence. By default the colours are set to 100 % weighting and the pure base colours (red, green, blue) are defined.

The weight can be any value between 0 % and 200 %. The colour can be redefined by clicking on the coloured button on the right of the dialog. The standard Windows colour selection dialog is opened. The solution is done by clicking on one of the colours or by entering appropriate numbers in the corresponding edit boxes.

Pressing the **OK** button will close the colour selection dialog and update the **Display window** immediately.

Only those channels, which are available in the image sequence, can be defined.

Parameters:

Image	Image sequence to edit
Weight	Colour weighting for each channel
Colour	Base colour for each channel

6.1.3   Functions

6.1.3.1 Functions in the File Menu

Open Image

This function reads a Zeiss LSM (*.lsm), Zeiss LSM TIFF (*000.tif) or Carl Zeiss Vision (*0.img) image sequence from a disk or network drive.
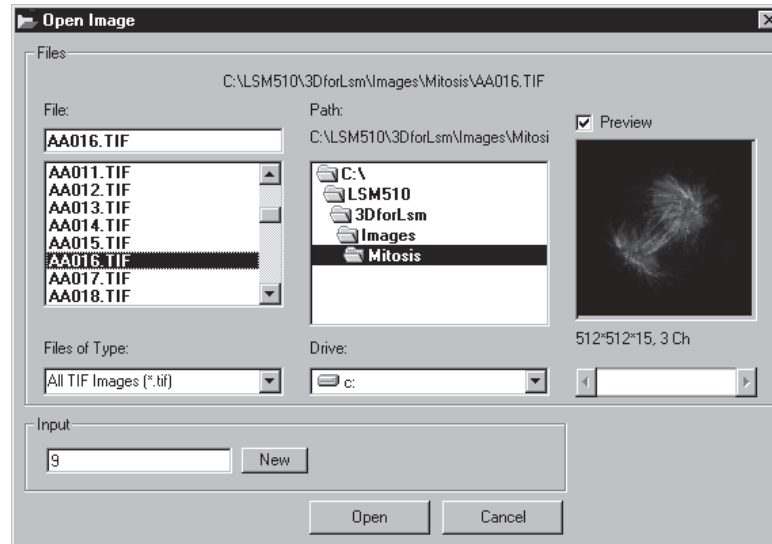
Fig. 6-6

The individual files of a Zeiss TIFF image sequence are read and saved as an image sequence in image memory. In addition, the image properties are read out of the TIFF files and allocated to the image sequence **Input**.

The directories of the current drive are listed in the **Directories** list box. Use the **Drives** list box to choose a different drive.

In case of choosing the TIFF-format in the **Files of Type** box, three number characters are always expected before the dot in the filename extension. The first number must be 000 at the end of the filename. From a complete sequence only this file is listed in the dialog, if "LSM TIF Images (*000.tif)" is selected in the **Files of Type** box. To view all TIFF files "All TIF Images (*.tif)" in the **Files of Type** box must be selected. This selection enables to start with a different file than with the very first (named *000.tif) at the end of the filenames three number digits.

Currently the Carl Zeiss Vision file format "KE Images (*0.img)" is supported. Two files per channel are saved.

Carl Zeiss Vision image sequences must have a number digit at the end of the base filename. They are used to indicate the different channels in a multichannel sequence. The numbering starts with zero (0). If a sequence is saved in the Carl Zeiss Vision format the numbers are generated automatically. To load such an image sequence "KE Images (*0.img)" in the **Files of Type** box must be selected.

The window incorporates the usual file selection controls. The bottom half displays a selection of the image properties that are stored in the image sequence.

Parameters:

      BaseName   Base name of the TIFF files (image sequence) to be loaded. Only the letters before the first number are stated.

      Input   Name of the resulting image in which the image sequence will be saved.

Save Image As

This function saves an image or image sequence to disk or network drive.
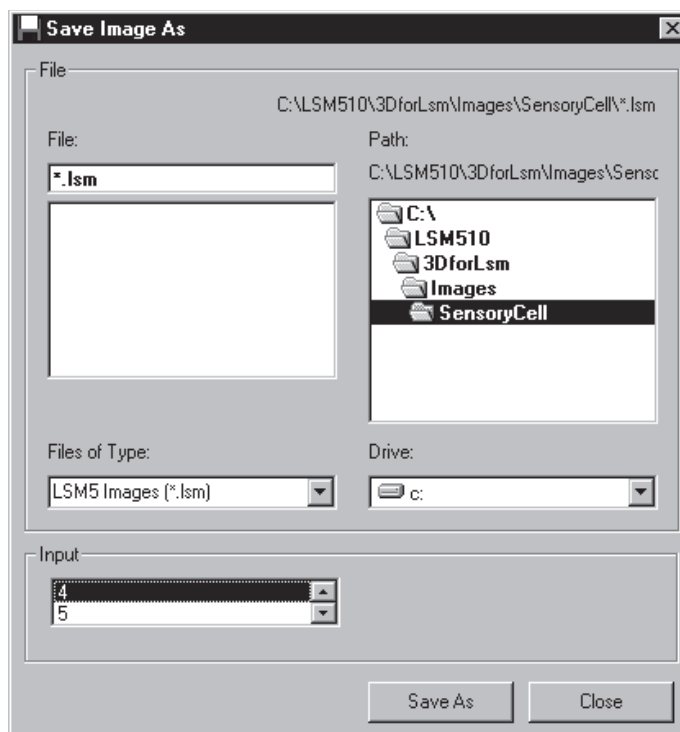
Fig. 6-7

All the files in the current directory that have the selected image format are listed in the **File Name** list box.

The directories of the current drive are listed in the **Directories** list box. Use the **Drives** list box to choose a different drive.

Use the list box **Files of Type** to select the image format. Currently the LSM image format (*.lsm) and the Carl Zeiss Vision file format "KE Images (*0.img)" is supported.

By choosing the Carl Zeiss Vision file format "KE Images (*0.img)", two files per channel are saved. On one hand the Carl Zeiss Vision type image sequence file, on the other hand the file with the image properties. One pair of files is written per channel. They are numbered automatically, starting with zero. A one number digit is added to the end of the filenames. The two files share the same filename but have different filename extensions (*.img and *.3d).

The content of the **Gallery** is shown in the **Input** section. The selection of the sequence to save is done by highlighting one of the provided names or by drag and drop from the **Gallery**.

Parameters:

      Input   Name of the image sequence to be saved

      Filename   Name of the file to be used on disk

Save Display As

This function saves the current **Display window** contents to a disk or network drive.
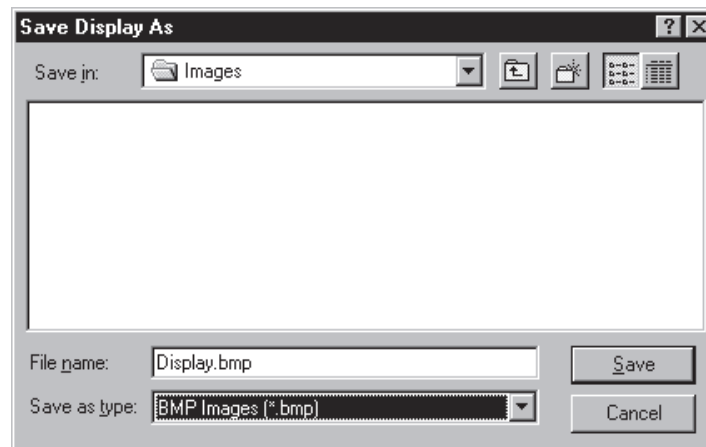
Fig. 6-8

Before the execution of this function any image or image sequence can be selected to be displayed. From a multichannel sequence any channel status (on or off) combination can be defined. The colours of the shown channels can be set with the function **Set Channel Colour**.

The current zoom factor of the **Display window** is not taken into account, the image is saved without any zoom.

The image is saved as a true colour image with 24-bit resolution. From the **Save as Type** list box one of the provided formats can be selected.

Parameters:

      None

Print

This function prints the current **Display window** contents.

The standard Windows print dialog is opened.

Before the execution of this function any image or image sequence can be selected to be displayed. From a multichannel sequence any channel status (on or off) combination can be defined. The colours of the shown channels can be set with the function **Set Channel Colour**.

Parameters:

> **None**

Exit

This function terminates the application completely.

All images and image sequences shown in the **Gallery** will be deleted from the memory. Save those images which might be used for any further processing.

Parameters:

> **None**

6.1.3.2 Functions in the Edit Menu

Copy

This function copies the current **Display window** contents to the clipboard. No dialog is shown.

Before the execution of this function any image or image sequence can be selected to be displayed. From a multichannel sequence any channel status (on or off) combination can be defined. The colours of the shown channels can be set with the function **Set Channel Colour**.

The current zoom factor of the **Display window** is not taken into account; the image is copied without any zoom.

The image is copied as a true colour image with 24-bit resolution. Afterwards the contents can be pasted to any other Windows application.

Parameters:

> **None**

Edit Channels

This function allows to add or to remove channels to a single or multichannel image.

On the **Add Channel** tab sheet the channels of (different) **Input** sequences can be defined to add (combine) channels to an **Output** sequence.
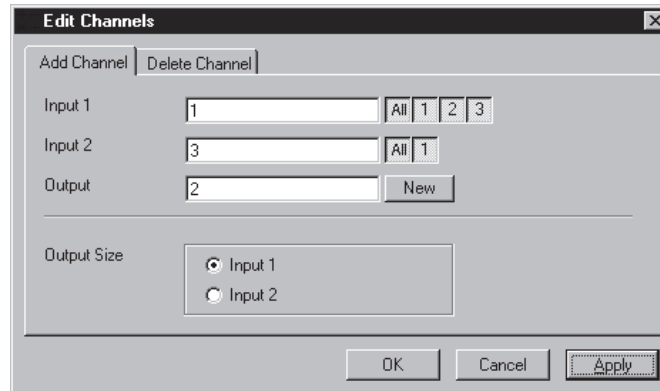
Fig. 6-9

This operation is useful to add a segmented channel (or any other result of a function) to the original image sequence. The selected channels of **Input 1** and **Input 2** are copied to **Output**. The maximum number of channels in an image sequence is eight.

If the image sequences do not have the same extents **Output Size** defines which input is taken as a reference. This selection also defines the properties for scaling and units in the output image sequences.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Output image sequence
Output size	Defines source image sequence for size, scaling, and units

On the **Delete Channel** tab sheet channels of the **Input 1** image sequence can be selected to delete channels.
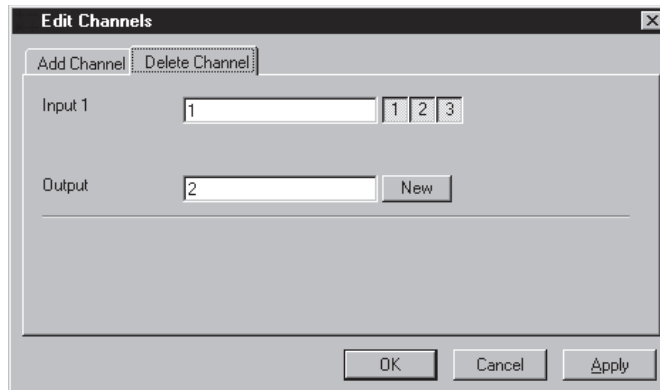
Fig. 6-10

This operation might save time and memory for further processing if not all channels are needed.

Only the selected channels of **Input 1** are copied to **Output**.

Parameters:

Input 1	Input image sequence
Output	Output image sequence

Delete All Images

This function deletes all images and image sequences from the memory (**Gallery**).

The function is used whenever a completely new image sequence should be processed. In order to drop the images item by item to the wastebasket all of them can be deleted by a single function.

If any image or image sequence is needed for further use save them first.

Parameters:

        None

6.1.3.3 Functions in the Process Menu

Arithmetics - Add

This function adds two image sequences.
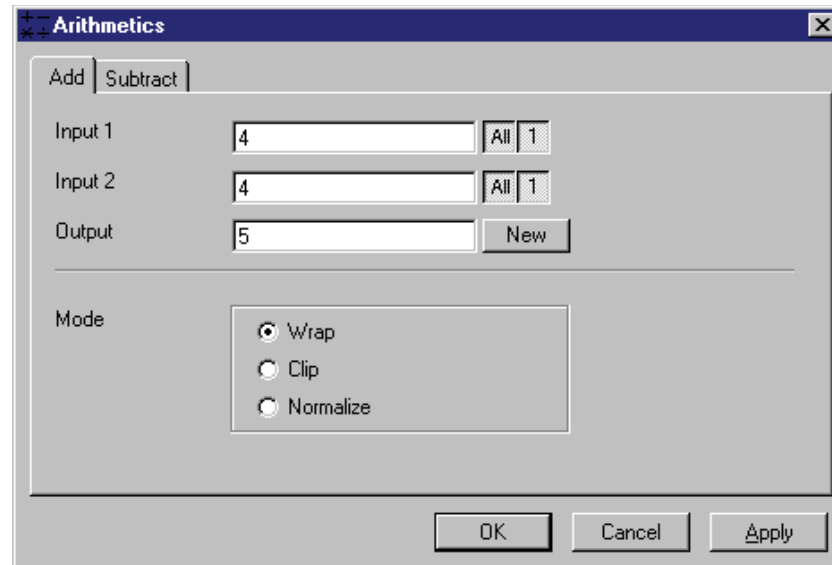
Fig. 6-11

The **Add** tab sheet of the **Arithmetics** dialog window must be selected.

If one or both input sequences are multichannel sequence, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

This function adds the two image sequences **Input 1** and **Input 2** voxel by voxel and generates the image sequence **Output**. Note that a resulting grey value may be greater than 255 (4095). The parameter **Mode** determines how a range overflow is handled:

1 - Wrap   No normalization - the grey values are displayed modulo 256 (4096). If the result is greater than 255 (4095), the value 256 (4096) is subtracted from it.

2 - Clip   Grey values which exceed 255 (4095) are replaced with 255 (4095).

3 - Normalize   The resulting grey value range is scaled to the range 0...255 (0...4095).

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Output image sequence
Mode	1 - Wrap 2 - Clip 3 - Normalize

Arithmetics - Subtract

This function subtracts two image sequences.
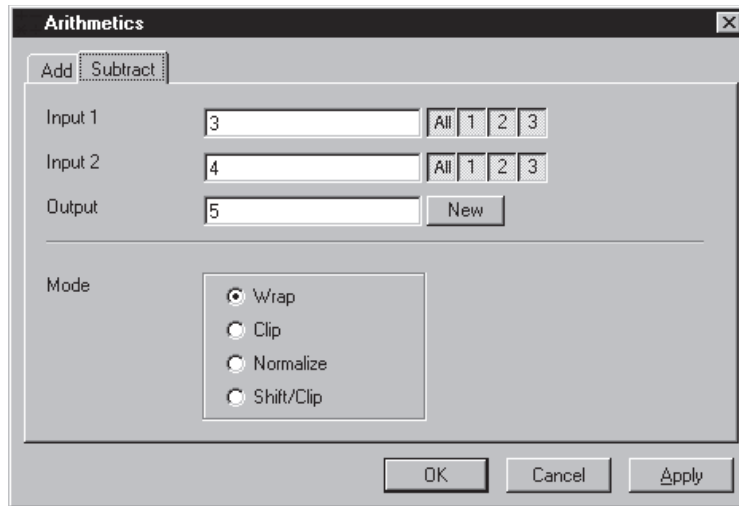
Fig. 6-12

The **Subtract** tab sheet of the **Arithmetics** dialog window must be selected.

If one or both input sequences are multichannel sequence, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

This function subtracts the two image sequences **Input 1** and **Input 2** voxel by voxel and generates the image sequence **Output**. Note that a resulting grey value may be less than 0. The parameter **Mode** determines how a range overflow (negative values) is handled.

1 - Wrap	No normalization - the grey values are displayed modulo 256 (4096). If the result is less than 0, the value 256 (4096) is added to it.
2 - Clip	Negative values are set to 0.
3 - Normalize	The resulting grey value range is scaled to the range 0...255 (0...4095).
4 - Shift/Clip	128 (2048) is added to the difference, then negative values are set to 0. Values greater than 255 (4095) are set to 255 (4095).

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Output image sequence
Mode	1 - Wrap
	2 - Clip
	3 - Normalize
	4 - Shift/Clip

Contrast - Interactive

This function allows interactive changes of the contrast of an image sequence.
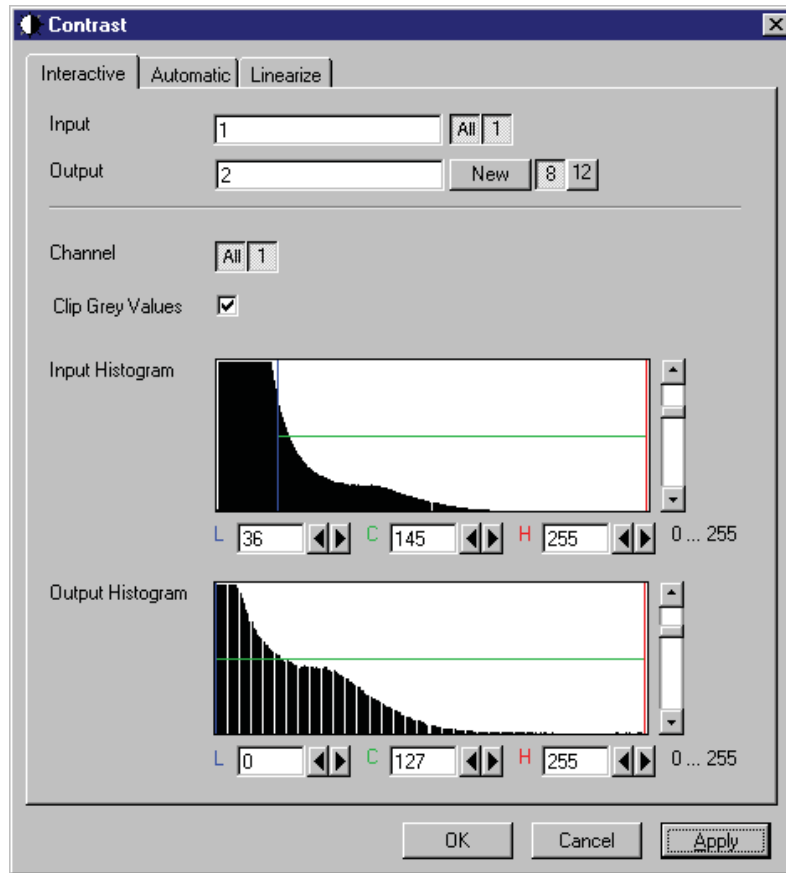
Fig. 6-13

The **Interactive** tab sheet of the **Contrast** dialog window must be selected.

A grey value range of the **Input** image sequence is scaled to another range in the **Output** image sequence. Both ranges can be edited interactively. This function is used to achieve a better view of an image sequence, or to scale a range of grey values to single value for a special coding in an image sequence. The function does not improve the result of the linear segmentation function **Segment**.

Input indicates the sequence to enhance. If it is a multichannel sequence, a single channel, all channels, or any number can be selected. The **Input** histogram shows the grey value distribution of the selected channels of the **Input** image sequence.

Output defines the name of the result sequence. It will get only those channels which are chosen by the **Input** parameter. The buttons labeled with 8 and 12 define the grey value (intensity) resolution in bit. Normally the result will get the same resolution as the **Input** sequence. A change will be needed if image sequences with different resolutions should be combined. Rising the grey value range to 12 bit will not enhance the display quality or measurement accuracy. The smooth and morphology functions will produce results with finer gradations.

If **Clip Grey Values** is selected, the output grey values are clipped to the **Low (L)** and **High (H)** values. If **Clip Grey Values** is not selected, output grey values beyond the **Low** and **High** value range are possible.

The **Output** histogram shows the resulting histogram. The horizontal axis represents the grey values from 0 to the maximum, which is either 255 or 4095, depending whether the input is 8 bit or 12 bit. The vertical axis represents the pixel count. The selected range is marked by the borderlines in the histogram. The blue line or **L** indicates the lower boundary, the red line or **H** the upper one, **C** indicates the center of the range.

There are three ways to change the range: clicking and dragging the borderlines with the mouse.

Entering a new value in the appropriate text boxes, clicking on the buttons ◆ or using the arrow keys from the keyboard. To alter the values within the histogram move the mouse pointer over one of the three coloured lines until the shape changes. Press and hold the left mouse button to move the line to a new position. To change the values with the arrow keys click once into the histogram. Using the left or right arrow key by its own will move the whole range. Pressing the **Shift** key additionally moves the lower boundary, the **Control** key the upper boundary.

The vertical scale of the histogram is set using the scroll bar. The units are percents of the maximum grey value distribution. This setting has no influence on the function.

Parameters:

Input	Input image sequence
Output	Output image sequence
Channel	Selection of the channel numbers for the **Output** image after contrast enhancement
Clip Grey Values	Clipping of grey values to the **Low (L)** and **High (H)** output grey values boundaries
Input L	Lower boundary of grey value range **Input**
Input C	Center of grey value range **Input**
Input H	Upper boundary of grey value range **Input**
Output L	Lower boundary of grey value range **Output**
Output C	Center of grey value range **Output**
Output H	Upper boundary of grey value range **Output**

Contrast - Automatic

This function scales the grey values of an image sequence to the maximum possible range.
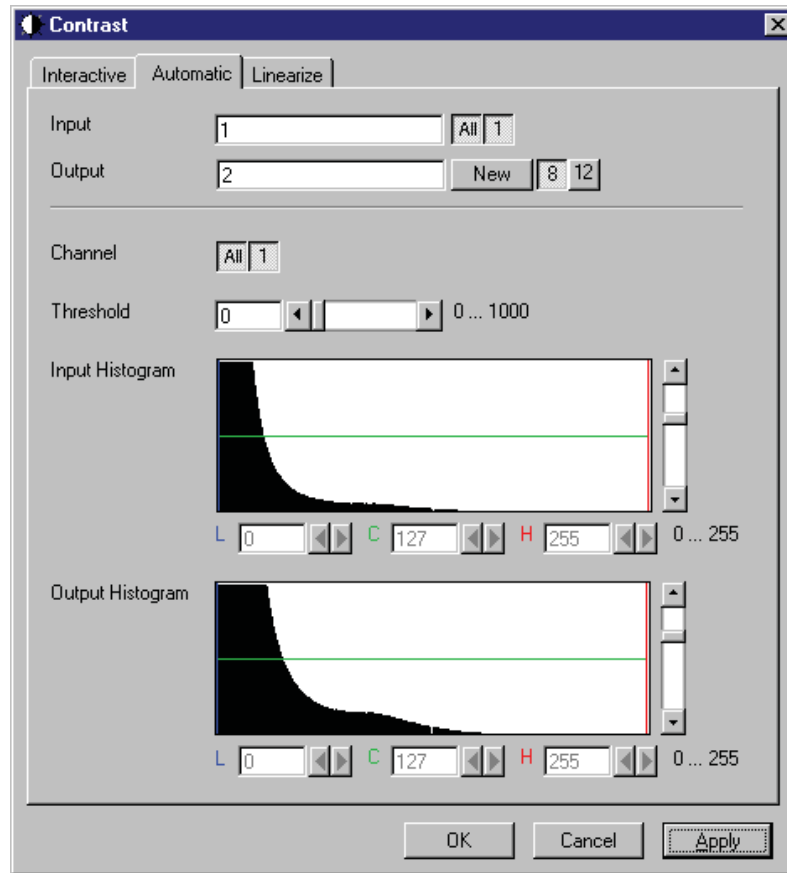
Fig. 6-14

The **Automatic** tab sheet of the **Contrast** dialog window must be selected.

This function enhances the contrast of an image sequence by spreading the grey value distribution over the maximum possible range. This function is used to achieve a better view of an image.

The light and dark grey value ranges with a low share of pixels are excluded from the operation by the parameter **Threshold**. The **Threshold** units are in thousandths of the total number of voxels. Using a value of 10 means that the scale interval is set so that 5/1000 of the total number of voxels on the light side, and 5/1000 of the total number of voxels on the dark side of the grey value distribution are excluded.

Input indicates the sequence to enhance. If it is a multichannel sequence, a single channel, all channels, or any number can be selected. The **Input** histogram shows the grey value distribution of the selected channels of the **Input** image sequence.

Output defines the name of the result sequence. It will get only those channels which are chosen by the **Input** parameter. The buttons labeled with 8 and 12 define the grey value (intensity) resolution in bit. Normally the result will get the same resolution as the **Input** sequence. A change will be needed if image sequences with different resolutions should be combined. Rising the grey value range to 12 bit will not enhance the display quality or measurement accuracy. The smooth and morphology functions will produce results with finer gradations.

The **Output** histogram shows the resulting histogram. They are not editable. The horizontal axis represents the grey values from 0 to the maximum, which is either 255 or 4095, depending whether the input is 8 bit or 12 bit. The vertical axis represents the pixel count. The vertical scale of the histogram is set using the scroll bar. The units are percentages of the grey value distribution maximum. This setting has no influence on the function.

Parameters:

Input	Input image sequence
Output	Output image sequence
Threshold	Exclusion value - 0...1000
Input L	Lower boundary of grey value range Input
Input C	Center of grey value range **Input**
Input H	Upper boundary of grey value range **Input**
Output L	Lower boundary of grey value range **Output**
Output C	Center of grey value range **Output**
Output H	Upper boundary of grey value range **Output**

Contrast – Linearize

This function scales a range of grey values of an image sequence to equal area fractions in the histogram.
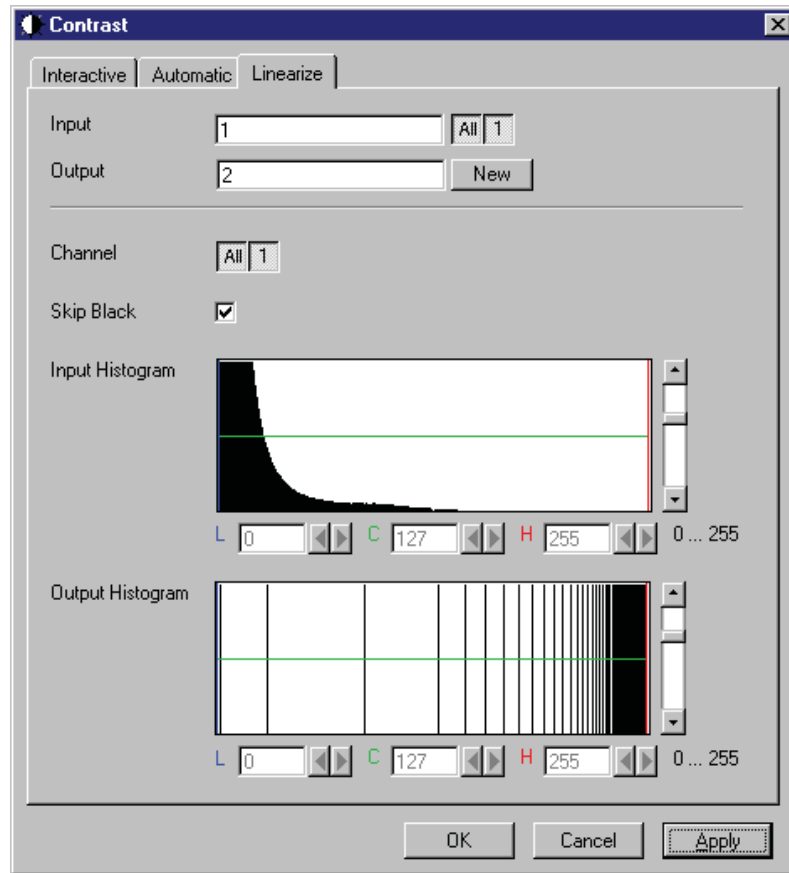
Fig. 6-15

The **Linearize** tab sheet of the **Contrast** dialog window must be selected.

This function enhances the contrast by linearizing the histogram of the image sequence to equal area fractions in the histogram. The areas (voxel count multiplied by grey value range) of all grey values in the **Output** histogram are the same. This function is used to achieve a better view of an image sequence. When **Skip Black** is checked the grey value 0 will not be taken into account for linearization.

Input indicates the sequence to enhance. If it is a multichannel sequence, a single channel, all channels, or any number can be selected. The **Input** histogram shows the grey value distribution of the selected channels of the **Input** image sequence.

Output defines the range of the result sequence. It will get only these channels which are chosen by the **Input** parameter. The grey value (intensity) resolution will be the same as the one from **Input**.

The **Output** histogram shows the resulting histogram. The horizontal axis represents the grey values from 0 to 255. The vertical axis represents the pixel count. The vertical scale of the histogram is set using the scroll bar. The units are percentages of the grey value distribution maximum. This setting has no influence to the function.

Parameters:

Image	Input image sequence
Output	Output image sequence
SkipBlack	0 - Grey value black is ignored 1 - Grey value black is taken into account
Input L	Lower boundary of grey value range Input
Input C	Center of grey value range Input
Input H	Upper boundary of grey value range Input
Output L	Lower boundary of grey value range Output
Output C	Center of grey value range Output
Output H	Upper boundary of grey value range Output

Smooth (Gauss)

This function performs a Gauss filter.
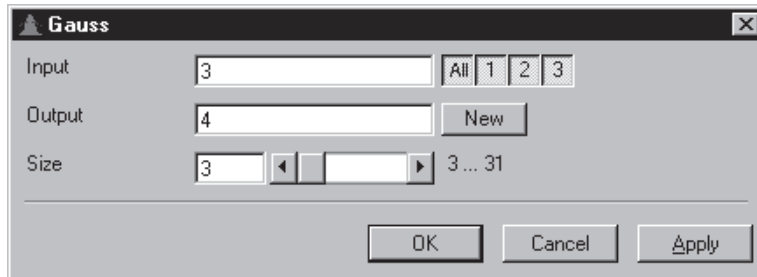
Fig. 6-16

The noise in the image sequence is reduced, the edge shape is nearly unchanged, local maxima are leveled, the dynamic range is reduced.

Image sequences should be smoothed before they are reconstructed or segmented. For most sequences a **Size** value of 3 is sufficient enough. If **Input** is a multichannel sequence, any number and combination of channels can be selected. **Output** will only get the selected channels as results.

The grey value of every pixel is substituted by a weighted average of its surrounding neighbors. The neighbors are defined by a cube. The affected pixel is the central pixel of the filter cube. The weighted filter cube is approximated by a binomial distribution. The size of the filter cube is set using the **Size** scroll bar. Even numbers are set to the next odd value. The **Size** defines the strength of the smoothing.

Parameters:

Input	Input image sequence
Output	Output image sequence
Size	Filter size (3...31, only odd numbers)

Morphology

The following four functions perform basic operations of mathematical morphology on image sequences.
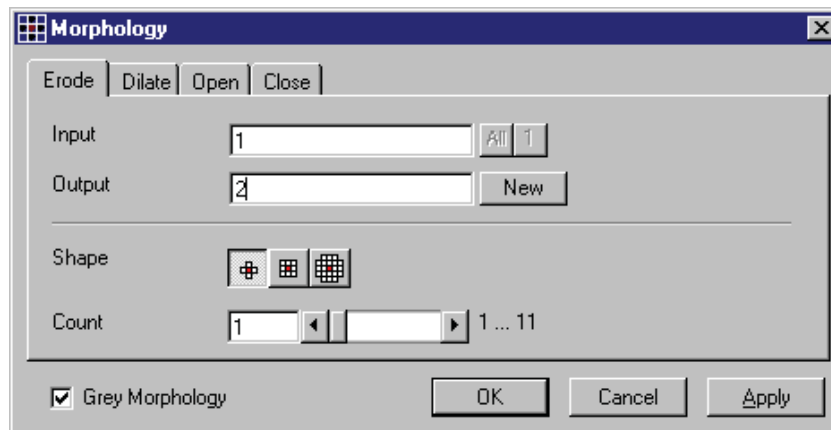
Fig. 6-17

As generalization of the morphology of two-dimensional images to three dimensions the structural elements are small volumina.

Literature

Bomans, M.; Höhne, K.-H.; Tiede, U.; Riemer, M.:
3D-Segmentation of MR Images of the Head for 3-D Display
IEEE Transactions on Medical Imaging 9, 1990, 177-183
Schiemann, T.; Bomans, M.; Tiede, U.; Höhne, K.-H.:
Interactive 3D-Segmentation of Tomographic Image Volumes
14. DAGM-Symposium Mustererkennung, Springer-Verlag 1992, 73-80

The input image sequence is analyzed voxel by voxel with a selected shape (**Shape**). The voxel to be analyzed is always the central voxel of the shape. The shape type determines which neighboring voxels are used to compute the resulting voxel.

The following structural elements are available for all morphological operations. They represent approximated spheres with an increasing radius.

Sequential image: Volume view: Cross shape
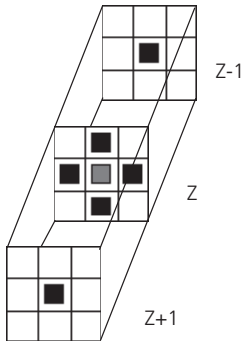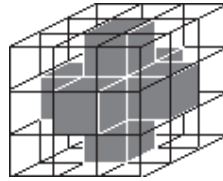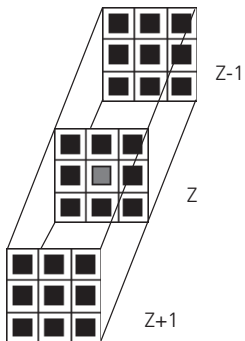
Sequential image: Volume view: Cross shape
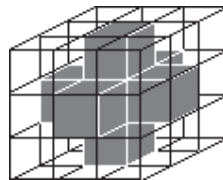
Sequential image:
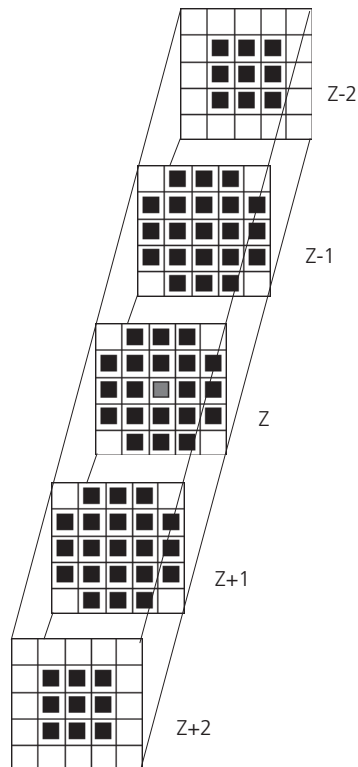
Volume view:
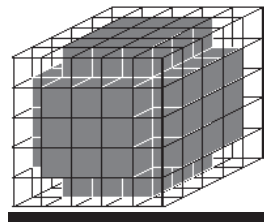
Cube cross shape: created through application of "cube" and "cross" one after the other.

For regions (voxels) that are at the edge of the image sequence, it assumed for erosion that there are white voxels with a grey value of 255 (4095) outside the edge. For dilation, it is assumed that there are black voxels with the grey value 0 outside the image sequence.

If the **Grey Morphology** tickbox is activated, erosion sets the grey value of the central voxel to the minimum of all neighboring voxels affected by the structural element; dilation sets the grey value of the central voxel to the maximum.

If the **Grey Morphology** tickbox is not activated, the neighboring voxels are only distinguished by grey value 0 and non-0. For erosion the central voxel is set to 0 if any of the neighbors is 0. It is set to 255 (4095) if any neighbor is not 0. For dilation the central voxel is set to 255 (4095) if any of the neighbors is not 0. It is set to 0 if all neighbors are 0.

Erosion reduces the size of bright regions, separates thin connections between them, and makes small regions disappear. Dilation, on the other hand, makes bright regions of the image grow in size, fills gaps, and smoothes small contour details.

The result of erosion and dilation is called opening. On the one hand, this maintains to some extent the original size of the regions while not losing the smoothing effect of erosion on the image. This name stands for the operation of reducing convex bulges in the contour of the region. Thin connections between regions are eliminated, broken borders between regions are connected, and small regions disappear.

The opposite operation (first dilation, then erosion) is called closing. Concave bulges in the contours of regions are filled in; connections are formed between adjacent regions.

The following example illustrates the operations "Open" and "Close" in two dimensions:

Open = Erosion + Dilation

Fig. 6-18

Close = Dilation + Erosion

Fig. 6-19

The "cube cross" shape was used for the operations shown.

Morphology - Erode

This function erodes structures in an image sequence.
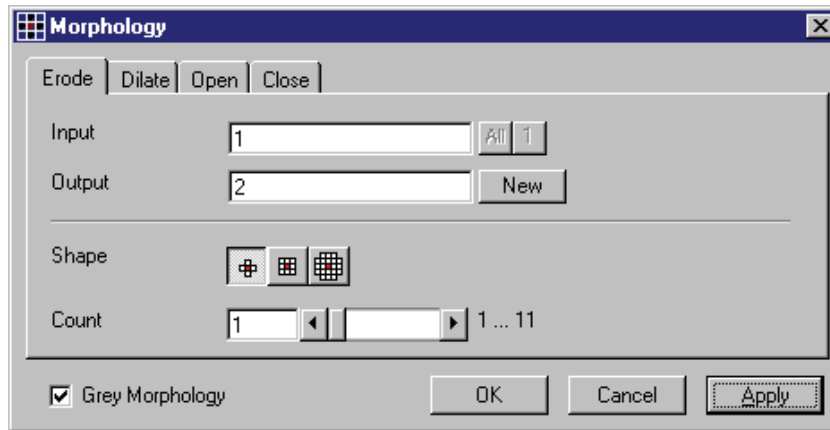
Fig. 6-20

In the **Morphology** dialog window, the tab sheet **Erode** must be selected.

Erosion makes bright regions smaller on a dark background. It also results in separation of thin connections between regions. Small regions disappear entirely.

If **Input** is a multichannel sequence any number and combination of channels can be selected. **Output** will only get the selected channels as results. The Input image sequence is eroded **Count** times with the shape **Shape**. The **Count** scroll bar determines the number of recursive operations.

The following shapes (numbered 1 to 3 from left to right) are available:

If **Grey Morphology** is selected the function will respect all grey value shades of the sequence **Input**. If **Grey Morphology** is not selected the function will distinguish between 0 and non-0 only. The result **Output** will be a binary sequence.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Shape	Shape used 1 - cross 2 - cube 3 - cube cross
Count	Number of recursive operations
Grey Morphology	0 - Distinguish between 0 and non 0 only 1 - All grey value shades are taken into account

Morphology - Dilate

This function dilates structures in an image sequence.
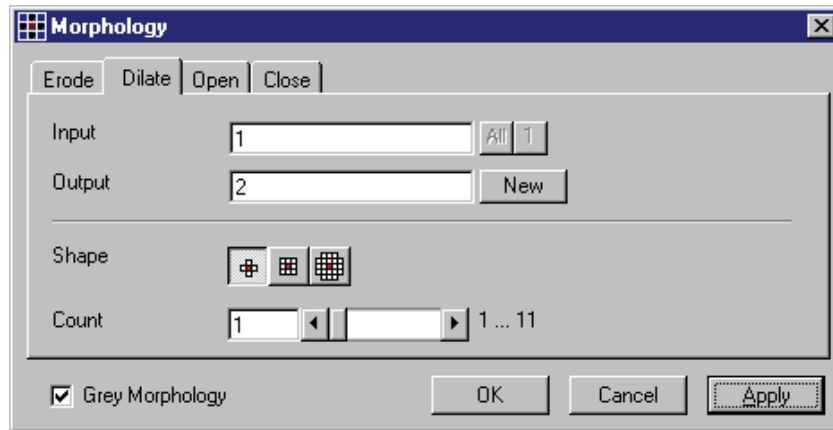
Fig. 6-21

In the **Morphology** dialog window, the tab sheet **Dilate** must be selected.

Dilation makes bright regions larger on a dark background. It also results in the filling of gaps and smoothing of small contour details.

If **Input** is a multichannel sequence any number and combination of channels can be selected. **Output** will only get the selected channels as results.

The **Input** sequential image is dilated **Count** times with the shape **Shape**. The **Count** scroll bar determines the number of recursive operations.

The following shapes (numbered 1 to 3 from left to right) are available:

If **Grey Morphology** is selected the function will respect all grey value shades of the sequence **Input**. If **Grey Morphology** is not selected the function will distinguish between 0 and non-0 only. The result **Output** will be a binary sequence.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Shape	Shape used 1 - cross 2 - cube 3 - cube cross
Count	Number of recursive operations
Grey Morphology	0 - Distinguish between 0 and non 0 only 1 - All grey value shades are taken into account

Morphology - Open

This function carries out an opening.
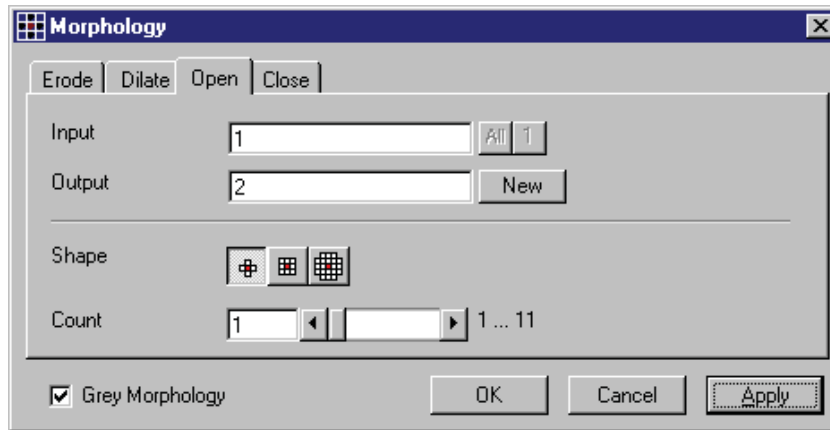
Fig. 6-22

In the **Morphology** dialog window, the tab sheet **Open** must be selected.

This function carries out an erosion followed by a dilation. For the most part, the opening maintains the original size of the regions. Thin connections between regions and small regions themselves disappear. Convex bulges in the contours of the regions are reduced. The opening is applied to the grey value image sequence **Input Count** times with the shape **Shape**. If **Input** is a multichannel sequence any number and combination of channels can be selected. **Output** will only get the selected channels as results.

The **Count** scroll bar determines the number of recursive operations.

The following shapes (numbered 1 to 3 from left to right) are available:

If **Grey Morphology** is selected the function will respect all grey value shades of the sequence **Input**. If **Grey Morphology** is not selected the function will distinguish between 0 and non-0 only. The result **Output** will be a binary sequence.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Shape	Shape used 1 - cross 2 - cube 3 - cube cross
Count	Number of recursive operations
Grey Morphology	0 - Distinguish between 0 and non 0 only 1 - All grey value shades are taken into account

Morphology - Close

This function carries out a closing.
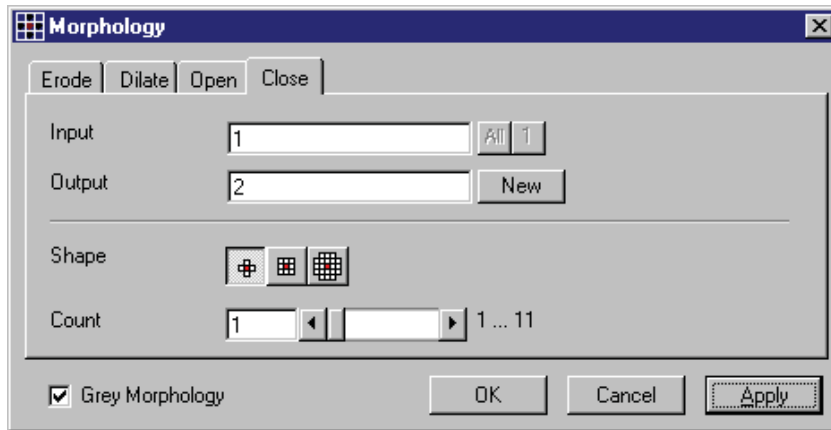
Fig. 6-23

In the **Morphology** dialog window, the tab sheet **Close** must be selected.

This function carries out a dilation followed by an erosion. For the most part, the closing maintains the original size of the regions. Connections are formed between adjacent regions; gaps and bright concave bulges in the contours of regions are filled in. The closing is applied **Count** times to the grey value image sequence Input with the shape **Shape**. If **Input** is a multichannel sequence any number and combination of channels can be selected. **Output** will only get the selected channels as results.

The **Count** scroll bar determines the number of recursive operations.

The following shapes (numbered 1 to 3 from left to right) are available:

If **Grey Morphology** is selected the function will respect all grey value shades of the sequence **Input**. If **Grey Morphology** is not selected the function will distinguish between 0 and non-0 only. The result **Output** will be a binary sequence.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Shape	Shape used 1 - cross 2 - cube 3 - cube cross
Count	Number of recursive operations
Grey Morphology	0 - Distinguish between 0 and non 0 only 1 - All grey value shades are taken into account

Segment - Interactive

This function carries out a grey value segmentation by means of thresholding.
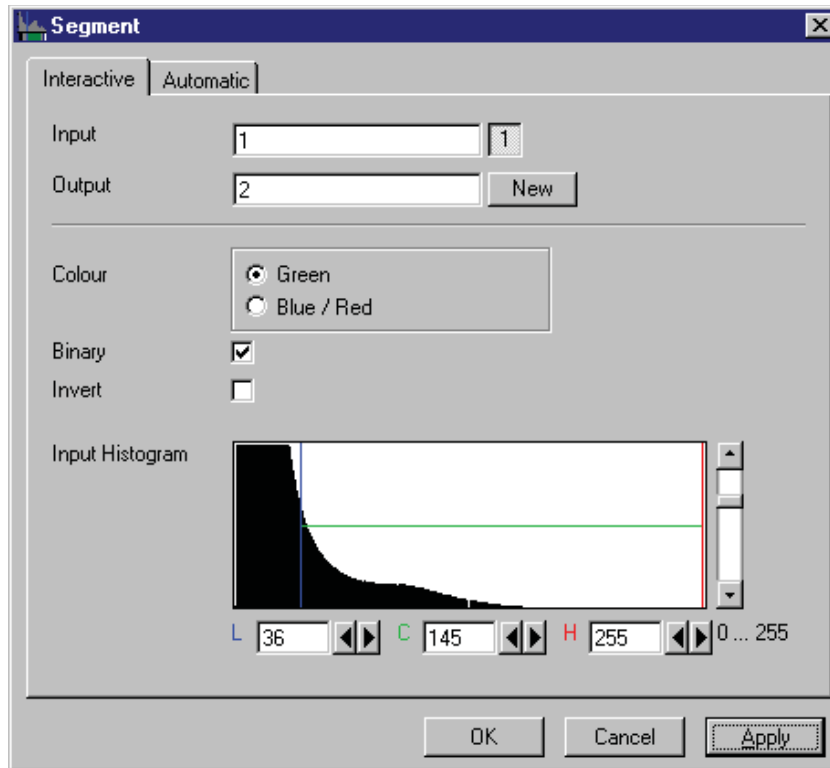
Fig. 6-24

The **Interactive** tab sheet of **Segment** dialog window must be selected.

Segmentation is especially used to generate binary regions. These are required for the measurement.

Two threshold values determine which grey value range of the **Input** image sequence is preserved and/or deleted in the **Output** image sequence. Only one channel of a multichannel sequence can be selected as **Input**. **Output** will always be a single channel sequence.

The vertical scaling of the histogram can be adjusted with the scroll bar at the right edge of the histogram. This setting has no influence on the function.

The thresholds **L**ow and **H**igh are determined either by moving the borderlines in the grey value histogram or by the scroll bars underneath. Furthermore, the values for **L**ow, **C**enter and **H**igh can be set through entry in the corresponding fields.

To move the lower (**L**) and upper (**H**) thresholds at the same time, move the vertical line in the grey value histogram or set the scroll bar (**C**).

The **Green** and **Blue/Red** option buttons of the parameter **Colour** determine whether the voxels within (**Green**) or outside (**Blue/Red**) of the grey value interval [**L, H**] are displayed with the corresponding colour.

If **Green** is selected, the voxels within the selected interval are highlighted in green. The rest of the image retains its original grey values. The voxels with the grey values **L**ow and **L**ow+1 are displayed in blue. The voxels with the grey values **H**igh and **H**igh-1 are displayed in red.

If **Blue/Red** is selected, the voxels with grey values within the interval **L**ow, **H**igh remain unchanged. Voxels with grey values less than **L**ow are highlighted in blue; those with grey values higher than **H**igh are highlighted in red.

If the **Invert** option is selected, the grey values outside the defined interval will be segmented.

If the option **Binary** is selected, then all grey values in the range from **L**ow to **H**igh will be set to white (grey value 255) in the **Output** image sequence, while all others will be set to black (grey value 0). If the option is not selected, the grey values within the selected interval remain unchanged, while those outside the range will be set to black. The measurement function accepts both results without any difference in the results.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Colour	Green - Selected interval is displayed in green
Blue/Red	Grey values below the selected interval are displayed in blue, grey values above in red
Binary	0 - Selected voxels retain the original grey value 1 - Selected voxels are set to grey value 255, the rest to grey value 0
Invert	0 - Grey values inside the selected interval are segmented 1 - Grey values outside the selected interval are segmented
L	Low grey value threshold
C	Center of threshold interval
H	High grey value threshold

Segment - Automatic

The function carries out an automatic grey value segmentation by means of thresholding.
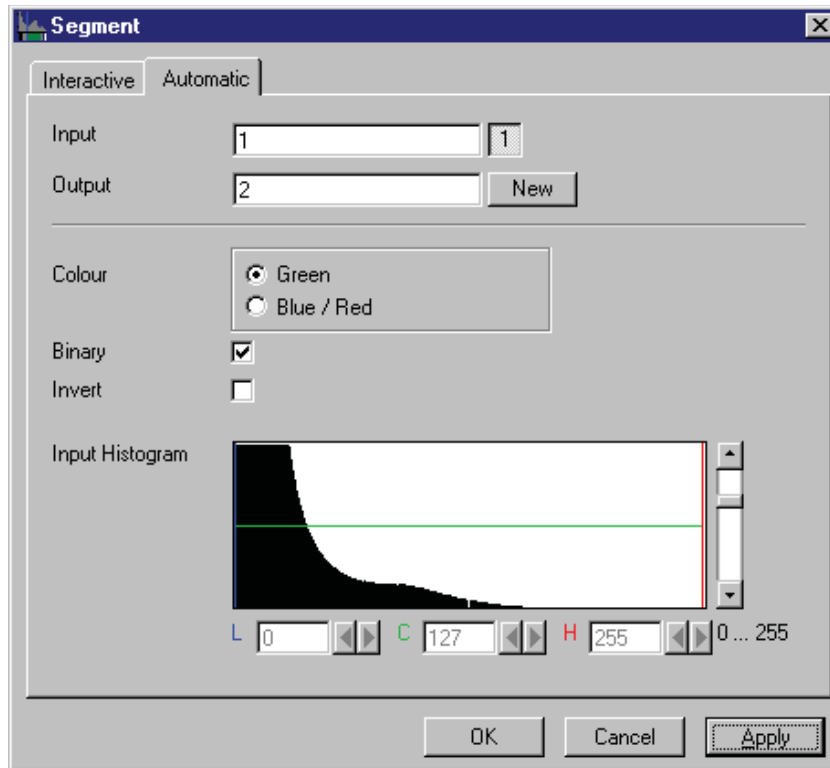
Fig. 6-25

The **Automatic** tab sheet of the **Segment** dialog window must be selected. Segmentation is especially used to generate binary regions. These are required for the measurement.

The function calculates the two strongest local minimums in the histogram of the **Input** image sequence. These values are used for the discrimination. Only one channel of a multichannel sequence can be selected as **Input**. **Output** will always be a single channel sequence. The vertical scaling of the histogram can be adjusted with the scroll bar at the right edge of the histogram. This setting has no influence on the function.

The **Green** and **Blue/Red** option buttons of the parameter **Colour** determine whether the voxels within (**Green**) or outside (**Blue/Red**) of the grey value interval [**L, H**] are displayed with the corresponding colour.

If **Green** is selected, the voxels within the selected interval are highlighted in green. The rest of the image retains its original grey values. The voxels with the grey values **L**ow and **L**ow+1 are displayed in blue. The voxels with the grey values **H**igh and **H**igh-1 are displayed in red.

If **Blue/Red** is selected, the voxels with grey values within the interval **L**ow, **H**igh remain unchanged. Voxels with grey values less than **L**ow are highlighted in blue; those with grey values higher than **H**igh are highlighted in red.

If the **Invert** option is selected, the grey values outside the defined interval will be segmented.

If the option **Binary** is selected, then all grey values in the range from **L**ow to **H**igh will be set to white (grey value 255 (4095)) in the **Output** image sequence, while all others will be set to black (grey value 0). If the option is not selected, the grey values within the selected interval remain unchanged, while those outside the range will be set to black.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Colour	Green - Selected interval is displayed in green Blue/Red - Grey values below the selected interval are displayed in blue, grey values above in red
Binary	0 - Selected voxels retain the original grey value 1 - Selected voxels are set to grey value 255 (4095), the rest to grey value 0
Invert	0 - Grey values inside the selected interval are segmented 1 - Grey values outside the selected interval are segmented
L	Low grey value threshold
C	Center of threshold interval
H	High grey value threshold

Boolean - And

This function carries out a bit-by-bit **And** calculation for the image sequences **Input 1** and **Input 2**.
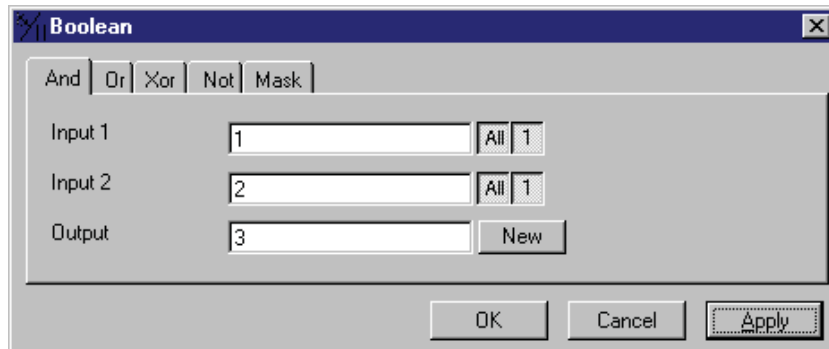
Fig. 6-26

The **And** tab sheet of the **Boolean** dialog window must be selected.

This function is especially well suited for masking images.

If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Resulting image sequence

Boolean - Or

This function carries out a bit-by-bit **Or** calculation for the images **Input 1** and **Input 2**.
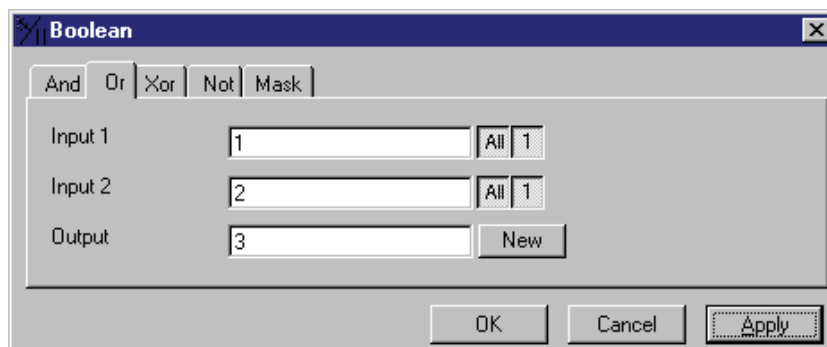
Fig. 6-27

The **Or** tab sheet of the **Boolean** dialog window must be selected.

This function can be used to combine binary masks or regions.

If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Resulting image sequence

Boolean - Xor

This function carries out a bit-by-bit **Xor** calculation for the images **Input 1** and **Input 2**.
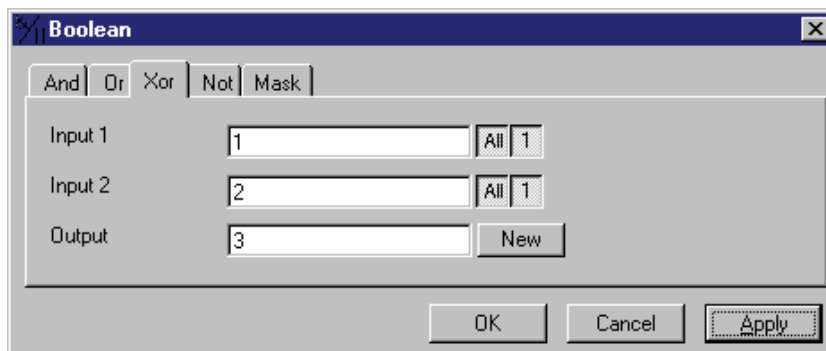
Fig. 6-28

The **Xor** option button of the **Function** option group in the **Boolean** dialog window must be selected.

This function can be used to combine binary masks or regions.

If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Resulting image sequence

Boolean - Not

This function carries out a bit-by-bit negation of an image.
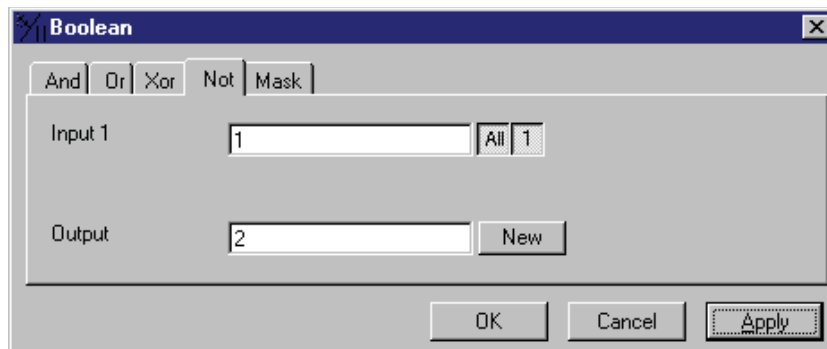
Fig. 6-29

The **Not** tab sheet of the **Boolean** dialog window must be selected.

If **Input** is a multichannel sequence any number or combination can be selected.

Parameters:

Input	Input image sequence
Output	Resulting image sequence

Boolean - Mask

This function masks a grey value image sequence.
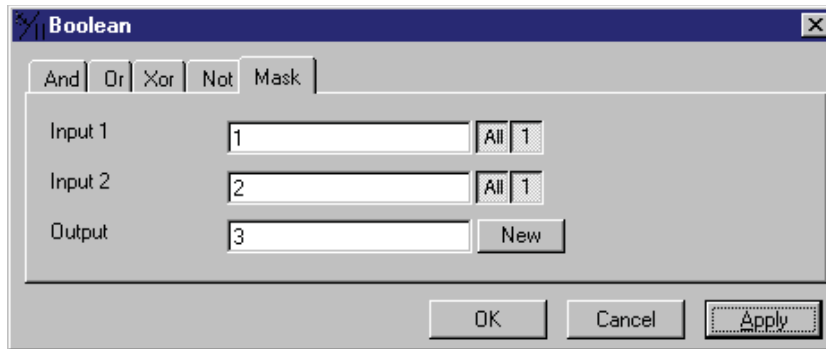
Fig. 6-30

The **Mask** tab sheet of the **Boolean** dialog window must be selected.

This function modifies the **Output** image sequence depending on the mask image sequence used.

If the grey value in **Input 2** is higher than 0, then the voxel values are copied from **Input 1** to the image sequence **Output**. If the grey value of the voxel is 0, then the voxel value of the **Output** image sequence is taken over.

If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 2** must be 1 or the same as for **Input 2**. They will be combined from left to right.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Resulting image sequence

Scrap

This function deletes or selects objects in a specified size range.
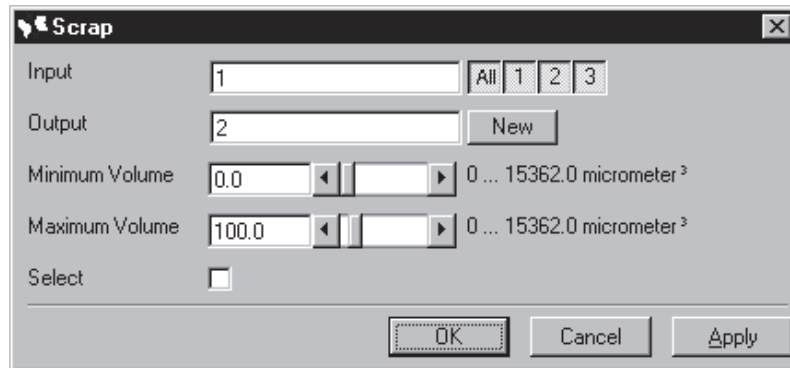
Fig. 6-31

The operation deletes or selects objects on the basis of their total volume in voxels. Objects with a volume within the range **MinVolume** to **MaxVolume** are effected.

To delete objects outside the range, the parameter **Select** must be active. If the parameter is not activated objects outside the defined volume range are deleted.

Parameters:

Input	Input image sequence
Output	Output image sequence
MinVolume	Minimum object size
MaxVolume	Maximum object size
Select	0 - Select the objects outside the size range 1 - Select the regions within the size range

Fill Holes

This function fills holes in all objects.
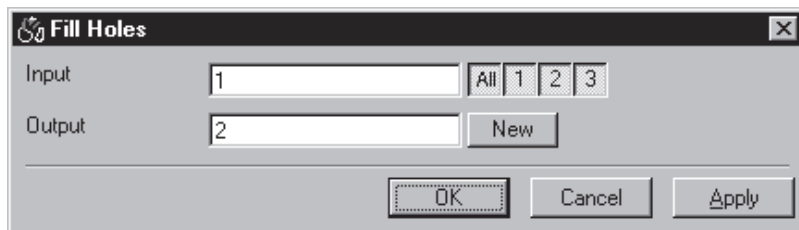
Fig. 6-32

All holes in objects are filled by this operation. Holes are structures, which have a grey value of 0 and are surrounded completely by voxels with a grey value not equal to 0. It is assumed that regions outside the image are black. Holes, which touch the image border, are retained.

Parameters:

Input	Input image sequence
Output	Output image sequence

6.1.3.4 Functions in the View Menu

Render - Surface

This function displays an image sequence according to the **gradient shading method**.
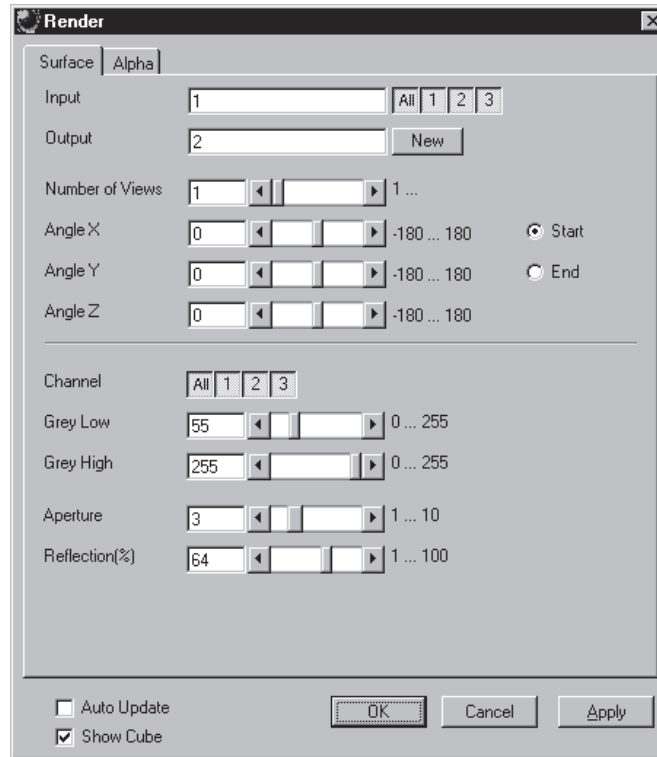
Fig. 6-33

The **Surface** tab sheet of the **Render** dialog window must be selected.

Method

The **Input** sequence defines the data to be reconstructed. If it is a multichannel sequence one or all channels can be selected for the reconstruction.

Output sets the name of the result image (sequence). If the sequence exists it is overwritten. Pressing the button **New** will generate a new name (number). The size of the sequential images in **Output** is determined by the size of the sequential images in **Input**.

Number of Views determines the number of reconstructions which should be computed. The radio buttons **Start** and **End** define which angle settings are currently shown. A definition for the angle **End** is only necessary if **Number of Views** is higher than 1. If this is true the result sequence will get views from the **Start** to the **End** angle definition. The other reconstructions are determined through the linearly interpolated intermediate angles. The direction of view is determined from the angles as follows:

The angle **Angle Z** determines the rotation of the direction of view on the Z-axis. The angle **Angle Y** determines the rotation of the direction of view on the Y-axis that has been rotated by the angle **Angle Z**. The angle **Angle X** determines the rotation of the direction of view on an X-axis that is rotated by **Angle Z** and **Angle Y**.

Channel defines if the following parameters are valid for **All** or just for one. Defining the thresholds for the channels independently is useful if the grey value boundaries of the objects differ too much in the different channels. The thresholds **Grey Low** and **Grey High** define the grey value range of the objects.

The parameter **Aperture** is a measure of the size of the highlights. Small values generate large highlights. Large values generate small highlights (similar to a spot).

Use the parameter **Reflection** to control the ratio of diffuse and reflective brightness components, i.e., the overall basic brightness compared with the highlights. When the value of **Reflection** is low, the highlights predominate; when the values are high, the region appears to be uniformly illuminated and the highlights are not so pronounced. When **Auto Update** is selected, the reconstruction is updated automatically whenever a parameter is modified (except **Input, Output**, or **Number of Views**). **Show Cube** defines whether a wire frame cube is shown in the **Display window** or not.

Application

This method can be applied, if the structures in the **Input** sequence can be segmented by grey value thresholding. Because the gradient is calculated for every pixel, the **Output** appears in very fine detail.

Noisy **Input** sequences must be smoothed (function **Smooth**) before rendering, otherwise the surface appears rough.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Number of Views	Number of reconstructions to be calculated
Angle X	Angle of rotation on the X-axis, start position
Angle Y	Angle of rotation on the Y-axis, start position
Angle Z	Angle of rotation on the Z-axis, start position
Channel	All - The following parameters are valid for all channels X - The following parameters are valid for the selected channel only
Grey Low	Low grey value threshold of the region to be displayed

Grey High	High grey value threshold of the region to be displayed
Aperture	Measure of the extent of the highlights
Reflection	Weight of the defuse brightness components in comparison to the highlights
Auto Update	0 - Function execution is performed on **OK** or **Apply** 1 - Function execution for the current angle is performed on any parameter change
Show Cube	0 - The wire frame cube is not shown 1 - The wire frame cube is shown in the **Display window**

Render - Surface: Method Description

This method displays the surface of structures in the **Input** sequence shaded as if a light illuminated it. The position of the light is behind the view point with parallel rays in the direction of the sequence.

The input sequence is segmented into object and background by grey value thresholding: object voxels are within the grey value range **Grey Low** to **Grey High**.

Each **Output** pixel corresponds to a point at the surface at which the ray in view direction through the **Output** pixels hits the surface. All rays are parallel.

The surface normal required for shading in this gradient renderer is the grey value gradient in the **Input** volume at the surface voxel position. It is not the geometric surface normal. The grey value gradient is determined from the grey values in a 3x3x3 cube around the surface voxel by averaging e.g. the x-gradient in y- and z-direction [4].

There is no depth cueing (far objects would appear darker).

The illumination model is a Phong model [1] (surface normal is determined for each **Output** pixel) with diffuse reflection and specular reflection. Diffuse reflection means that the surface reflects light with equal intensity in all directions. The brightness of a given surface patch depends not on the view-direction, but only on the angle between light and surface normal. Specular reflection is observed on shiny surfaces as a highlight. The light is reflected as from a mirror. The maximum intensity is observed when the view direction is the one of the mirrored light direction.

Render - Alpha

This function displays an image sequence according to the **alpha rendering method**.
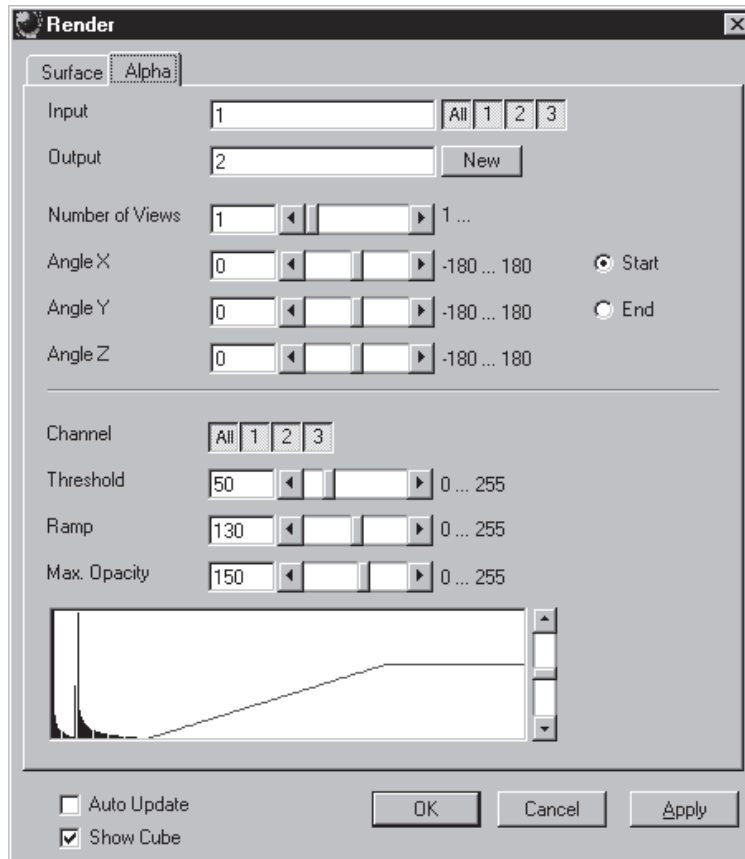
Fig. 6-34

The **Alpha** tab sheet of the **Render** dialog window must be selected.

One or more reconstructions of the input image sequence are computed according to the alpha rendering method. This type of reconstruction should be used if there is no possibility to segment the structures in the image sequence and also if the objective is to make deeply layered structures visible.

Method

The **Input** sequence defines the data to be reconstructed. If it is a multichannel sequence one or all channels can be selected for the reconstruction.

Output sets the name of the result image (sequence). If the sequence exists it is overwritten. Pressing the button **New** will generate a new name (number). The size of the sequential images in **Output** is determined by the size of the sequential images in **Input**.

Number of Views determines the number of reconstructions which should be computed. The radio buttons **Start** and **End** define which angle settings are currently shown. A definition for the angle **End** is only necessary if **Number of Views** is higher than 1. If this is true the result sequence will get views from the **Start** to the **End** angle definition. The other reconstructions are determined through the linearly interpolated intermediate angles.

The direction of view is determined from the angles as follows:

The angle **Angle Z** determines the rotation of the direction of view on the Z-axis. The angle **Angle Y** determines the rotation of the direction of view on the Y-axis that has been rotated by the angle **Angle Z**. The angle **Angle X** determines the rotation of the direction of view on an X-axis that is rotated by **Angle Z** and **Angle Y**.

Channel defines if the following parameters are valid for **All** or just for one. Defining the opacity for the channels independently is useful when the brightness and contrast of the channels differ too much. **Threshold** defines the range with no opacity. It is completely transparent. The range starts at grey value 0.

The length of slope is defined by **Ramp**. The maximum opacity value is set with the parameter **Max. Opacity**. This range ends at the maximum grey value. The **Opacity Table** shows the grey value histogram of Input with the opacity definition as a red line.

When **Auto Update** is selected, the reconstruction is updated automatically whenever a parameter is modified (except **Input, Output**, or **Number of Views**). **Show Cube** defines whether a wire frame cube is shown in the **Display window** or not.

Application

1. This method can be applied, if the structures in the Input sequence are unsharp so that objects are poorly defined by their grey value.

2. In this case, the Opacity Table is defined as a ramp. Low grey values have weight 0 to suppress the background voxels. The opacity rises with increasing grey values, depending on the parameter Ramp. The value of Max. Opacity defines the weight of the high grey values. High grey values above a threshold have weight 255 to show the "object" voxels unsuppressed. Of course a smooth step can be used.

3. The result is a display with inside structures shining through. A 3D impression can be obtained by rendering with several view directions.

4. In contrast to this, a voxel renderer like the gradient renderer would display only the surface of objects that are defined by grey value-thresholds. This surface would appear shaded as if illuminated by a light.

5. The method can also be applied to visualize pronounced structures within other enclosing structures, if the structures have different grey value ranges.

6. In this case, the Opacity Table is defined as a step. Low grey values (background) have weight 0. High grey values (inside structures) have maximum weight.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Number of Views	Number of reconstructions to be calculated
Angle X	Angle of rotation on the X-axis, start position
Angle Y	Angle of rotation on the Y-axis, start position
Angle Z	Angle of rotation on the Z-axis, start position
Channel	All - The following parameters are valid for all channels X - The following parameters are valid for the selected channel only
Threshold	Grey value where the opacity starts rising
Ramp	Length of the opacity slope
Max. Opacity	Maximum opacity value
Opacity Table	Maximum opacity value
Auto Update	0 - Function execution is performed on OK or Apply 1 - Function execution is performed on any parameter change
Show Cube	0 - The wire frame cube is not shown 1 - The wire frame cube is shown in the Display window

Render - Alpha: Method Description

Each **Output** pixel is a weighted sum of the **Input** voxels along a ray in view direction through the **Input** sequence. Each **Input** voxel has an opacity value, dependent only on its grey value. The opacity values are defined by the parameters **Threshold**, **Ramp**, and **Max. Opacity**.

Accumulation of pixels proceeds along the ray from back to front, i.e. from far pixels to near pixels. If a new pixel is added, it increases the result intensity by its grey value weighted by the opacity value, and attenuates the previously accumulated intensity according to the opacity value. Full intensity stops accumulation.

This calculation must be repeated for each pixel of the ray to generate one **Output** pixel. Then for each **Output** pixel to produce a 2D **Output** image for the selected view-angle. Then for each view-angle to produce an output sequence for **Number of Views** different view angles.

Render - References

[1] J.D. Foley,A.van Dam, S. K. Feiner, J.F.Hughes, Computer Graphics: Principles and Practice, Addison Wesley, Reading, MA, 1990.

[2] M. Levoy, Display of Surfaces from Volume Data, IEEE Computer Graphics & Applications, May 1988, 29-37.

[3] J. Ylä-Jääski, F.Klein, O. Kübler, Fast Direct Display of Volume Data for Medical Diagnosis, VGIP:Graphical Models and Image Processing 53,1991,7-18.

[4] K.H. Höhne, R. Bernstein, Shading 3D-Images from CT Using Gray-Level Gradients, IEEE Transactions on Medical Imaging, 5, 1986, 45-47.

[5] D.Gordon, R.A. Reynolds, Image Space Shading of 3-Dimensional Objects, CVGIP 29, 1985, 361-376.

6.1.3.5 Functions in the Measurement Menu

Measurement Concept

Measurement is based on regions (objects) in three-dimensional space. Segmenting an image sequence generates these. The image segmentation process produces a mask image that defines the region.

A region is a group of voxels that touch at the surfaces or at the edges, but not at the corners (18 voxel neighborhood).

This is illustrated by the following example. The voxels marked black in sequential image Z-1, Z, Z+1 all belong to the same region as the grey central voxel in sequential image Z. The volume view shows the neighborhood interrelationships as a 3D projection.
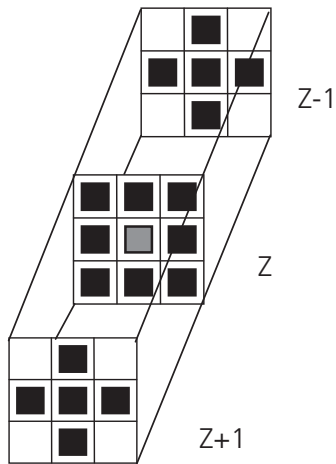
Sequential image: Volume view:
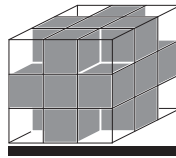
Fig. 6-35

Measurement Process

The measurement process consists of three steps: region definition, checking of the validity of the regions, and feature calculation.

Region definition: - Automatically from the mask image

Region validation check depends on: - Minimum volume
- Measurement condition

Feature calculation depends on - Shape of the region
- Densitometric value distribution of the region
- Feature parameters
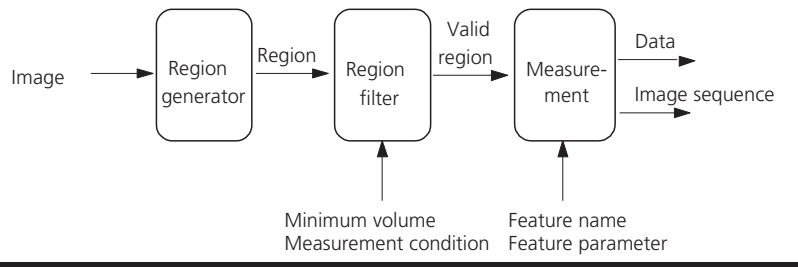
Fig. 6-36

All regions found are checked according to certain conditions. The voxel volume of each region must be equal to or greater than **MinVolume**. The measurement condition must be fulfilled. Only those regions that meet all the conditions are valid for the measurement. The region can be measured or labeled. Measurement is a process that produces data. Labeling is a process that generates an image volume.

Automatic Object Measurement – Object Features

A measurement feature describes a region characterized by a number (e.g. volume, area or a densitometrical statistic). The features can be selected on the **Object Features** and **Volume Features** tab sheets.
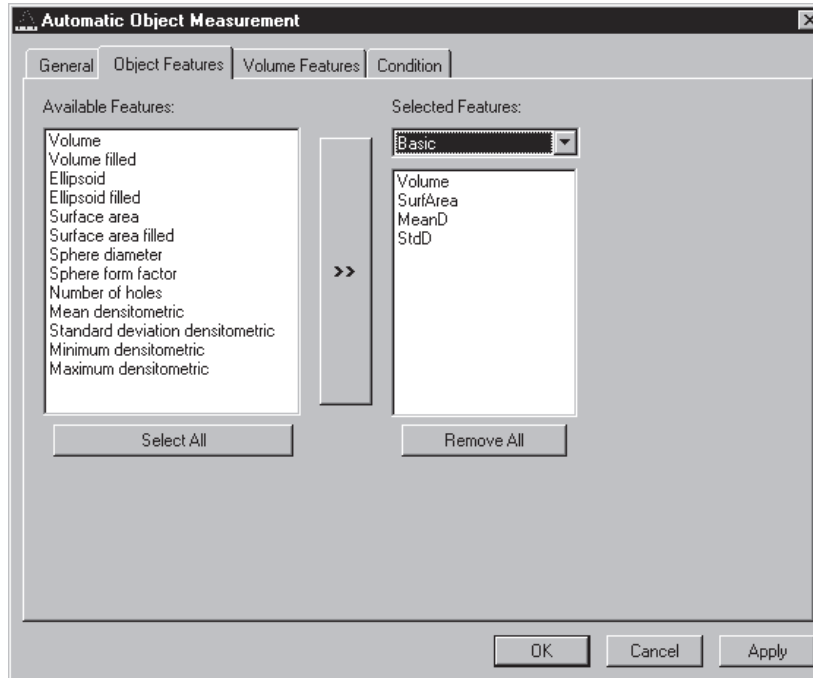
Fig. 6-37

The scalings and units are taken automatically from the assigned sequence.

The measurement features can be selected individually for each measurement. The object features generate a result value for every single object.

The dialog shows two lists. One shows the **Available Features** as groups (on the left). The other one shows the **Selected Features**. Double-clicking on items of the left list will add the **Selected Features** to the right list. Double-clicking on an item of the right list will remove this item from the list. **Selected Features** can also be transferred by clicking on the button in the middle (<< / >>) of the dialog.

The combo box above the right list represents predefined feature lists. Selecting one of the entries will fill the right list with these features; previously selected features will be overwritten.

The button **Select All** will copy all features to the list of selected features.

The button **Remove All** will clear the list of selected features.

Clicking on the **Apply** button will execute the measurement process and switch to the **General** tab sheet of the dialog.

Parameters:

Available Features	List of available object features
Selected Features	List of selected object features
Select All	Select all available object features for measurement
Remove All	Remove all object features from the selected features list

The following sections describe all measurement features which are defined in the system.

Object Features (geometric)

If **Object Features** are selected, one set of measurement data is calculated for each object.

Group Name	Name	Description
Volume	Volume	Volume of the object.
Volume Filled	VolumeF	Volume of the filled object.
Ellipsoid	EllipseA	Length of the main axis of the ellipsoid with the same geometrical moment of inertia as the object.
	EllipseB	Length of the middle axis of the ellipsoid with the same geometrical moment of inertia as the object.
	EllipseC	Length of the minor axis of the ellipsoid with the same geometrical moment of inertia as the object.
Ellipsoid filled	EllipseAF	Length of the main axis of the ellipse with the same geometric moment of inertia as the filled object.
	EllipseBF	Length of the middle axis of the ellipse with the same geometric moment of inertia as the filled object.
	EllipseCF	Length of the minor axis of the ellipse with the same geometric moment of inertia as the filled object.
Surface Area	SurfArea	Surface area of the object.
Surface Area Filled	SurfAreaF	Surface area of the filled object.
Sphere Diameter	Dsphere	Diameter of the sphere with the same volume. $\sqrt{6 * VOLUMEF / \pi}$
Sphere Form Factor	Fsphere	Form factor of the object. $6 \cdot \sqrt{\pi} \cdot \dfrac{VOLUMEF}{\sqrt{SURFAREAF^3}}$
Number of Holes	Nparts	Number of holes within an object.

Object Features (densitometric)

Group Name	Name	Description
Mean Densitometric	MeanD	Densitometric mean value of an object.
Standard Deviation Densitometric	StdD	Standard deviation of the densitometric values of an object.
Minimum DensitometricMinD		Minimum grey value of an object.
Maximum Densitometric	MaxD	Maximum grey value of an object.

Automatic Object Measurement - Volume Features

A measurement feature describes a region characterized by a number (e.g. volume, area, or a densitometrical statistic). The features can be selected on the **Object Features** and **Volume Features** tab sheets.
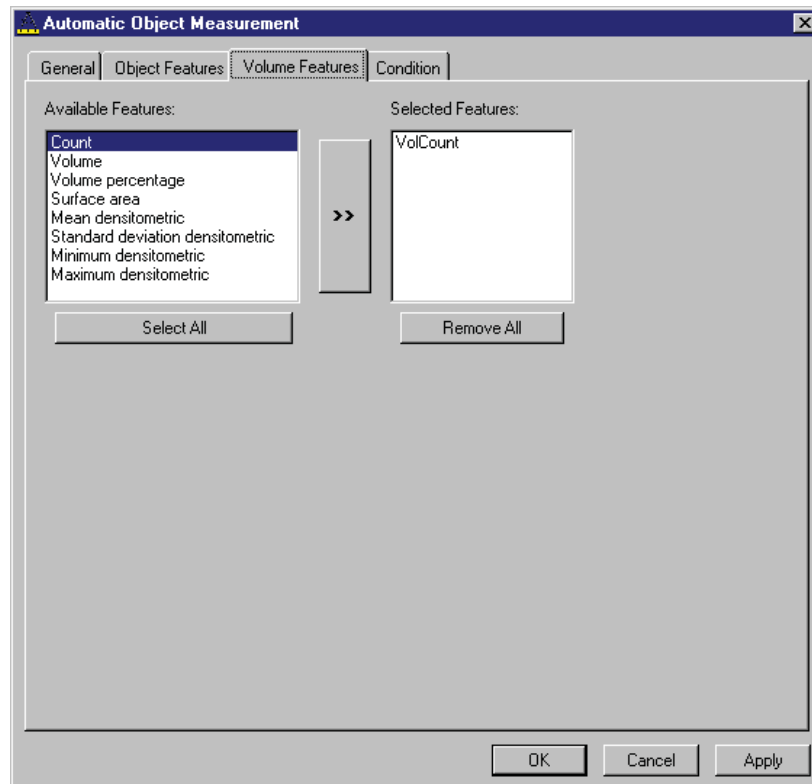
Fig. 6-38

The measurement features can be selected individually for each measurement. The object features generate a result value for every single object.

The dialog shows two lists. One shows the **Available Features** as groups (on the left). The other one shows the **Selected Features**. Double-clicking on items of the left list will add the **Selected Features** to the right list. Double-clicking on an item of the right list will remove this item from the list. **Selected Features** can also be transferred by clicking on the button in the middle (<< / >>) of the dialog.

The combo box above the right list represents predefined feature lists. Selecting one of the entries will fill the right list with these features; previously selected features will be overwritten.

The button **Select All** will copy all features to the list of selected features.

The button **Remove All** will clear the list of selected features.

Clicking on the **Apply** button will execute the measurement process and switch to the **General** tab sheet of the dialog.

Parameters:

Available Features	List of available object features
Selected Features	List of selected object features
Select All	Select all available object features for measurement
Remove All	Remove all object features from the selected features list

Volume Features (geometric)

The volume-related measurement generates one measured value per image sequence. The following table contains the predefined volume characteristics.

Group Name	Name	Description
Count	VolCount	Number of regions measured.
Volume	VolVolume	Total volume of all regions.
Volume Percentage	VolVolumeP	Total volume of all regions, in relation to the volume of the image sequence.

Volume Features (densitometric)

Group Name	Name	Description
Surface Area	VolSurfArea	Total surface area of all regions.
Mean Densitometric	VolMeanD	Mean grey value of all regions.
Standard Deviation Densitometric	VolStdD	Grey value standard deviation of all regions.
Minimum Densitometric	VolMinD	Minimum grey value in the image sequence.
Maximum Densitometric	VolMaxD	Maximum grey value in the image sequence.

Automatic Object Measurement - Condition

The measurement conditions are used to limit the objects to be evaluated (e.g. only objects with defined minimum value). All objects are tested against the defined conditions. If the conditions are fulfilled the feature values are written to the data table.
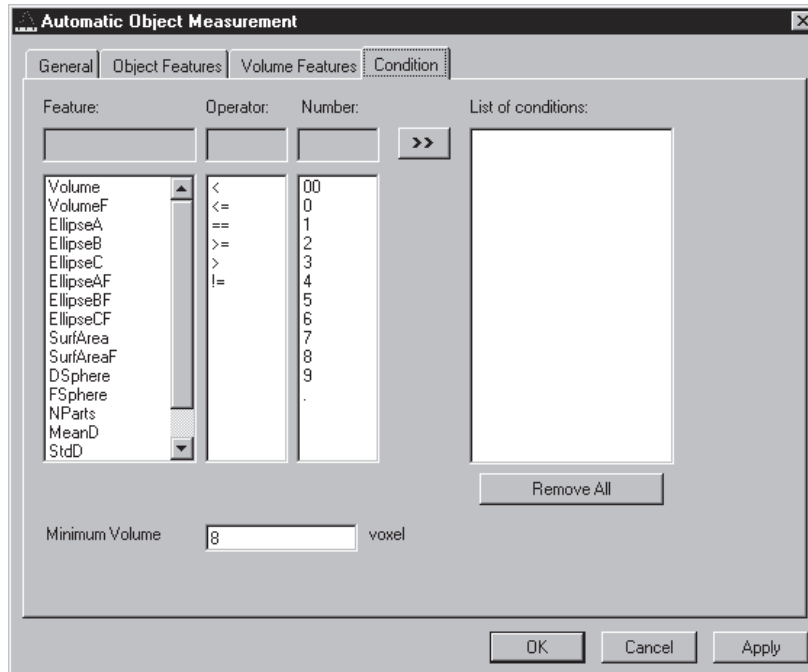
Fig. 6-39

To define the following parameter select the **Condition** tab sheet of the **Automatic Object Measurement** dialog window.

The list on the very left at the dialog shows all the measurement **Features**. The second list provides the comparison **Operators** and the next **Numbers** to define a value. This gives the possibility to compose an expression to test a feature value against a constant value. The fields above the lists will show the composed (selected) string. Clicking on the desired list entry does the selection. The button with the „>>„ characters adds this string to the **List of Conditions**. All lines of the **List of conditions** are combined with the AND expression automatically. To remove a condition line double-click on it.

The parameter **Minimum Volume** defines the minimum voxel volume for the measurement. This is an easy way to eliminate very small regions caused by noisy sequences and segmentation process.

The button **Remove All** will clear the list of defined conditions.

Clicking on the **Apply** button will execute the measurement process and switch to the **General** tab sheet of the dialog.

Parameters:

Feature	List of available object features
Operator	List of available condition operators
Number	List of numbers to compose the value
List of conditions	Defined condition list
Remove All	Remove all entries from the **List of conditions**
Minimum Volume	Minimum object volume in voxel

Automatic Object Measurement - General

This function carries out an automatic measurement and labeling.
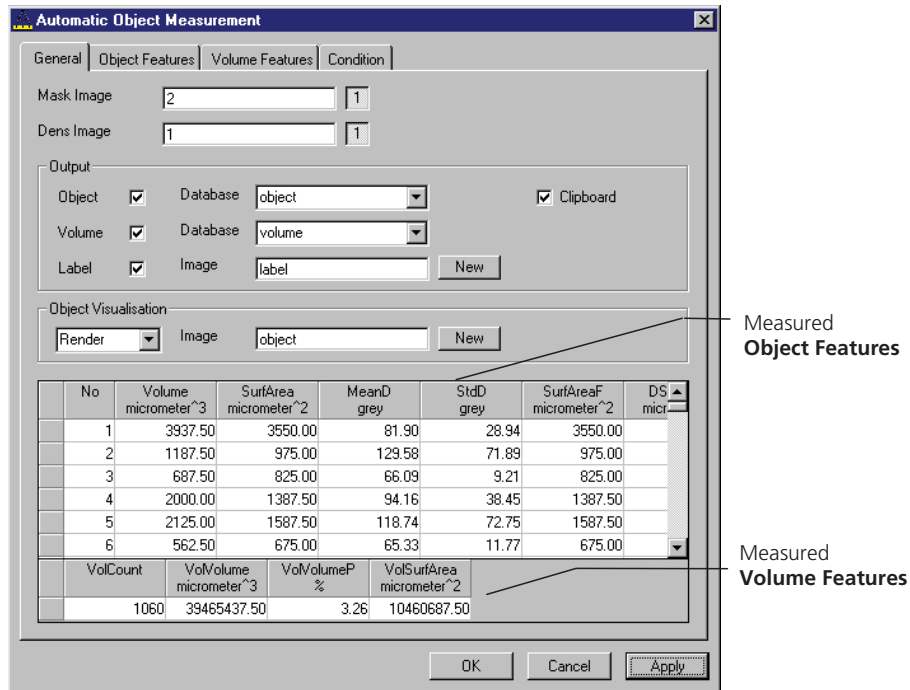
Fig. 6-40

The regions must be defined by an image sequence **Mask Image** (the objects must be separated from one another by black voxels with the grey value 0). This sequence is generated with the function **Segment**. If it is a multichannel sequence a single channel has to be chosen.

The image **Dens Image** is needed for the measurement of the densitometric features. Image sequence properties like scaling and unit are taken from **Dens Image**. A single channel of this sequence (if it is multichannel) must be selected with the buttons to the right of the parameter.

The measurement results can be stored to database files. These files are tab delimited ASCII files which can be easily imported to major Windows programs like text processing or spreat sheet application. Writing database files are independently supported for object and volume features. Activating the corresponding check boxes enables it. The name of the database is defined with the field **Database**. The files will be located in the subdirectory DATA of the main installation directory. The filename extension TXT will be added automatically.

If the check box **Label** is activated a single channel sequence will be generated. It contains all the measured objects, each object is coloured homogeneous but in different colours. To copy all measurement values to the clipboard activate the check box **Clipboard**.

A single object of interest can be visualized. Clicking on a specific row in the data grid chooses the object. By selecting a row in the data grid a new image is created with the object of interest visualized. The visualization depends on the settings in the **Object Visualisation** field. If **Render** is chosen, the object of interest is displayed with the **Surface Rendering** method. If **Mask** is chosen, the object is labelled in a pseudo colour in a new image stack.

Parameters:

Mask Image	Single channel mask image sequence that defines the objects
Dens Image	Image sequence for densitometric measurement and property source
Object	Stores measurement values of objects, including database filename
Volume	Stores volume measurement values of objects, including database filename
Label	Generates an image sequence with all objects labelled in different pseudo colours
Clipboard	Measurement values are automatically written to the clipboard

6.2   Change Filters

The **Change Filters** tool is used to update the filter data in the software after a change of filters in the reflector turret.

- Close the ZEN software program.

- Insert the new filter module in the reflector turret.

- Double-click on the **Change Filters** icon on the desktop.

  - The **Emission Filter & Beam Splitter Control** window appears on the screen. The name of the currently used database is displayed in the **System Database** box, with the filter type being indicated below for checking purposes.

  - The **Filter Cubes Stand** panel shows the **Filter-Wheel No.** and the filter positions available.

  - Use the **Name** and **ID** selection boxes to enter the filters installed in the individual positions of the filter wheel.

- Open the **Name** (or **ID**) selection box of the relevant filter position and select the new filter set from the list.

- Click on the **Store** button to accept the new settings.

- Click on the **Close** button to close the **Emission Filter & Beam Splitter Control** window.

☞   All available filter sets have to be registered in the filter list (see **Edit Filter List** function, next page).
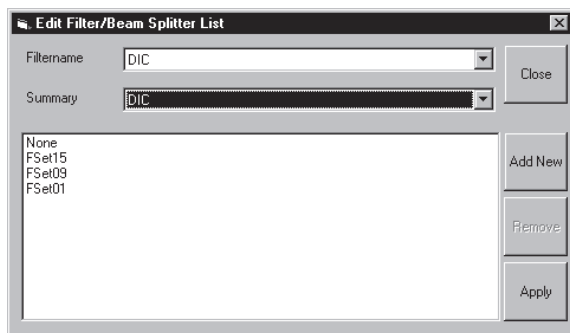
Fig. 6-41   Edit Filter/Beam Splitter List window

Edit Filter List

The **Edit Filter List** function permits updating of the filter data in the software after a change of filters on the stand.

- Close the LSM 5 Rel 4.2 or ZEN software program.

- Double-click on the **Change Filters** icon on the desktop.

- Click on the **Edit Filter List** button in the **Emission Filter & Beam Splitter Control** window.

  - The **Edit Filter/Beam Splitter List** window is opened.

This window permits a list of the most frequently used filter sets to be compiled.

- Click on the arrow button in the **Filtername** list box to open it.

- Select the filter set which shall be included in the list.

- Click on the **Apply** button.

The selected filter set is included and displayed in the list (below the **Sumary** list box).

This filter set is now also available in the **Name** selection boxes of the **Filter Cubes Stand** panel and can be assigned to a filter wheel position.

To remove a filter set which is no longer needed from the list, proceed as follows:

- Click on the name of the filter set concerned in the list box of the **Edit Filter/Beam Splitter List** window.

- Click on the **Remove** button. The filter set is deleted from the list and is then no longer available in the **Filter Cubes Stand** panel of the **Emission Filter & Beam Splitter Control** window.

Add New

This function permits new filter sets to be added to the database.

For this, proceed as follows:

- Click on the **Add New** button on the **Edit Filter/Beam Splitter List** window.

  – The **Add New Filter/Beam Splitter** window is opened.

- Enter the data of the new filter set in the **Filter Cubes Stand Description** panel, then click on the **Apply** button.

The new filter set is stored in the database and included in the **New Filter Cubes Stand** panel. You can now activate the filter for a filter wheel position using the procedure described above.

☞   If you have activated the **Non Zeiss** check box, filter sets from other manufacturers can also be included in the database.
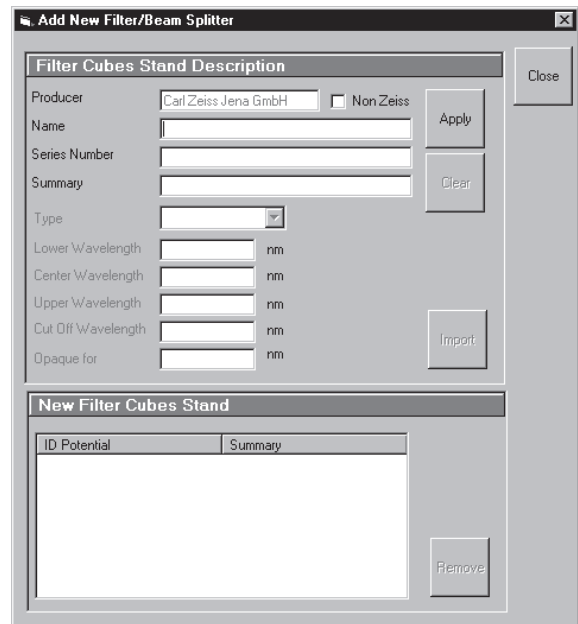
Fig. 6-42   Edit Filter/Beam Splitter List window

- To remove an new filter set from the database, select it with a click of the mouse in the **New Filter Cubes Stand** panel and then click on **Remove**.

- Click on **Close** to close the **Add New Filter/Beam Splitter** window.

- Click on **Close** to close the **Edit Filter/Beam Splitter** List window.

- Click on the **Store** button to accept the new settings.

- Click on the **Close** button to close the **Emission Filter & Beam Splitter Control** window.

When you start the ZEN software, the filter data are updated.
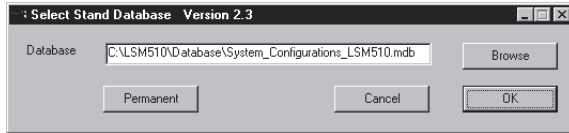
Fig. 6-43   Select Stand Database ... window

6.3   Stand Select

The **Stand Select** tool permits a new or updated database to be assigned to the LSM 5 Rel. 4.2 or ZEN software program. This function should preferably be performed by authorized service personnel.

If this is not possible, proceed as follows:

- Close the LSM 5 Rel. 4.2 or ZEN software program and double-click on the **Stand Select** icon on the desktop.

  – The **Select Stand Database** window appears on the screen. The currently used database is displayed in the **Database** box.

Fig. 6-44   Open window

- Click on the **Browse** button to activate the new database.

  – The **Open** window appears on the screen.

- Select the directory where the new database is stored.

- Click on the name of the database (file extension: ***.mdb**) and then on the **Open** button.

  – The **Open** window is closed and the name of the new database appears in the **Database** box.

- Click on the **Permanent** button. The **Select Name** window appears.

- Select the relevant stand icon from the **Icon** list box and click on **OK**. The **Select Name** window is closed and the desktop icon is updated.

- Then click on the **OK** button in the **Select Stand Database ...** window to accept the new settings and to close the window. (Clicking on **Cancel** will cancel the procedure.)

  – After the next restart of the LSM 5 Rel. 4.2 or ZEN software, the new database will be automatically loaded.

Fig. 6-45   Select Name window

CHAPTER 7   ANNEX

CONTENTS

7   ANNEX

7.1   Software and Hardware Options

This section describes optional software and hardware configurations. Depending on your configuration, the content of dialogue and function may differ.

7.1.1   Software

The following software packages for ZEN are available:

– Software "Physiology "
– Software "Topography "
– Software "Macro Recorder and Editor"
– Software "3D for LSM"
– Software "Multiple Time Series"
– Software "Image VisArt plus"
– Software "Deconvolution" (available with LSM 5 software Rel. 4.2 only, see respective manual)
– Software "StitchArt plus"
– Software "FRAP"
– Software "FRET"
– Software "Visual Macro Editor"

If your configuration does not include any of the SW packages "Physiology", "FRAP" or "FRET", the following functions are not available:

– Mean of ROI view type and analysis functions

If your configuration does not include the "Physiology" software package, the following functions are not available:

– Mean of ROI scan button in Time Series Control
– Ion Concentration analysis in the Process tab

If your configuration does not include the "FRAP" software package, the following functions are not available:

– Kinetics analysis view type.

If your configuration does not include the "FRET" software package, the following functions are not available:

– **FRET** Macro button in the **Macro** Menu and the FRET view button.

If your configuration does not include the "Visual Macro Editor" software package, the following functions are not available:

– **VME** button in the **Macro** Menu

If your configuration does not include the "Topography" software package, the following functions are not available:

– **Topo** view type in the **Image Display** document.

If your configuration does not include the "Macro Recorder and Editor" software package, the following functions are not available:

– **New**, **Save** and **Save as** buttons in the **Macro Control** window

– **Edit**, **Step**, **Delete**, **Editor** buttons in the **Macro Control** window

If your configuration does not include the "3D for LSM" software package, the following separate application is not available:

– **3D for LSM**

If your configuration does not include the "Multiple Time Series" software package, the following function is not available:

– Macro: "Advanced Time Series"

If your configuration does not include the "Image VisArt plus" software package, the following functions are only available in a basic, stripped down version.

– **3D** view type in the **Image Display** document

If your configuration does not include the "Deconvolution" software package, the following functions are not available

– **DCV Settings** button in the **Ortho** function of the **Image Display** window

– **DCV** button in the **Process** menu

If your configuration does not include the "StitchArt plus" software package, the following function is not available:

– Macro: "**StitchArt plus**" or Tile scan with overlap as Acahisition mode and Stitch function in the processing tab.

7.1.2   Hardware

Depending on whether the following hardware components are available or not, the content of the screens may differ:

– Piezo objective focusing device / Z-Piezo stage

- X-Y scanning stage (different types available)

- Axio Observer.Z1 / Axio Imager.Z1

- Depending on the configuration the scan head equipment may differ in filters, beam splitters and the number of photomultiplier

- Transmitted-light PMT

- Non Descanned Detectors (NDD)

- Monitor diode

- LSM *DuoSan*

If your configuration does not include the Piezo objective focusing device or the Z-Piezo stage, the following functions are not available:

- **Hyperfine Z Sectioning** in the **Z Stack** tool in the left tool area

- **HRZ** parameters in the **Stage and Focus Control** window

If your configuration does not include a X-Y scanning stage, the following functions are not available:

- **Stage** tool, **Tile Scan** tool and Positions tool in the left tool area

Depending on the used microscope stand: Axio Imager.Z1, Axio Imager.M1, Axioskop 2 FS MOT or Axio Observer.Z1, the following dialogue and available functions may differ:

- Context and accessibility of the **Ocular** tab in the **Light Path Tool.**

If your configuration does not include scan head META, monitor diode, transmitted light PMT and/or NDD, the following functions may differ:

- Context and accessibility of the **Light Path** tool

If your configuration does not include an AxioCam, the following functions are not available:

- **Camera** in the **Light Path** tool, **Channel** tool

If your configuration does include the LSM DuoScan the content of the Bleach tool varies.

7.2   Courses on "How to Operate the System in an Optimized Way"

Carl Zeiss is offering training courses on how to operate the system in an optimized way.

- Courses are held in our application center in Jena, Germany.

- Courses are held in English or German language, respectively.

Check out:

www.zeiss.de/lsm

for the latest dates and ask your Zeiss representative for a quotation on courses.

7.3   Beam path Configuration Guide for Fluorescence Microscopy with the LSM 5

7.3.1   Optical elements in the Configuration Control window

All wavelength values given in Nanometer [nm].

7.3.1.1   Main Dichroic Beam Splitter (HFT)

- A **HFT XXX[/YYY/...]** deflects the indicated laser lines onto the specimen and allows the emitted fluorescent light to pass through.
  Example: HFT 458/514, HFT UV/488/543/633 (deflects also UV excitation light)

- A **HFT KP XXX** (KP = Short Pass) is a special type of a main dichroic used for IR multiphoton excitation. The **HFT KP 650**, deflects laser light with a wavelength longer as 650 nm onto the specimen and allows fluorescent emission light in the visible range below 650 nm to pass through.

  Example: HFT KP 650

- A **HFT KP XXX_YYY** is a combination of a HFT YYY and HFT KP XXX used for simultaneous IR multi photon and single photon excitation.
  Example: HFT KP 700_488.

7.3.1.2   Secondary Dichroic Beam Splitter (NFT)

- The **NFT XXX** is used to split the emitted light which will be guided into separate channels. Light with shorter wavelength than XXX nm is deflected, light with longer wavelength passes the NFT. A cascade of NFTs allows to distribute the emission light to more than two channels/detectors.

- The **NFT KP YYY** treats the emission light the other way round: it transmits light shorter than YYY nm and deflects above YYY nm.

7.3.1.3   Emission Filters (EF)

- A **LP XXX** (Long Pass) transmits emission light with wavelengths longer than the indicated threshold value XXX.

- A **KP XXX** (Short Pass) transmits emission light with wavelengths shorter than the indicated threshold value XXX.

- A **BP XXX-YYY** (Band Pass) transmits emission light within the indicated wavelength band.

- A **BP XXX/BB** has a transmission band for emission light with a center wavelength of XXX nm and a width of BB nm.

- The **BG 39** (Blue Green glass) blocks infrared excitation light by absorption.

- **BP ... IR** (Band Pass – Infra Red) is a band pass suitable for detection of IR excited dyes. It blocks the IR light.

7.3.1.4   Plates and Mirrors

- **Plates** do transmit light 100%. They are used for a correct beam guidance and will be set automatically.

- **Mirrors** do deflect 100% over the whole spectral range and can be used to guide the emission light to selected detectors.

7.3.2   Setup of Tracks Using Single Detectors (channel mode)

a)   One track only – simultaneous acquisition of channels

- Switch on the suitable lasers for excitation of the dyes in the specimen. For the UV laser and the Argon laser set the tube current of the laser to a value of app. 50% (as described in chapter 4, section 4.5.2.2 of this manual). Example: for Alexa 488 and CY3 switch on **Argon** (blue excitation) and **HeNe1 or DPSS 561** (green excitation).

- Activate the proper laser lines in the **Line Active** check box, set **Transmission [%]** for each active line.
  Example: Select 488 to 4% and 543 to 50%

- Select a main dichroic beamsplitter (HFT) which deflects the selected laser lines to the specimen.
  Example: HFT 488/543
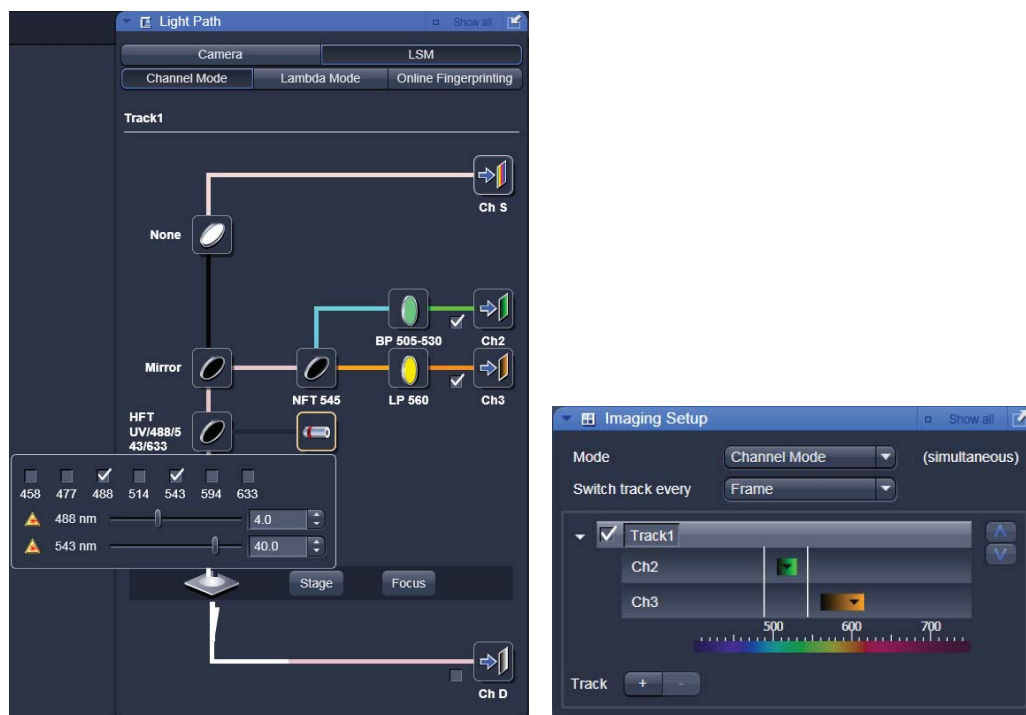
Fig. 7-1   *Light Path* tool, *Imaging Setup* tool and laser panel

- Check the available emission filters (EM 1- 4) for transmission of fluorescent light from the specimen, in order to identify the channels for detection.
  Example: BP 505-530 in channel Ch 2 for acquisition of green emission and LP 560 in channel Ch 3 for acquisition of red emission

- Use the secondary beam splitters (NFT 1/2/3) to split and guide the emitted fluorescent light to the detectors (PMTs) of the selected channels (see above).
  Example: a mirror in NFT 1 position will deflect all emission light to pass to Ch 2 and 3. NFT 545 will allow light longer than 545 nm to pass to channel Ch 3 and deflects light shorter then 545 towards channel Ch 2.

- Select the proper emission filters in front of the channels and activate channels.
  Example: select LP 560 in front of Ch 3, and BP 505-530 in front of Ch 2.

- Make sure that the active detection bands do not include any of the active laser lines
  Example: do not use a LP 505 for detection of green emission when using the 543 nm line for green excitation

Additional hints:

- Do not forget to turn on the corresponding lasers and detectors.

- Assign appropriate colors to these activated channels. Example: Ch 2 - green (for Alexa 488 emission, Ch 3 - red (for CY3 emission)

- The Spectra display in the *Imaging Setup* tool is a big help for checking if the configuration of the beam path was successful. It shows activated laser lines and for each channel the emission range that can be "seen" by the detector indicated by the corresponding channel color. If the detector is not switched on, the respective channel is not displayed in the *Imaging Setup* tool.

- When simultaneously detecting more than one fluorescent dye use channel Ch 1 for detection of the emission with long wavelength, then channels Ch 4 and Ch 3 (if available) for medium wavelengths and channel Ch 2 for short wavelengths.

- Use NFT 3 for separating emission into channels Ch 1 and Ch 4 and NFT 2 for separating emission into channels Ch 2 and Ch 3

b) Multiple tracks – sequential acquisition of channels

Multi-tracking is the method of choice for multi-fluorescence imaging. It allows to avoid artifacts from emission crosstalk that can occur in simultaneous excitation and detection of multiple dyes. To sequentially acquire multiple channels, laser lines are switched very fast and channels are recorded quasi-simultaneously.

The configuration of multiple tracks follows the same rules described above for a single track. The main difference is that each track is configured to excite and detected only one fluorescent dye to prevent cross talk (or two dyes with non overlapping emission spectra).

- Create a track for Alexa 488 as described above.

- Add a new track by clicking the [+] button and edit it as described above..

- The configuration shown in the *Light Path* tool refers to the activated track. To activate a track click on the grey bar on the right hand side of the track name. To include a track in the scanning process, activate it by ticking the box on the left hand side of the track name

- To extend the detection band for the green light it is now reasonable to use the LP 505 instead of BP 505-530 in track one. This is now possible since the green laser line is turned off during detection of the green fluorescence emission.

- Use the Spectra display in the *Imaging Setup* tool to check the proper settings for each individual track as described above.
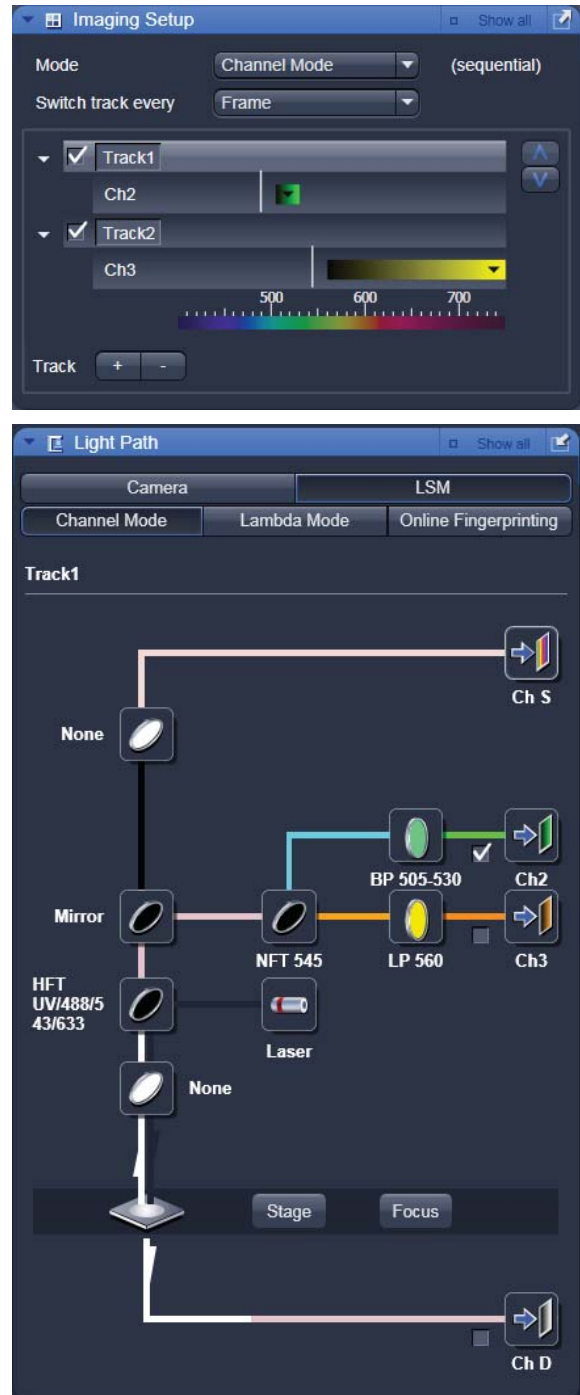
Fig. 7-2   Image Setup and Light path tools

Line and Frame Mode of Multitracking

- Settings can be used for **Line** or **Frame** wise multi-tracking acquisition.

- In Line mode the lines are scanned in turns for all tracks with the corresponding laser lines turned on exclusively. Preferred for living samples with moving objects. Acquisition time can be reduced using bidirectional scan mode.

- In Frame mode, the whole frames are scanned in turns for all tracks with the corresponding laser lines turned on exclusively. This mode can be advantageous for dyes that tend to bleach and need time to recover.

- There are parameters that can be changed quickly in a line wise acquisition: Amplifier Gain, Amplifier Offset, Laser Line Attenuation,

- Any other changes of track settings of the selected tracks, e. g. different filters, dichroics or Detector Gain settings, need a bit more time to be changed and therefore should be changed only when Frame mode is used.

- There is a fast Frame mode, which requires identical settings of these parameters.

- In our example it is now useful to use the BP 505-530 also in track two. It has no function in track two, but it guarantees equal settings in both tracks/channels, which in turn allows line wise Multi-tracking.

7.3.3   Beam path Configuration for Multi Photon Excitation

- The beam path configuration for multi photon excitation follows the same rules as described for a single and multi track configuration (see above, paragraph 7.3.2)

- Use a KP 650 or KP 700 as main dichroic beam splitter to deflect the IR excitation light (700-1050 nm) onto the specimen.

- On detection side, set always a BG 39 in the beam path or use a IR suitable band pass filters (BP XXX-YYY IR) to prevent a bleed through of IR excitation light to the detector.

7.4   Recommendations for excitation laser lines and emission filters of dyes

Dye	Laser line/HFT	Emission/EM
DAPI	364 or 405	> 385/420, max. at 461
EBFP	364 or 405	> 385/420, max. at 447
Hoechst	364 or 405	> 385/420, max. at 440
Fluoro-Gold	405 or 458	> 420/475, max. at 536
ECFP	405 or 458	> 420/475, max. at 501
Lucifer Yellow	458	> 475, max. at 536
EGFP	477 or 488	> 505, max. at 507/516
FM 1-43™	477 or 488	> 505, max. at 598
Alexa Fluor 488™	488	> 505, max. at 520
Calcium Green	488	> 505, max. at 531
Cy2™	488	> 505, max. at 508
DiO (DiOC18(3))	488	> 505, max. at 508
Fluo-3	488	> 505, max. at 520
Fluorescein (FITC)	488	> 505, max. at 520
Cy3™	514/532	> 530, max. at 566
EYFP	488 or 514/532	> 530, max. at 535
Oregon Green	514/532	> 530, max. at 535
SYTOX Green	514/532	> 530, max. at 536
FM 4-46	514 or 532/543	> 560, max. at 640
Alexa Fluor 546™	532/543	> 560, max. at 572
Calcium Orange	532/543	> 560, max. at 575
DiI (DiIC18(3))	532/543	> 560, max. at 565
DsRed	532/543 or 561	> 560, max. at 583
Tetramethylrhodamine (TRITC)	532/543 or 561	> 560, max. at 576
Rhodamine B	532/543	> 560/585, max. at 625
Texas Red™	561 or 594	> 570/600, max. at 620
Alexa Fluor 633™	633/635	> 650, max. at 654
Cy5™	633/635	> 650, max. at 666

Here you can note your specific combinations:

Dyes	Laser/HFT	EM1	NFT	EM2

Example:

Dyes	Laser/HFT	EM1	NFT	EM2
FITC/Cy3	488/543	BP 505-530	545	LP 560

7.5   Filter Change in the Detection Beam Path of LSM 510 Channels 1 and 2

For optimum investigation of specimens it is useful to employ filter wheels permitting the motor-controlled change between different filters for narrow-band or broad-band detection depending on the wavelength. The number of filters is limited by the capacity of the filter wheel. The change of the filter wheel as a whole involves complete readjustment.

The filter wheels of channels 1 (upper cover cap) and 2 (lower cover cap on the right side) of the Scanning Module have a change position in which a filter, including its mount, can be changed in a reproducible position without requiring readjustment. The filters can be rotated in their cells, and with the light path being eccentric relative to the filter center, the best transmission area of the filter for the respective wavelength or pass range can be found by rotating the filter. This is very important for the investigation of specimens of low emission.

Filter change

- By software control, move filter wheel (7-3/**5**) to the change position.

- Pull cover cap (7-3/**1**) off the Scanning Module.

- Use the filter tool (7-3/**2**) to pull the filter mount (7-3/**4**) with the filter (7-3/**3**) out of the guide well.

- Change filter to suit the application.

☞   The filter can be rotated in its mount, allowing adjustment for finding the best transmission area of the filter for the wavelength used.

- Enter the designation of this particular filter into the System Software database.
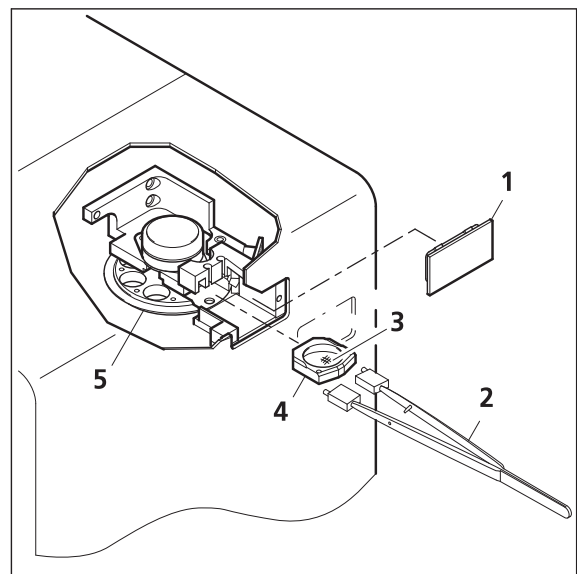
Fig. 7-3   Filter change

7.6   Changing Filters in the LSM 5 *LIVE* Scanning Module

For optimum investigation of specimens it is useful to employ filter wheels permitting the motor-controlled change between different filters for narrow-band or broad-band detection depending on the wavelength. The number of filters is limited by the capacity of the filter wheel. The change of the filter wheel as a whole involves complete readjustment.

The filter can be exchanged as a whole. Please ask your local LSM specialist to do this exchange in order to maintain your warranty.

Filter-wheel change

- Pull the filter-wheels carefully out of the appropriate guide well, see Fig. 7-4:
  - **1** – filter-wheel 1 for emission filters (EM 1)
  - **2** – filter-wheel for secondary dichroic beam splitters (NFT)
  - **3** – filter-wheel 1 for emission filters (EM 2)
- Use the filter exchange tool carefully and applying as little force as possible to pull the filter-wheel.
- Do not touch the filters.
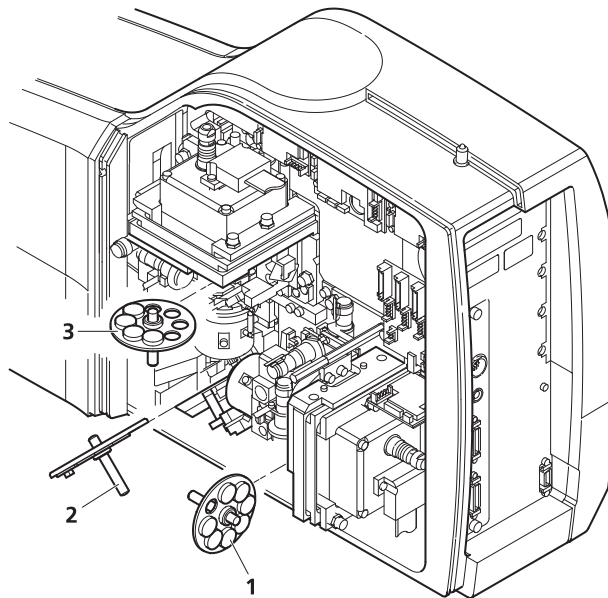
Fig. 7-4   Change-over of the filter-wheels

- Change filter-wheel to suit the application. Make sure that the filter-wheel is correctly fitted in the mount.
- Enter the designation of this particular filters into the System Software database.

7.6.1   Mounting and Dismounting the LSM 510 Scan Head

The scan head is connected to the microscope via an integrated safety interlock. It can be moved between two microscopes. Make sure the system is shut off completely before starting the following procedure:

☞   Be aware that the scan head weights up to 15 kg.

☞   Please see chapter 1 for safety relevant cables and connections.

Moving the scan head between Axio Observer.Z1, Axioskop 2FS MOT and Axio Imager.Z1:

- LSM 510 META scan head: Remove the blue cover from the back of the scan head (Fig. 7-5 and Fig. 7-6).
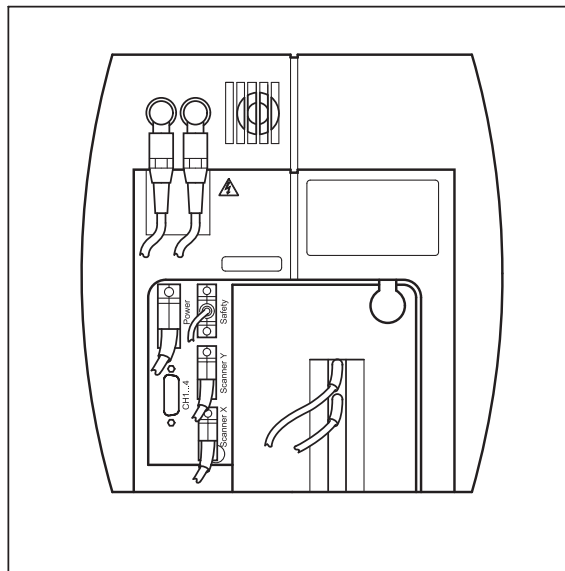
Fig. 7-5   **Rear view of META scan head**
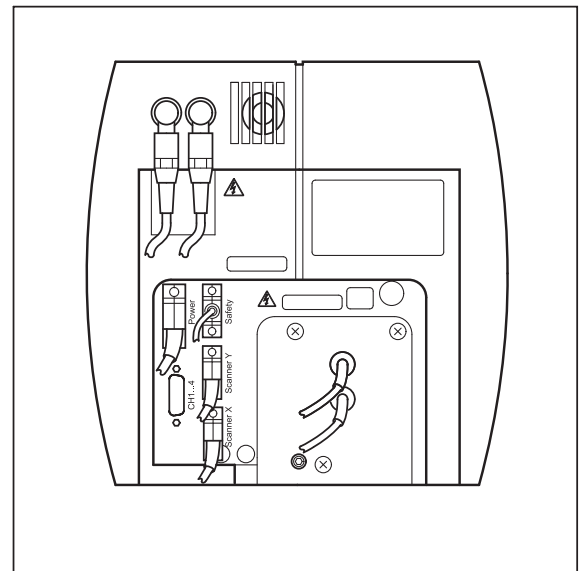
Fig. 7-6   **Rear view of META scan head with blue cover removed**

• Loosen the screw on the lower end of the scan head (Fig. 7-7/**1** and Fig. 7-8/**1**)
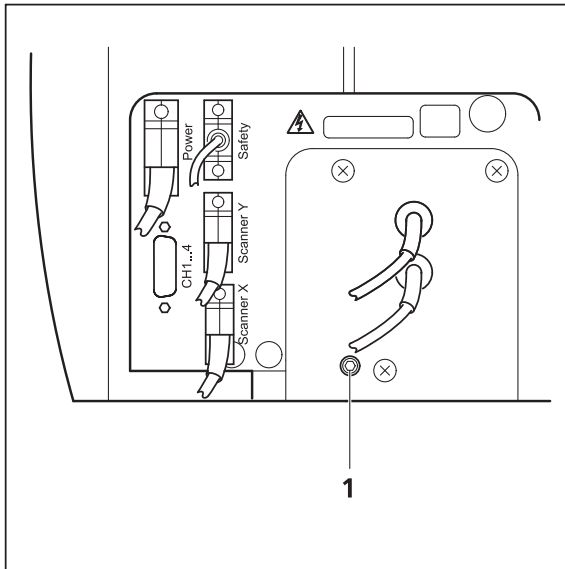
Fig. 7-7   Position of the screw on the LSM 510 META scan head
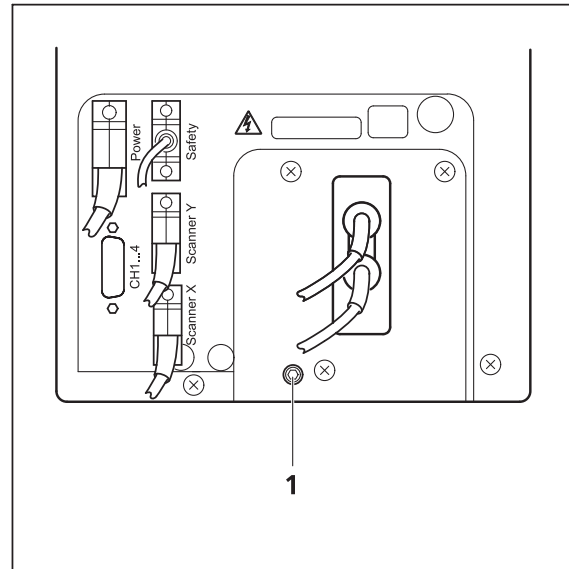
Fig. 7-8   Position of the screw on the LSM 510 scan head

• Loosen the screws on the port to the microscope for Axio Observer.Z1 (Fig. 7-9/**1**) or to the tube on Axio Imager.Z1 and Axioskop 2 FS MOT (Fig. 7-10/**1**).
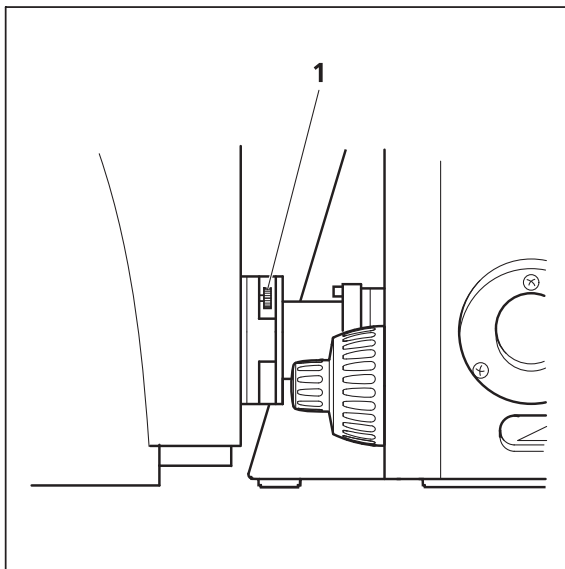
Fig. 7-9   Port connection between LSM and Axio Observer.Z1
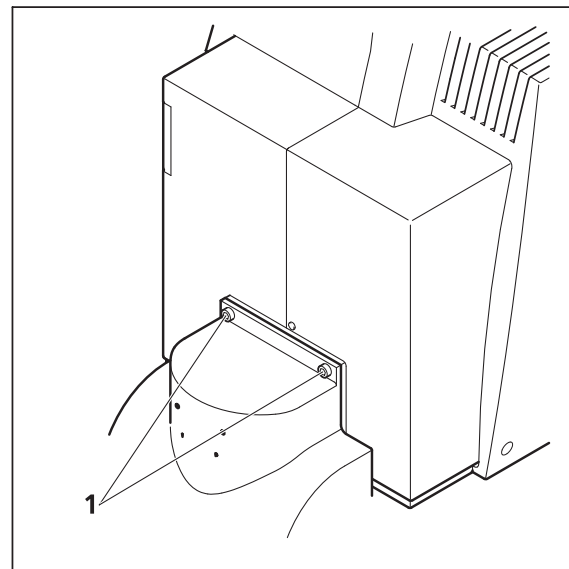
Fig. 7-10   Fastening screws of the scan head at the front of the tube on Axioskop 2 FS MOT and Axio Imager.Z1

• Slowly pull the scan head away from the microscope port or the tube. For mounting the scan head onto a microscope, make sure the pins and the electronic connections of the safety interface match closely. Fasten the screws on the front of the tube (Fig. 7-10/**1**) or at the microscope port (Fig. 7-9/**1**) followed by fastening the screw on the back of the scan head (Fig. 7-7/**1** and Fig. 7-8/**1**).

7.7 Detaching / Attaching the LSM 5 *LIVE* Scanning Module from / to Microscope Stands

Tool needed: 3 mm Allen key

☞ The user can remove the Scanning Module from one microscope and attach it to another within a few minutes. Described below is the change-over from an Axioskop 2 FS MOT to an Axio Observer.Z1 in sideport configuration.

⚠ **Before** the change-over, **shut down** the system as described in chapter 4 in order to avoid damage to the system and loss of data.

- Loosen the three screws (7-11/**1**) at the Scanning Module (7-11/**2**) fitted to the Axioskop 2 FS MOT.

- Cautiously pull Scanning Module off the Axioskop 2 FS MOT stand.

- Attach Scanning Module to the left sideport of the Axio Observer.Z1, minding the guide pins (7-11/**5**), and secure it with the three screws (7-11/**1**).

☞ As the Scanning Module is heavy, weighing about 19.5 kg, it is easier if the changeover is carried out by two persons.

- Pull off covering caps (7-11/**3**) from the CAN-BUS and RS232 interface ports at the rear of the Axio Observer.Z1, remove the two cables 457411-9011 (CAN-BUS) and 457411-9012 (RS232) from the Axioskop 2 FS MOT, plug them into the Axio Observer.Z1 and secure them there.

- Switch the LSM 5 *DUO* on.

- Click on the **Stand select** icon to update the system database with the new database of the Axio Observer.Z1 microscope.

- Restart the LSM 5 *DUO* program.

☞ Please see chapter 1 for safety relevant cables and connections.

☞ For optimal performance, an optical realignment of the LSM 5 *LIVE* scanhead is mandatory. Use the **Kollimatic** macro for this procedure, as described in chapter 4.

1   Screw
2   Scanning module
3   Cover cap
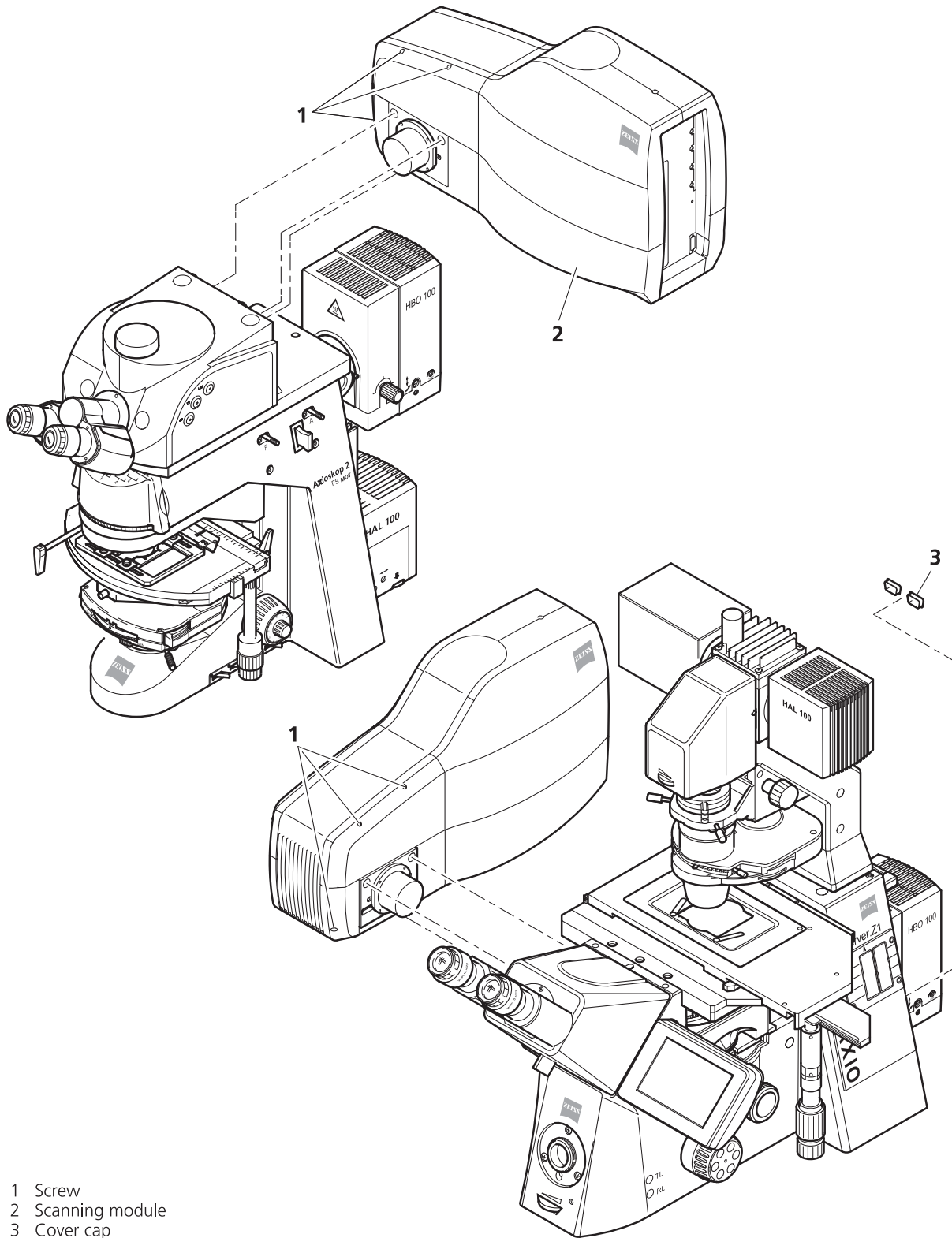
Fig. 7-11   **Change-over of the Scanning Module**

7.8   Changing Filters and the HFT Main / NFT Secondary Dichroic Beam Splitters in the Scanning Module of the LSM 5 *EXCITER*

For optimum investigation of specimens it is useful to employ filter wheels permitting the motor-controlled change between different filters for narrow-band or broad-band detection depending on the wavelength. The number of filters is limited by the capacity of the filter wheel. The change of the filter wheel as a whole involves complete readjustment.

The filter wheels of channels 1 (vertical filter wheel) and 2 (horizontal filter wheel) of the Scanning Module have a change position in which a filter, including its mount, can be changed in a reproducible position without requiring readjustment. The filters can be rotated in their cells, and with the light path being eccentric relative to the filter center, the best transmission area of the filter for the respective wavelength or pass range can be found by rotating the filter. This is very important for the investigation of specimens of low emission.

Removing the cover from the scanning module

- Close the software and switch off the instrument.

- Unscrew the two hex socket screws (7-3/**3**) on the customer side of the scanning module (7-3/**1**). The screws are secured against falling out and remain in the cover.

- Pull cover cap (7-3/**2**) off the Scanning Module.

Filter change

- Turn the filter wheel required into a position where the filter to be changed is accessible.

- Use the filter tool (7-4/**4**) carefully and applying as little force as possible to pull the filter mount with the filter (7-4/**1**) out of the guide well.

- Change filter to suit the application. Make sure that the filter is correctly fitted in the mount.

- Enter the designation of this particular filter into the System Software database.
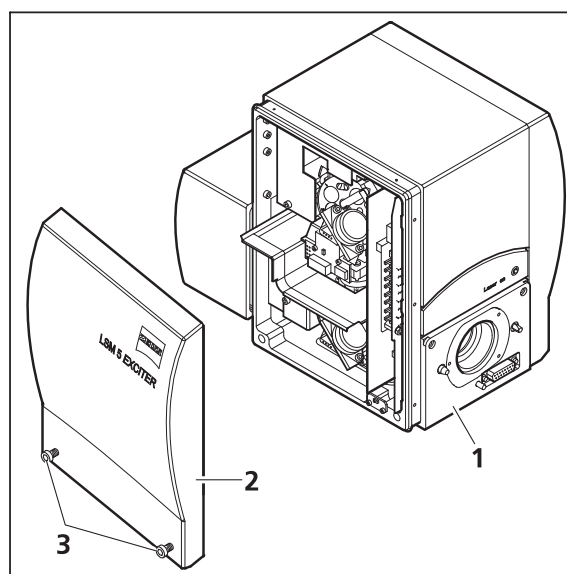
Fig. 7-12   **Scanning Module**

Changing the HFT main and NFT secondary dichroic beam splitters

The main (7-4/**2**) and secondary (7-4/**3**) dichroic beam splitters are identically mounted in the scanning module. Therefore, the changing procedure is also identical for both dichroic beam splitters. However, since the main dichroic beam splitter is more difficult to access, it should not be changed unless absolutely necessary.

- Carefully press the dichroic beam splitter (7-4/**3**) to the top left out of its bracket and remove it from the scanning module.

- Insert the new dichroic beam splitter, making sure that it is correctly fitted in the bracket.

☞   The white mark on the underside of the dichroic beam splitter is used for software initialization and must not be removed.

- After changing the filters or dichroic beam splitters, reattach the cover to the scanning module and tighten the two hex socket screws.
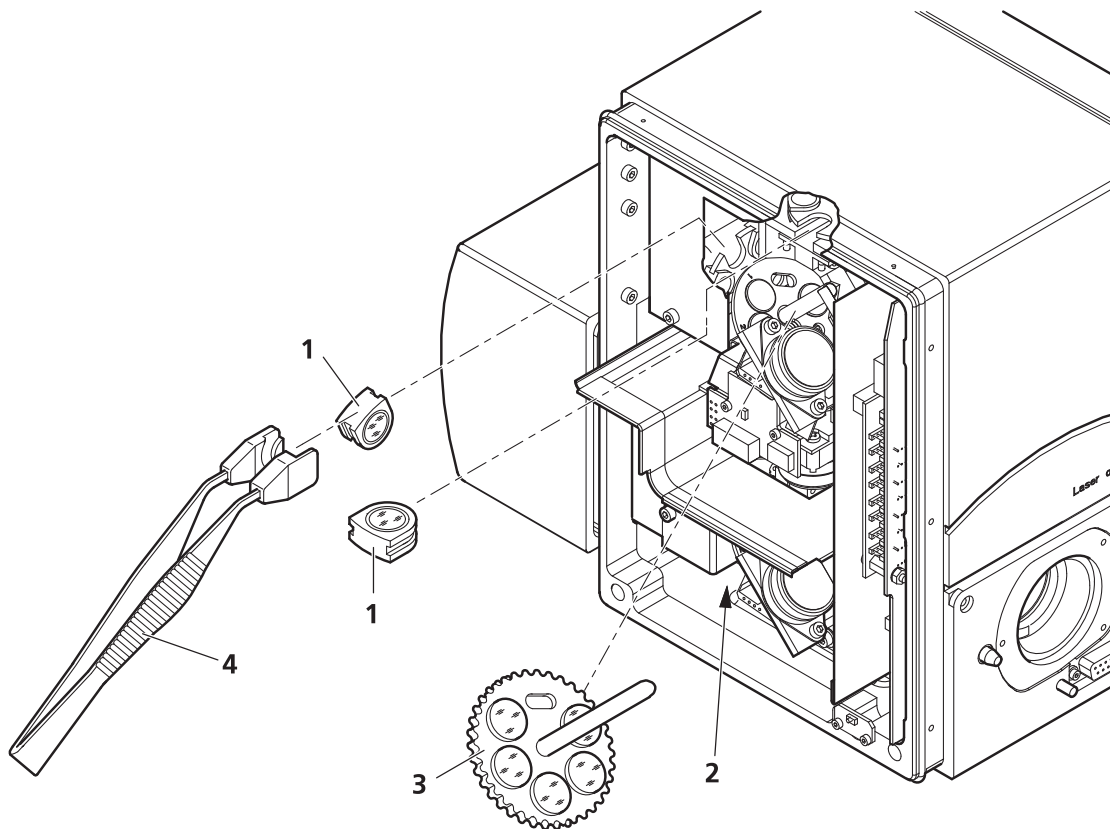
Fig. 7-13   Change-over of the Scanning Module

- Switch on the instrument again.

- Double-click the **Change Filters** icon in the PC desktop.

- Update the relevant filter data in the **Emission Filters & Beam Splitter Control** window.

- Start the LSM 5 *EXCITER* software program.

7.9 Detaching / Attaching the LSM 5 *EXCITER* Scanning Module from / to Microscope Stands

Tool needed: 3 mm Allen key

☞ The user can remove the Scanning Module from one microscope and attach it to another within a few minutes. **No adjustment** is required after the change-over. Described below is the change-over from an Axio Imager.Z1 to an Axio Observer.Z1 in baseport configuration.

⚠ **Before** the change-over, **shut down** the system as described in chapter 4 in order to avoid damage to the system and loss of data.

- Turn out both knurled-head screws (7-11/**1**) at the Scanning Module (7-11/**2**) fitted to the Axio Imager.Z1.

- Turn out M3 hexagon socket screw (7-11/**3**) with the Allen key.

- Cautiously pull Scanning Module off the Axio Imager.Z1 stand.

- Attach Scanning Module to the baseport of the Axio Observer.Z1, minding the guide pins (7-11/**6**), and secure it with the M3 hexagon socket screw (7-11/**3**).

- Fasten Scanning Module to the baseport with two hexagon socket screws (7-11/**5**), using an offset Allen key.

☞ As the Scanning Module is heavy, weighing about 14 kg, it is easier if the changeover is carried out by two persons.

- Pull off covering caps (7-11/**4**) from the CAN-BUS and RS232 interface ports at the rear of the Axio Observer.Z1, remove the two cables 457411-9011 (CAN-BUS) and 457411-9012 (RS232) from the Axio Imager.Z1, plug them into the Axio Observer.Z1 and secure them there.

- Switch on the LSM 5 *EXCITER*.

- Click on the **Stand select** icon to update the system database with the new database of the Axio Observer.Z1 microscope.

- Restart the LSM 5 *EXCITER* program.
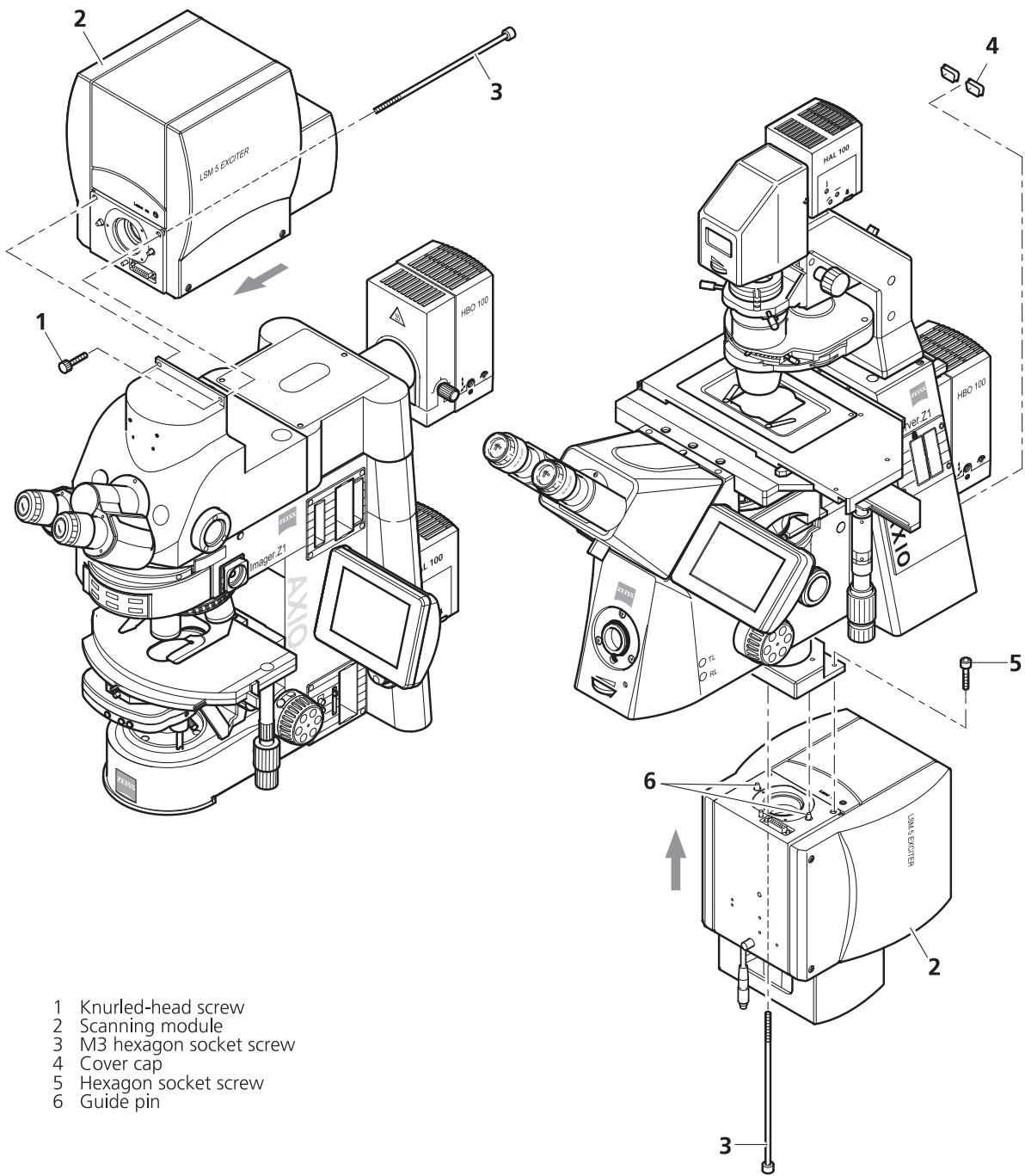
1 Knurled-head screw
2 Scanning module
3 M3 hexagon socket screw
4 Cover cap
5 Hexagon socket screw
6 Guide pin

Fig. 7-14   **Change-over of the Scanning Module**

7.10   The Use of the Piezo Fine Focusing Stage

7.10.1   General Description

The Piezo fine focusing stage is a compact attachment for the Axio Imager.Z1, Axioskop 2 FS MOT and Axio Observer.Z1 microscope stages, which allows the particularly fast and high-precision fine focusing of the object. The piezo stage permits fine focusing over a range of up to 250 µm (200 µm guaranteed), with the smallest step width being less than 10 nm, reproducibility better than 40 nm, and the maximum speed amounting to 60 Hz. The stage allows the use of specimens with a weight of less than 100 g.

The piezo stage is not used if manual coarse focusing is performed. To position the objective in relation to the optical Z-axis, the standard XY-microscope stage is used.

The piezo stage features a mount for standard object carriers of 76 mm x 26 mm x 1 mm and a milled-out receptacle for ∅ 36 mm x 1 mm Petri dishes.

7.10.2   Applications of the Piezo Fine Focusing Stage

 – High-precision fine focusing and translation of the object along the optical axis.

 – Fast and high-precision mounting of one-dimensional Z-line sections.

 – Fast and high-precision mounting of two-dimensional R-Z-longitudinal sections.

 – Fast and high-precision mounting of XY-Z-Stacks for the three-dimensional reconstruction of the object.

 – Exact measurement of Point-Spread-Functions for deconvolution.

7.10.3   Additional Information on the Operation

The piezo fine focusing stage is a high-precision, sensitive accessory for the LSM 5 systems from Carl Zeiss and must therefore be treated carefully.

High mechanical stress, such as the use of specimens weighing more than 100 g or the application of pressure or knocks on the movable stage tongue, can result in damage and therefore in failure of the stage function.

To be able to fully utilize the outstanding precision attainable with the fine focusing stage, anything which could interfere with its operation, especially mechanical knocks and impact of the LSM 5 system components, should be avoided. We would recommend you to always use the actively vibration-damped table.

The specifications of the stage are obtained only after a warm-up phase of approx. 30 minutes. Furthermore, the installation conditions for the LSM 5 system must be observed.

The maximum reproducibility (better than 40 nm) for moving to an absolute position in Z is achieved by always moving to the required position from below.

Fine focusing is performed mechanically via an inclined position of the stage tongue. Therefore, the lifting range Z at the location of the image field depends on the position of the piezo stage in relation to the optical axis. This means: if the user shifts the object on the microscope stage to the right via the piezo stage, the lift will be different from the one in the zero position of the stage (max. 250 µm) and also from the one after a shift of the stage to the left.

If the LSM 5 system is equipped with a motorized scanning stage, this shift is read back to Δx and the lift is calibrated automatically if the zero position of the piezo stage has been matched to the zero position of the scanning stage via an initialization run. For this, activate the **Stage** button of the **Acquire** toolbar. Then position the scanning stage in such a way that the optical axis of the microscope corresponds to the zero position of the piezo stage, i.e. to the center of the specimen holder in the stage tongue. Then perform initialization by pressing the **piezo Null** button. This step must be repeated after every new start of the system. Also see the notes on the operation of the motorized scanning stages.

If the system is equipped with a manual microscope stage, the user has the option of performing the calibration by entering the Δx shift in mm via the **Calibration** slider.

The shift is read off from the microscope stages. In the case of the manual AxioImager stage, Δx can be read directly from the scale adhered to the front of the stage. In the case of the manual Axio Observer.Z1 stage, a scale is located on the right of the knob, where the 45 mm Δx shift relative to the zero position of the microscope stage can be read off. The Δx value is positive for both stages if shift from the zero position is made to the right and negative if the shift is made to the left.

On account of the inclined position of the stage tongue, the object is also shifted laterally during the fine focusing motion. This lateral shift is negligibly small if, as recommended by us, specimen carriers with thickness 1.0 mm are used exclusively. Otherwise, the marked lateral shift of the object during fine focusing can result in image distortion. For the same reason, Petri dishes without fixation ring must be used exclusively.

The nosepiece of the Axio Observer.Z1 stand is moved to the load position prior to switching off the LSM 5 system and the piezo stage is then moved to the lowest position to avoid damage of the objective or object by a possible collision. The user must refocus after start-up of the system. Before an objective change in the Axio Observer.Z1 or the Axio Imager.Z1 the nosepiece and the microscope stage must be moved to the load position by the user, and then back to the work position to prevent the objectives from hitting the piezo components. This is performed automatically if the objectives are changed menu-controlled via the relevant buttons of the ZEN program.

7.11   Piezo Objective Focusing Device

For Axio Imager.Z1, Axio Imager.M1 and Axio Observer.Z1

Range: $\geq$ 200 µm

Minimum step size: 15 nm

Speed:

		Piezo objective focussing device
Slices	Step size [µm]	xz-lines / s
20	0.5	460

Objectives:

– W0.8/M27

– Modified Achroplan 40x / 0.8 W with reduced length to compensate for piezo height

Installation:

- Screw in your microscope objective into Piezo Objective Focusing Device (see Fig. 7-15/**1**).

- Screw the thread-ring into your microscope (see Fig. 7-15/**2**).

- Easy clamp the Piezo Objective Focusing Device on the thread-ring (see Fig. 7-15/**3**).
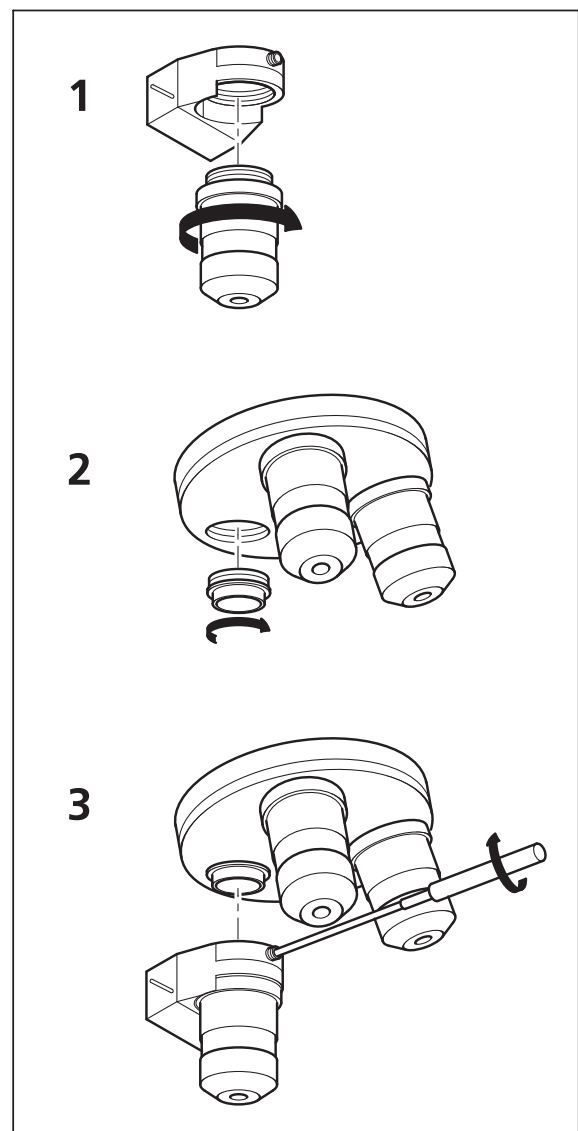
Fig. 7-15   Installation of the Piezo Objective Focusing Device

7.12 Z-Piezo Focussing Insert for Scanning Stages

For Axio Imager.Z1, Axio Imager.M1 and Axio Observer.Z1

Range: ≥ 200 µm

Minimum step size: 5 nm

Speed:

		Piezo objective focussing device
Slices	Step size [µm]	xz-lines / s
20	0.5	290
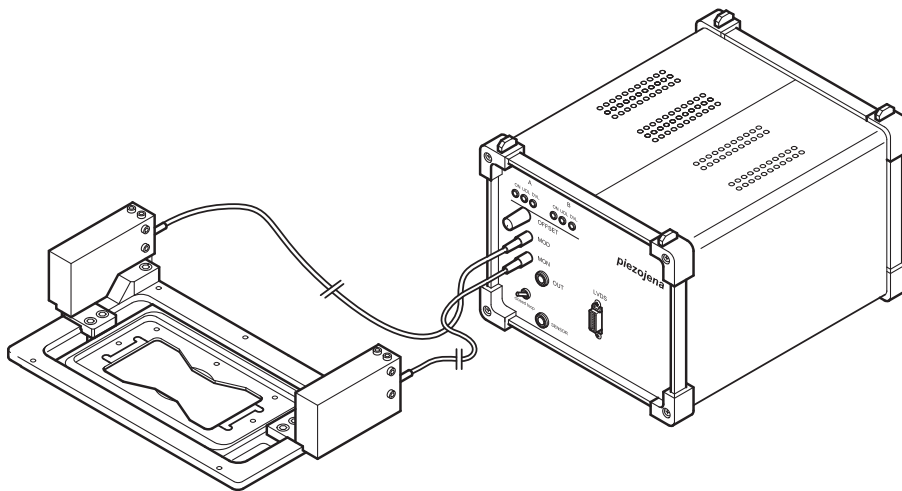
Fig. 7-16   Z-Piezo focusing insert for scanning stages

The Z-Piezo stage can only be mounted onto scanning stages. The insert is used instead of the standard inserts. The Z-Piezo insert needs to be srewed onto the scanning stage to ensure maximum fixation to the stage. It can hold classic objective slides and 35 mm Petri dishes.

7.13   Specifications of Trigger-Interface

Application:

With LSM 5 Family systems you can control various actions externally using Trigger-In or force external devices to work at a defined time depending on an action using Trigger-Out during time series.

These actions are: Scan-Start / Stop, Bleach, Change of Scan-Interval, end of a countdown, set marker into image or even a mouse-click on a button.

Interface:

– User Port Adapter on the back of the Real Time Electronics in the System Electronic Rack

– Connector 2x **15-point** high density D-Type plug, 1x coax with outer shield
  (Triax Lemosa Serie 00, EPL.00.650NLN)

Number:

– 8x signal IN (all able to generate an interrupt)

– 18x signal OUT (10 synchronous, 8 asynchronous relative scan position)

– 1x clock

Connector:

Coax: Triax Lemosa Serie 00, EPL.00.650NLN

Pin	Signal name	Description
Signal	PCLK_Out	PixelClock Output
Signal shield	GND	Signal ground
Outer shield	Shield	Chassis ground

The pixel clock is exactly symmetrical to the pixel. For example if the pixel rate is 10 kHz the high-level and the low lovel both have a time of 50 microseconds. The width of the TTL pulse therefore is 1/(2 x pixelrate).

15-point high density D-Type plug: Plug „A"

Pin	Signal name	Description	Software interface
1	SyncOUT0	Synchronous output	Trigger 1 out
2	SyncOUT1	Synchronous output	Trigger 2 out
3	SyncOUT2	Synchronous output	Trigger 3 out
4	SyncOUT3	Synchronous output	Trigger 4 out
5	GND	Signal ground	-
6	AsyncOUT0	Asynchronous output	-
7	AsyncOUT1	Asynchronous output	-
8	AsyncOUT2	Asynchronous output	-
9	AsyncOUT3	Asynchronous output	-
10	GND	Signal ground	-
11	AsyncIN0	input	Trigger 1 in
12	AsyncIN1	input	Trigger 2 in
13	AsyncIN2	input	Trigger 3 in
14	AsyncIN3	input	Trigger 4 in
15	Connector detector*	Input / Connector detector	-

15-point high density D-Type plug: Plug „B"

Pin	Signal name	Description	Hardware output only
1	SyncOUT4 **	Synchronous output	Sync. signal: Stack out
2	SyncOUT5	Synchronous output	-
3	SyncOUT6 / sync outline **	Synchronous output	Sync. signal: Line out
4	SyncOUT7 / sync outframe **	Synchronous output	Sync. signal: Frame out
5	GND	Signal ground	-
6	AsyncOUT4	Asynchronous output	-
7	AsyncOUT5	Asynchronous output	-
8	AsyncOUT6	Asynchronous output	-
9	AsyncOUT7	Asynchronous output	-
10	GND	Signal ground	-
11	AsyncIN4	input	-
12	AsyncIN5	input	-
13	AsyncIN6	input	-
14	AsyncIN7	input	-
15	Connector detector*	Input / Connector detector	-

*) If the pin is connected to ground, the software recognizes that at least one connector is connected to the UserPort2.
**) The position of the two switches on the UserPortAdapter inside the RT-PC must switch thrown towards enclosure = default position.

Timing of the synchronization signals Stack out, Line out and Frame out

These signals are directly generated by the hardware and cannot be influenced by the software. They are also not seen by the user software and cannot be used to generate any markers within the image acquistion. They are highly precise tools to control external hardware. The signals are precise to the pixel and depending on the chosen pixel time the time between the signal and the start of the image acquistion varies.

The Line out signal is set 44 pixels before the first image pixel of each line.

The Frame out signal is set 42 pixels before the first image pixel of each frame.

The Stack out signal is set 40 pixels before the first image pixel of each Z-stack.

The signal itself is identical to the signal of the pixelclock but is only set for one pixel.

Type/Voltage Range:

– TTL signal level 3.3 V, CMOS low power consumption

– 5.0 V tolerant input/output for interfacing with 5 V logic

Load:

Output: <50 mA (internal serial 68 ohm resistor)

Input: 4.7 kOhm input impedance (internal 4.7 kOhm pullup to 3.3 V)

Trigger pulse description:

Signal output:

– Low level <0.4 V, high level >2.7 V

– Slew rate 10 ns/V

Signal input:

– Low level <0.5 … 0.8 V, high level >2.0 … 5.5 V

– Falling edge force interrupt

– Pulse width to detect signal >50 ns

Caution:

– Never apply more than 5 V or negative voltages to avoid any damage.

– In and outputs are not galvanically decoupled.

– Therefore proper measures for galvanic decoupling of external devices have to be taken (opto-coupler etc.).
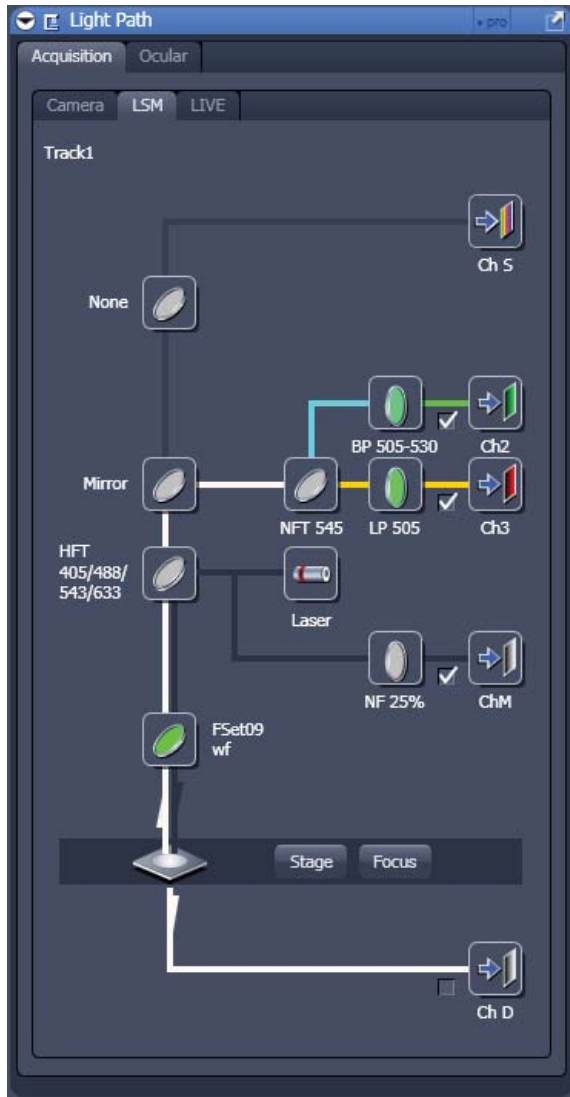
Fig. 7-17   Light Path tool with Monitor Diode

7.14   The Monitor Diode

The monitor diode is placed in the excitation ray path of the LSM 510 behind the beam splitter combining the visible and the UV ray path and in front of the main beam splitter. It allows to monitor the laser power input to the system and the noise over time. An attenuation filter wheel is placed in front of the diode. It is not possible to select one line out of a few excitation wavelengths to be detected by the diode. The Monitor Diode is displayed only in **Show all** mode.

Proceed as follows to activate the diode as a detector:

- Click on the corresponding button in the **Light Path** tool of the ZEN software (**Show all** mode).

- Choose either **Frame** or **Line** scan.

- Start a **continuous** scan: the system will scan with the diode as an additional channel.

- Choose the right amplification of the signal in the monitor diode channel by using the special neutral density filters or / and by using the setting of the **Amplifier Gain** and **Amplifier Offset** value. **(Channels ChM-1).**

Application examples:

a)   Checking the laser power

This function is not automated so far. To qualitatively measure the laser power, the diode can be used in such a way that the gray level obtained in the **Line Scan** mode at a certain setting of the whole system is stored as a text overlay together with the image (manually done by user). As the diode setting (Ampl. Gain, Ampl. Offset, ND filter) is stored together with the image, the setting is automatically reloaded when using the **REUSE** button. If deviations can be observed it is easy to set the laser power to the old value by means of the AOTF transmission.

b)   Noise Reduction by Ratio

Contrary to the PMT signal, the signal of the monitor diode is not modulated by any specimen information. Thus it can be used to ratio the PMT signal to get rid of the laser noise (due to any laser as a physical fact) and thereby improve the signal to noise ratio of the fluorescence or reflectance image. The major condition which has to be fulfilled to use the monitor diode for this purpose is that the dominating source of noise is laser noise. The signal of the monitor diode will always be dominated by laser noise (independent of the power set at the laser, or the transmission set at the AOTF), whereas the dominating source of noise in the PMT signal can also be the shot noise of light (shot noise especially occurs in low light fluorescence application; as a rule of thumb, the shot noise is limiting the signal to noise ratio, if the PMT voltage has to be set to a value > 400 V).

Fig. 7-18   **Acquisition Mode and Channels tool showing the Amplifier Gain and Offset.**

☞   Any kind of noise which can not be observed in both channels at a time will be amplified and not reduced by the ratio process. Low or high frequency laser noise is the only source of noise which is correlated in the PMT signal and the signal of the monitor diode.

Low or high frequency laser noise is mainly introduced if the Ar lasers are used at a tube current lower than 8 A (Ar-Vis) or 20 A (Ar-UV) respectively.

Fig. 7-19   Light Path tool with Monitor Diode and Ratio Channels

To use the monitor diode for ratio application, proceed as follows:

- Open the **Ligh Path** tool in **Show all** mode and open the **Ratio** channels on the bottom of the **Light Path** tool.

- Activate the ratio channel R1 or R2 in addition to the monitor diode channel (ChM-1) and one PMT channel.

- Choose the appropriate PMT channel as source 1 in the **Ratio Settings** panel and ChM-1 as source 2. If this numbering is changed (inverted), the ratio image will show an inversion of gray levels if compared to the PMT image.

☞   It is not possible to do the ratio between an on-line ratio image generated with two PMT channels (as in ion-concentration sensitive ratio imaging) and the signal of the monitor diode.

The following image is an example of the reduction of correlated noise. The low frequency noise has been generated artificially.
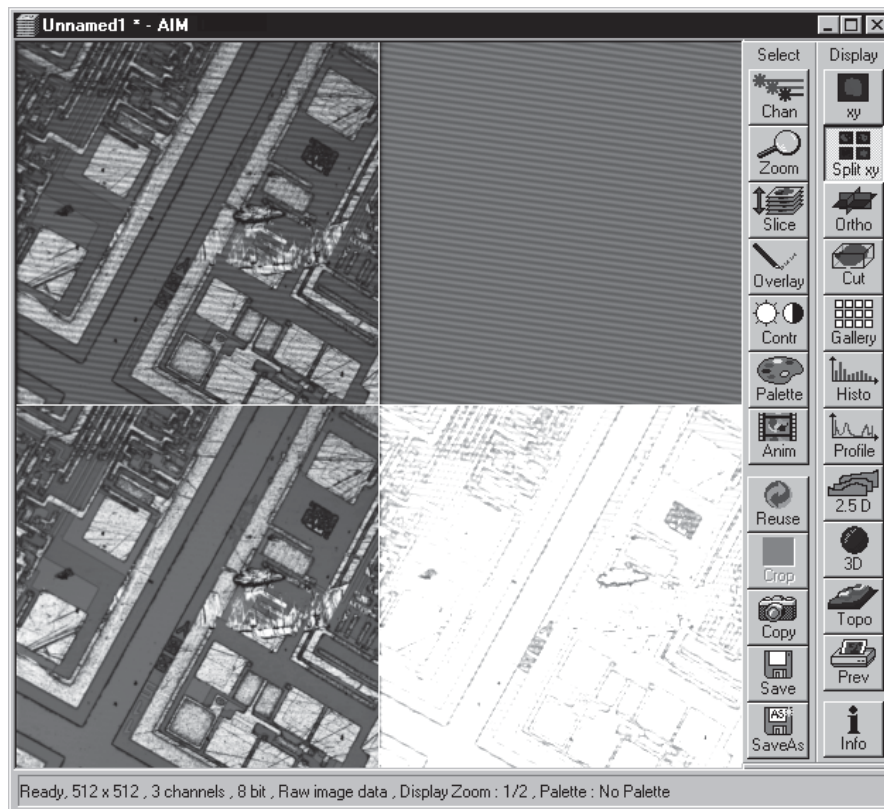
Fig. 7-20   Image Display from LSM 5 software Rel. 4.2 – equivalent in ZEN

The image in the upper left corner shows the PMT image plus noise, the image beneath this (upper right corner) shows the signal of the diode expanded to 512 x 512 pixels (noise without object information). The two images below show the ratio of the PMT and diode signal (left) and the sum of all signals (right). The sum-image does not contain any information and can therefore be neglected.

To get a ratio image like the one shown here, Detector Gain, Amplifier Gain, Amplifier Offset of the PMT channel, Gain and Offset of the diode channel, Gain and Offset of the ratio channel must be set in the correct way.

Each of the parameters summarized effects either the amplification of the ratio image, or the contrast of the ratio image, or the quality of the noise reduction.

The single steps to find the right setting of all the parameters to be set are listed in the following:

- Activate the Range Indicator.

- Adjustment of **Amplifier Offset**: the Offset of the PMT channel and diode channel have to fit to each other to guarantee the best noise reduction.

The best way to do the adjustment is the following:

- Choose different colors in the **Configuration Control** window for PMT and diode channel.

- Activate **Line Scan**.

- Switch off all laser lines in the **Excitation** window.

- Activate **Continuous**

- Set values for **Ampl. Gain** to 1 in each channel.

- Set the lines visible to the same level as close to the ground level as possible; the values you find for the Offset in each channel should be negative.

A final adjustment of the offset adjustment is done by visually evaluating the noise reduction in the ratio image. As the Offset value of the PMT channel influences the range setting of the ratio image much less than the Offset value of the diode channel, the fine tuning should be done via the PMT offset, if required.

☞ As mentioned before, the calculation of the ratio image is very sensitive to different signal offsets in the two channels used. As the offset is influenced by the scan speed as well as by the Amplifier Gain used, the offset calibration is not valid any more if the scan speed is changed, or the Ampl. Gain is set to a new value respectively. In most cases a new fine tuning is necessary. If this doesn't work, the complete calibration process has to be repeated.

Another possibility to calibrate the offset values is to set the values to -0.1 as default for both channels, then perform steps 3 and 4 and finally adjust the noise reduction by varying the PMT offset value.

If the ratio application is used and the offset has been set to the best reduction of noise in the ratio image, it is not allowed to change the offset of the PMT channel to change the reduction of background fluorescence, for example. This can be done only if the diode offset is corrected afterwards.

Adjustment of Detector Gain

The Gain of the PMT should be set with the help of the range indicator function. No 'red' and no 'blue' pixels should occur in the image of the PMT.

Amplifier Gain [Show All mode only]

The diode signal is set to the right range (gray level between 50 and 200 - 8 bit image / 750 and 3500 - 12 bit image) with the help of gray filters and amplifier gain. The use of a lower filter density should be prioritized against the use of a high gain value.

The value of the amplifier gain of both channels (PMT and diode) should be set to one, if possible. Because of an increasing amplifier noise, parallel to the gain factor, a gain value of more than 2 should be avoided. The most important thing is to avoid pixels below the zero level and beyond the maximum range respectively.

Gain and offset in Ratio channel

If the setting of the PMT channel is finished, the range of the ratio channel is adjusted by the parameters in the corresponding formula. Four types of formulas are offered when the **R1** button is pushed. The only formula needed for a ratio image with the monitor diode is type 1:

$$\frac{S1+n}{S2+m} * x+y$$

The values for n and m have to be zero, as well as the value for y. Any deviation from zero will decrease the contrast of the ratio image.

Fig. 7-21   Channel tool with Ratio channel active

Only the value of x shall be influenced by the user. Dependent on the choice of data depth (8 or 12 bit), x is between 0 and 256 (8 bit) or between 0 and 4096 (12 bit).

Default settings are 150 and 3000 respectively. With the help of the range indicator the default value is changed until pixel overflow ('red pixels') is no more available

☞   Any new value can be set by hand-typing and pressing the **ENTER** key while the scan is running. Any change in the setting parameters of PMT and diode signal will make a new Gain x in the ratio formula necessary.

If the adjustment of all parameters is finished, only the ratio image can be scanned or displayed by switching off the PMT channel and the diode channel in the **Configuration Control** window and leaving only the Ratio Channel turned on. As a result, only the ratio image is displayed; which can still be influenced by the settings in PMT and diode channel.

7.15   AxioCam High Resolution Digital Cameras

7.15.1   High Resolution Microscopy Camera AxioCam HSc

Cat. No.   426506-0000-000, incl. AxioVision driver, FireWire / IEEE1394 interface cable and IR barrier filter BG40

High Speed Color

Number of sensor elements: 660 (H) x 494 (V) = 330 K pixels

Pixel size: 9.9 um (H) x 9.9 um

Chip size: 6.5 mm x 4.9 mm, equivalent to 1/2"

Spectral range: With IR barrier approx. 400 nm to 720 nm

Live frame rates (depending on hardware and software configuration):

Mode	H	x	V	Max. frame rate @ 20 ms
Slow	660	x	494	27 frames/s
Medium	220	x	164	64 frames/s
Fast	132	x	98	64 frames/s

Hard disc recording: Inline recording of image data directly to harddisk at all speeds with AxioVision 4 Module "Fast Acquisition" available.

Frame rates for time series recording in AxioVision 4 module "Fast Acquisition" (depending on special hardware requirements):

Mode	Binning	H	x	V	Max. Frame Rate
Standard mono	1 x 1	660	x	494	60
Binned mono	2 x 2	328	x	246	108
Binned mono	3 x 3	216	x	164	143
Binned mono	4 x 4	160	x	122	174
Binned mono	5 x 5	124	x	98	198

Readout of Sensor Sub-Regions ("ROI"):   Adjustable

Digitization: 12 Bit / 24.57 MHz pixel clock

Dynamic Range: Typical 1:1800 at 17 e readout noise

Integration Time: 1 ms to 60 seconds

Cooling: One stage Peltier cooling

Control signals: TTL output for controlling of external shutters

Interface: FireWire / IEEE1394, 6 pin connector, cable 5 m, 400 Mbit/s

Optical Interface: C-mount

Thread depth for objectives: max. 5 mm

Row Data Rate: max. 28 MB/s or 1.6 GB/minute (adequate PC memory capacity required)

Size / Weight: Approx. 11 cm x 8 cm x 4.5 cm / 350 g

Power Supply: 10 to 33 VDC, 4 W by the FireWire cable (IEEE 1394 a) from the PC

7.15.2   High Resolution Microscopy Camera AxioCam HSm

Cat. No	426507-0000-000, incl. AxioVision driver, FireWire / IEEE1394 interface cable and protective cover glass BK 7

High Speed Monochrome

Number of sensor elements:	660 (H) x 494 (V) = 330 K pixels
Pixel size:	9.9 um (H) x 9.9 um
Chip size:	6.5 mm x 4.9 mm, equivalent to 1/2 "
Sensor:	Sony ICX 414 AL for monochrome, progressive readout
Spectral range:	With protective cover glass app. 350 nm - 1000 nm

Live frame rates (depending on hardware and software configuration):

Modus	H	x	V	Max. frame rate @ 20 ms
Slow	660	x	494	27 frames/s
Medium	328	x	246	64 frames/s
Fast	216	x	164	64 frames/s

Harddisc recording:	Inline recording of image data directly to harddisk at all speeds with AxioVision 4 Module "Fast Acquisition" available.

Frame rates for time series recording in AxioVision 4 Module "Fast Acquisition" (depending on special hardware requirements):

Modus	Binning	H	x	V	Max. Frame rate
Standard mono	1 x 1	660	x	494	60
Binned mono	2 x 2	328	x	246	108
Binned mono	3 x 3	216	x	164	143
Binned mono	4 x 4	160	x	122	174
Binned mono	5 x 5	124	x	98	198

Readout of Sensor Sub-Regions ("ROI"):	Adjustable
Digitization:	12 Bit / 24.57 MHz pixel clock
Dynamic Range:	Typical 1:1800 at 17 e readout noise
Integration Time:	1 ms to 60 seconds
Cooling:	One stage Peltier cooling
Control signals:	TTL output for controlling of external shutters
Interface:	FireWire / IEEE1394, 6 pin connector, cable 5 m, 400 Mbit/s
Optical Interface:	C-mount
Thread depth for objectives:	max. 5 mm
Row Data Rate:	max. 28 MB/s or 1.6 GB/minute (adequate PC memory capacity required)
Size / Weight:	Approx. 11 cm x 8 cm x 4.5 cm / 350 g
Power Supply:	10 to 33 VDC, 4 W by the FireWire cable (IEEE 1394 a) from the PC

7.15.3 High Resolution Microscopy Camera AxioCam HRm Rev.2/3.3V

Cat. No   426511-0000-000, incl. digital interface and cable

High Range Monochrome

Number of Pixels: 1388 (H) x 1040 (V) = 1.4 Mega pixel

Chip size: 8.9 mm x 6.7 mm, equivalent to 2/3"

Spectral range: With BK-7 protection glass up to 1000 nm, with IR barrier filter BG40 limited to about 350 nm to 700 nm

Selectable Resolution by Binning or Microscanning

H	x	V	Acquisition Time (s) @ 20 ms exposure
1388	x	1040	0.2 (5 images / s)
2776	x	2080	0.8 (1.25 images / s)
4164	x	3120	1.7

Dynamic Range: Better than 2000 : 1 @ 8 e readout noise

Integration Time: 1 ms to several minutes

Cooling: Single stage Peltier cooling

Optical Interface: C-Mount

Size: about 11 cm x 8 cm x 6.5 cm (2.3" x 3.2" x 2.6")

Registration: CE, cUL

Power Supply: 12 V DC, 1 A, 230 V/110 V, autodetecting

7.15.4 High Resolution Microscopy Camera AxioCam HRc Rev.2/3.3V

Cat. No   426510-0000-000, incl. digital interface and cable

High Range Color

Number of Pixels: 1388 (H) x 1040 (V) = 1.4 Mega pixel

Chip size: 8.9 mm x 6.7 mm equivalent to 2/3"

Spectral range: Limited by IR barrier filter BG40, about 400 nm to 700 nm

Selectable Resolution by Binning or Microscanning

H	x	V	Acquisition Time (s) @ 20 ms exposure
1388	x	1040	0.2 (Color interpolation)
1388	x	1040	0.8 (full resolution for color channels)
2776	x	2080	2.9 (full resolution for color channels)
4164	x	3120	6.5 (full resolution for color channels)
4164	x	3120	1.6 (Fast Scan)

Dynamic Range: Typical 2000 : 1 @ 9 e readout noise

Integration Time: 1 ms to several minutes

Cooling:	One stage Peltier cooling
Optical Interface:	C-Mount
Size:	about 11 cm x 8 cm x 6.5 cm (2.3" x 3.2" x 2.6")
Registration:	CE, cUL
Power Supply:	12 V DC, 1 A, 230 V/110 V, autodetecting

7.15.5 Microscope camera port adapters for the AxioCam

Adapter Video V200 C 2/3" 0.63x at frontport Axio Observer.Z1   Cat. No 000000-1071-171

This adapter is **needed for attachment** of the high-resolution AxioCam microscope cameras **on the Axio Observer.Z1**

Camera Adapter 60N C 2/3" 0.63x   Cat. No 426113-0000-000

This adapter is **needed for attachment** of the high-resolution AxioCam microscope cameras **on the Axio Imager.Z1, Axio Imager.M1, Axio Observer.Z1 SP** and **Axioskop 2 FS MOT**.

☞   **No other cameras are supported by the LSM Software!**

CHAPTER 8

MULTIPHOTON LASER SCANNING MICROSCOPY WITH LSM 5 FAMILY SYSTEMS

CONTENTS

Page

8   MULTIPHOTON LASER SCANNING MICROSCOPY WITH LSM 5 FAMILY SYSTEMS

8.1   NLO Non Linear Optics Laser for LSM 510 NLO and LSM 510 META NLO

The LSM 510 scanning module can be operated with a Titanium-Sapphire-Laser to perform Multiphoton Microscopy. Depending on the type of laser the following operation procedures apply:

- The operation of software controlled lasers (Chameleon laser from Coherent or Mai Tai laser from Spectra Physics) is integrated into the LSM software.

  – Both lasers should be kept in Stand by mode for daily or weekly operation. The cooling of the laser must not be shut down during stand by. Only in case of more than two weeks without operation the laser should be shut down completely. This has to be performed according to the operation manual for the laser provided by the manufacturer. The shut down and the start procedure can take up to one hour.

  – Specific for the operation of Chameleon lasers (Coherent; for further details please refer to the operators manual of the laser):

    The key on the front panel of the laser power supply is used for switching between Stand by mode and Operation mode.

☞ The laser has to be in Operation mode in order to control it with the LSM software.

  – Specific for the operation of Mai Tai lasers (Spectra Physics; for further details please refer to the operators manual of the laser):

    The laser is put to **Operation** mode or Stand by mode using the LSM software. Therefore no further operation of the laser via the laser power supply unit is needed. The key on the front panel of the laser power supply is an interlock key. When the key is removed the system cannot be operated. The power switch has to be kept **On** for leaving the laser in Standby.

- Operation of Verdi Mira lasers (Coherent)

  These lasers are not operated via the ZEN software. Refer to the operator manual of the manufacturer for using these lasers in combination with the LSM 510. However it is needed to indicate the tuned wavelength in the software to ensure the appropriate setting of the AOM frequency (as described below in section 8.1.1).

8.1.1   Laser Control tool

The **Laser Control** tool shows the NLO laser as an additional laser of the system.
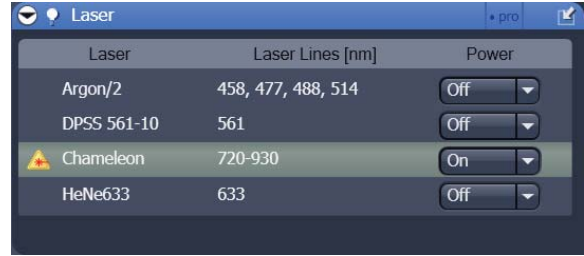
Fig. 8-1   Laser control tool

Operation of the Chameleon (Coherent) and Mai Tai (Spectra Physics) laser

- Click **On** to activate the laser for the software. The laser is put from Standby to Operation and the power ramps up. This takes a few minutes until the laser is ready. The system now has access to the laser and reads out the actual status of the laser.

- The wavelength and laser intensity (transmission through an Acousto-Optical Modulator, AOM) are controlled in the laser control pop up from the **Light Path** tool (Fig. 8-2).

- Enter the desired wavelength in the **Edit Laser Wavelength** input box.

Operating a Verdi Mira laser

- For other lasers: The laser itself is not switched on when clicking **On**, this has to be done manually on the laser control unit. The software is informed that the laser is switched on and the laser safety shutter in the AOM box can be opened.

- The wavelength has to be tuned manually and then entered manually in the **Edit Laser Wavelength** input box.

The **Fine Tuning of the AO-Frequency** is available through the LSM 4.2 software only and not accessible in the ZEN software. If needed, start the system with LSM 4.2 and click the **Modify** button in the **Laser control** window. There you find the slider to fine-tune the AO-frequency (Acousto Optical Modulator) during the continuous scanning procedure. However, this should only slightly influence the intensity of the signal because the automatic presetting is calculated exactly for each wavelength.

- Click **Store** to confirm the setting. The **Laser Modify Control** window is closed and the **Laser Control** window is updated.
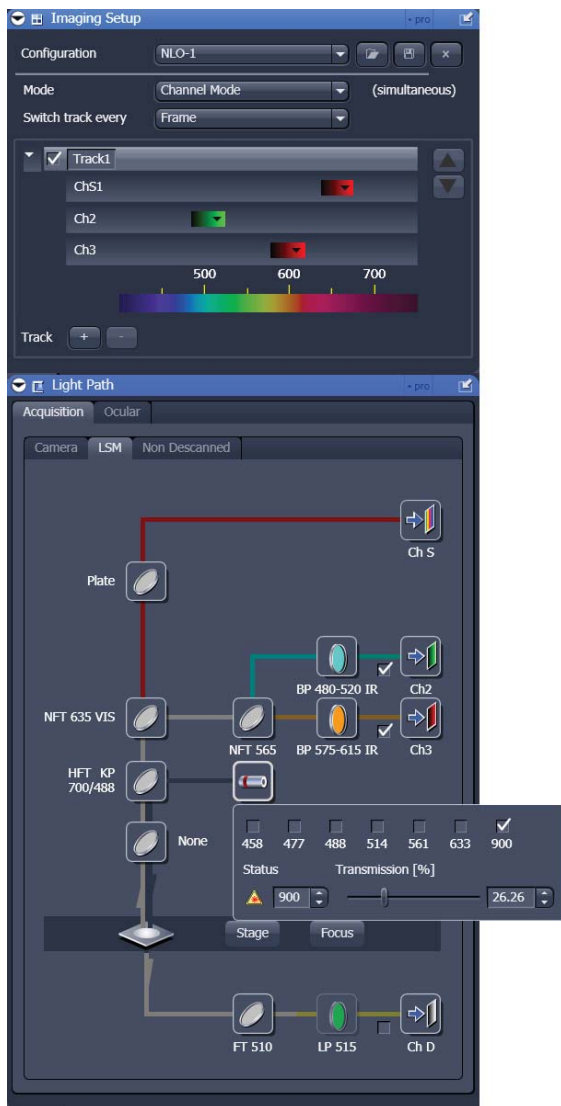
Fig. 8-2   Imaging Setup and Light Path tool

8.1.2   Imaging Setup and Light Path tool

Application of the NLO laser requires special main dichroic beam splitters and the respective emission filters to be activated in the **Light Path** tool.

NLO-excited signals can be detected in every channel. However, it is important to efficiently block NIR (near infrared) excitation stray light from the detector.

The following filters are designed for detection of NLO-excited fluorescence signals:

− **HFT KP 660:** main dichroic beam splitter reflecting NIR excitation longer than 680 nm, transmitting shorter wavelengths

− **HFT KP 700 / 488 (514, 543) or combinations of the KP 700 with more than one additional laser line in the visible range:** main dichroic beam splitter reflecting NIR excitation longer than 700 nm, transmitting shorter wavelengths and reflecting 488 nm (or any other wavelength/s indicated) excitation for simultaneous detection of NIR and VIS excitation

− **KP 660** (short pass filter): transmitting wavelengths shorter than 685 nm

− **BP 500-550 IR or other bandpass filters with indication of IR** are special bandpass filters with minimal transmission in the NIR range (extension IR). They minimize excitation stray light in the detection beam path.

− **BG 39** is a highly efficient block filter for NIR

8.1.3 Pinhole and Collimator Settings

- The pinhole can be fully opened for maximum detection efficiency due to the intrinsic optical sectioning capabilities of the NLO effect (see **Scan Control** window, **Channel Settings**).

- In the **Pinhole & Collimator Control** window, the **NIR** collimator can be used to align the overlap of the excitation planes within the object for VIS as well as NIR excitation light wavelength in the **Collimator** panel (see **Maintain** menu, **Pinhole** button).

8.2 Non Descanned Detection (NDD)

The application of Non-Descanned Detection with the LSM 510 is only useful in combination with the optional NLO laser.

The Non-Descanned Detection modules can be used on the reflected or transmitted-light beam path or simultaneously on both beam paths. This means that a maximum of four NDD channels can be configured. If two NDD channels have been assigned to the transmitted-light beam path, no transmission PMT can be implemented.

In Non-Descanned Detection, the radiation emitted by the specimen is directly guided onto the relevant detector without passing the scanner mirror again.

Non-Descanned Detection is set and configured in the **Light Path** tool by choosing the **Non-Descanned** tab next to the **LSM** tab. This tab is only available when NDD detectors are connected.

- Click on the **Non-Descanned** tab to change to Non-Descanned Detection.

- Configure the NDD channels analog to the Descanned Detection mode.

☞ The configuration of multiple tracks is possible for NDD applications, but they can not be combined with tracks which are set up with the standard channels.

- Before activating image acquisition close the shutter in front of the HBO or HAL illumination. Push in the pushrod (8-4/**1**) for closing the shutter. This enhances image quality. If the hardware is still version 3.0 or lower, make sure that the detector gain for any used NDD is set to the minimal value before re-opening the manual shutter in front of the HAL / HBO illumination.
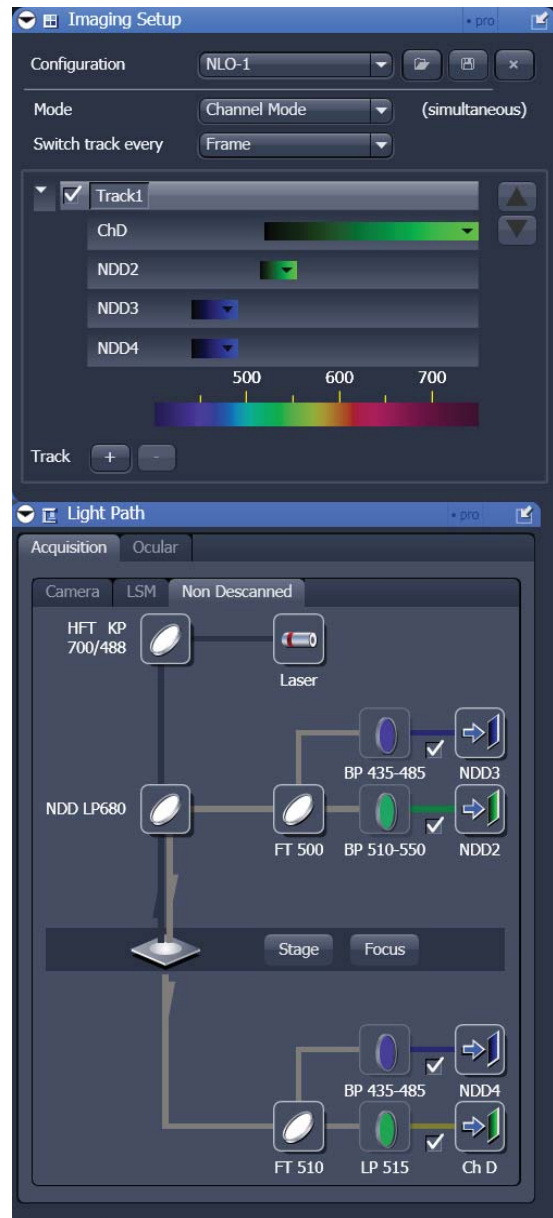
Fig. 8-3 **Imaging Setup and Light Path tool with NDDdetectors.**
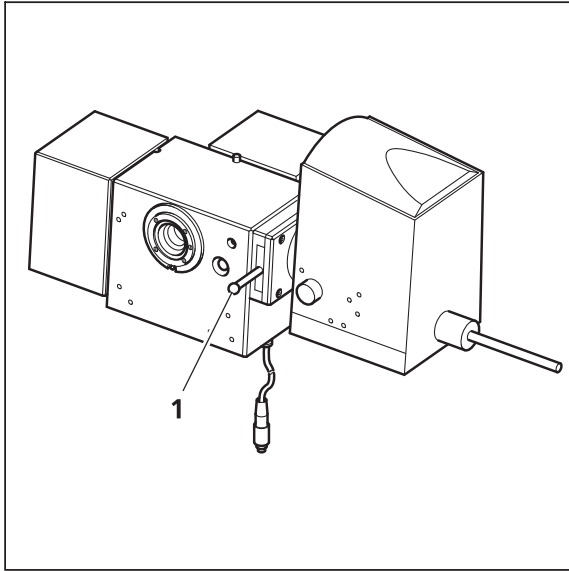
**Fig. 8-4   Non descanned detection module
with HBO 100 lamp**

- Switching from LSM to VIS mode automatically stops scanning. Open the shutter in front of the HBO or HAL illumination for conventional florescence or transmitted light microscopy.

☞ Be aware that the NDD reflector cube in the reflector turret leads to a strong back reflection of HBO light into the specimen plane and the eyepiece. When observing the specimen through the ocular lens the use of the NDD reflector cube should be avoided. The light flash is not harmful but unpleasant. To avoid this, an additional filter (#1261-345) can be mounted into the NDD reflector cube which greatly reduces the intensity of the back reflected light in the eyepiece. **Note**: This filter also reduces the intensity of the excitation light by 7 %.

8.3   Multiphoton Laser Scanning Microscopy Using the Zeiss LSM 510 META NLO

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February 2002

8.3.1   Preface

Safety Considerations when using ultrafast lasers coupled to a microscope.

Users operating ultrafast lasers have to observe all of the precautions specified in the Operating Manual for the laser and caution should be exercised when using the laser.

All users should be familiar with risks and good safety practices before access to the laser is granted.

Users must not look directly into the laser beam. Direct eye contact with the output beam from the laser will cause serious damage and possible blindness.

Every precaution should be taken to avoid exposing skin, hair or clothing to the laser, as this may cause burns.

Beware of reflected laser light and remove jewelry before working with the laser.

Avoid using organic solvents near the laser.

Protective housings should remain in place, when the laser is in use.

Safety signs have to be posted to inform people that lasers may be in use.

8.3.2   Introduction to Multiphoton Laser Scanning Microscopy

Multiphoton laser scanning microscopy (MPLSM) has become an important technique in vital and deep tissue fluorescence imaging. In MPLSM, fluorescent molecules are excited by the simultaneous absorption of two or more near infrared (NIR) photons. Multiphoton excitation has a quadratic dependence, producing excitation only at the focal plane; thus, out-of-focus fluorescence does not contribute to image background, and photodamage outside the plane of focus is greatly reduced. In practical terms, MPLSM makes it possible to acquire images with a high signal-to-noise ratio by using a wavelength that is less harmful to live cells. The use of NIR light makes it possible to image deeper in the specimen, due to less scatter and absorption of the incident light. However, multiphoton excitation depends on some special criteria that differ from those needed for single photon excitation events. Here we will provide a simplified explanation of the physics of multiphoton excitation.

8.3.2.1   Multiphoton excitation – How does it work?

In single photon excitation, a fluorescent molecule or fluorochrome (also called a chromophore) absorbs a high energy photon of light within a certain wavelength range and then, within nanoseconds, releases a photon of longer wavelength (lower energy). The absorption of a photon results in the excitation of the molecule, by displacing an electron within the molecule from the ground state to an excited state. Thus, for a single photon excitation event, excitation is directly proportional to the incident photon flux of the source, since each photon has an equal probability of exciting a molecule in the ground state. As the molecule relaxes back to the ground state, some energy is lost through non-radiative exchange (heat or vibration within the molecule), but the rest is shed as a photon of light.
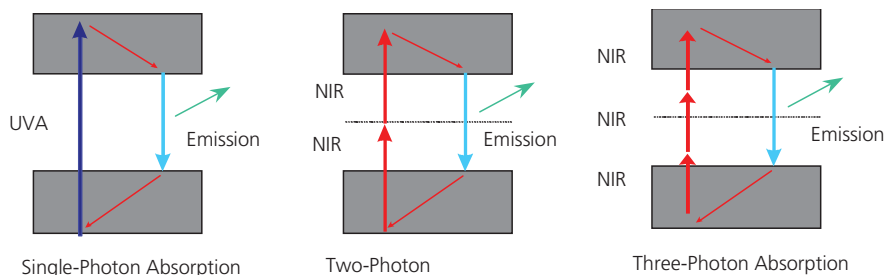
Fig. 8-5   The principle of multiphoton excitation

The energy loss accounts for the Stokes shift seen between the excitation and the emission wavelength and explains why the emission maxima is always of a lower energy, more red-shifted, from the excitation maxima. Multiphoton excitation of the fluorochrome is induced by the combined effect of two or more, lower energy, NIR photons. As a rule of thumb the energy of the two photons is roughly half the energy of the photons needed for single photon absorption, although there are clear exceptions to this rule. Multiphoton excitation can be achieved by two photons of the same or different wavelengths, but with a single laser source, two photons of the same wavelength are used.

The probability of multi-photon excitation is proportional to the incident photon flux density which is the intensity squared (I^2), because a quasi-simultaneous absorption of two photons is necessary. It follows, that for three-photon excitation, the probability of three-photon absorption is the intensity cubed (I^3). The emission characteristics of the excited fluorochrome is unaffected by the different absorption processes (Fig. 8-5).

While this appears conceptually simple, two difficulties, at the level of the fluorochrome, confound our understanding of this process. First, it is difficult to predict whether a molecule will efficiently absorb the two lower energy photons simultaneously. Drastic differences in multiphoton absorption between different molecules have been identified and it is difficult to predict by the structure of a molecule how well it will efficiently absorb simultaneous low energy photons, although some theories are emerging (see Albota et al., 1998; Rumi et al., 2000 for review). Second, the wavelengths for maximum multiphoton excitation are very difficult to predict. Deriving the multiphoton excitation wavelength maximum is clearly not as simple as doubling the single photon excitation wavelength maximum. Both of these criteria must be measured and are reflected in the multiphoton cross-section (usually referred to as δ) for a given fluorochrome (see Xu, 2000 for review).

The cross-section data indicate how well the molecule absorbs multiphoton energy at different wavelengths of NIR light. What is both interesting and perplexing about this data is that several molecules that all emit green light, i.e. excited at roughly the same wavelength via single photon absorption, can have multiphoton excitation maxima that are very different. For instance, although Fluorescein and GFP both emit green light, the multiphoton cross-section peak for Fluorescein is 770-790 nm, but is centered around 900 nm for GFP (S65T) (Xu, 2000) whereas, the single photon excitation maxima for these two molecules are both around 470-490 nm. These questions represent an intense area of investigation for physicists and chemists who specialize in multiphoton absorption (see Section 8.3.3.4 Choosing fluorescent probes for MPLSM).

8.3.2.2   Increased signal-to-noise, enhanced vitality, and deep optical sectioning in MPLSM

One of the greatest benefits of multiphoton excitation is that excitation is practically limited to the focal plane. This effect increases signal-to-noise and decreases phototoxicity. In single photon excitation, the excitation of a dye is directly proportional to the average power (Ex ~ P_{avg}). Thus, excitation takes place in the whole cone of focus and optical resolution is accomplished by using a confocal aperture. For multiphoton excitation, however, excitation of the dye is proportional to the squared intensity (Ex ~ I^2), as mentioned above. For a focused beam, intensity (I) can be described as average power (P_{avg}) divided by the cross-sectional area of the beam (A), so for multiphoton excitation, the excitation is proportional to the average power divided by the area of the beam, squared (Ex ~ $[P_{avg}/A]^2$). Thus, as the beam diameter becomes smaller (such as at the focal plane) excitation is increased and excitation out of the plane of focus becomes highly improbable and falls off with the axial distance from the focal plane with the power of 4. This explains why multiphoton excitation is mainly limited to the focal plane (Fig. 8-6). Moreover, the cross-sectional area of the beam is dependent on the NA of the objective. Objectives with a larger NA can focus light to a smaller beam waist, which is why high NA objectives are preferred for multiphoton excitation microscopy.

Since out-of-focus fluorescence, which usually contributes to background noise in the image, is created inefficiently, MPLSM can be a better technique for imaging fine structures masked by background noise. Optical sectioning can be performed without the use of the pinhole to eliminate out-of-focus fluorescence and nearly all of the fluorescence produced at the plane of focus can be used to make the image. Although a pinhole is not normally needed using MPLSM, it is possible to use the confocal pinhole together with multiphoton excitation to prevent highly scattered photons from reaching the detector and to improve optical sectioning. This can be done on an LSM 510 NLO by carefully adjusting the collimation lens and the z-position of pinhole 1 (Refer to the alignment protocol in subsequent sections).

It is tempting to think of the decrease in background signal as an increase in resolution. This is a common misconception. In fact, due the longer excitation wavelength the optical resolution along the optical axis is worse in comparison to the resolution in a classical confocal LSM. Objects obscured by background fluorescence, may appear brighter or more defined using MPLSM, but this is not due to an increase in resolution, but rather a reduction in background noise, resulting in better contrast.

One-Photon Excitation   Multiphoton Excitation

$$Ex \sim P_{avg} \qquad Ex \sim \left(\frac{P_{avg}}{A}\right)^2$$
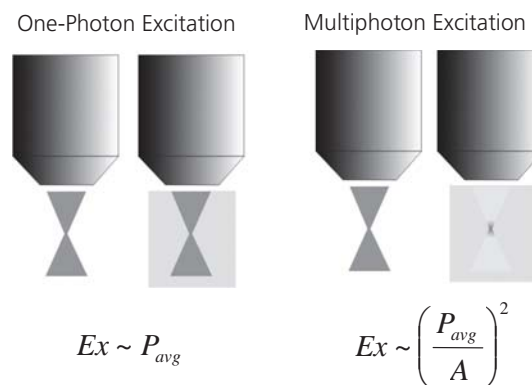
Fig. 8-6   One-photon vs. Multiphoton excitation. Two images of an objective are shown for each example. In each case, the first indicates the shape of the focused beam after passing through the objective. The second indicates the fluorescence that would be observed if the beam was focused through a cuvette containing a homogeneous solution of fluorescent dye.

The lack of out-of-focus excitation greatly reduces bleaching, and therefore, photodamage, throughout the sample. This reduces damage caused by repeated or slow scans; however, photobleaching at the focal plane is still present. In fact, some reports indicate that bleaching may be accelerated at the plane of focus using multiphoton excitation (Patterson and Piston, 2000). Some essential, endogenous molecules within the cell can absorb UV or visible range photons (such as NAD, FADH etc.), which can destroy and deplete these molecules. Thus, it can be safer for vital imaging to use an excitation source outside of the visible range like a NIR laser. A profound example of this effect is seen in a comparative study performed by Squirrell and colleagues (Squirrell et al. 1999), where the vitality of cleavage stage hamster embryos was assessed after repeated exposure to visible range laser light and pulsed 1047 nm laser light. In these experiments, confocal imaging resulted in arrested cellular division and embryo lethality, whereas imaging using multiphoton microscopy resulted in much less embryo lethality and better data collection. In fact, at least one embryo imaged in this way was able to develop into a completely normal adult hamster named laser, illustrating the strength of this technique.

The use of NIR light has the additional benefit of being able to penetrate deeper into tissue than visible wavelength light. Compared to confocal microscopy, excitation can be achieved in deeper positions of the specimen and more data along the z-axis can be obtained. However, particularly in deep tissue imaging, care must be taken to recover as many emission photons as possible. While the incident NIR light has an advantage over visible range excitation sources, the photons that are emitted are at visible wavelengths and have the potential to be scattered or absorbed by the tissue. To improve the efficiency of collection, high numerical aperture (NA) objectives should be used, although it is often difficult to obtain lenses that have NAs above 1 with a working distance longer than 250 µm. In addition, non-descanned detectors (NDDs), which collect photons at a point closer to the specimen and do not require the emission to be focused back through the scan mechanism, can be used to improve deep tissue imaging by improving the collection efficiency of scattered photons

8.3.2.3   Drawbacks of using NIR light for microscopy

Although the use of NIR photons has many advantages, there is a distinct disadvantage to this mode of excitation.

Absorption of NIR light by water and some other particular molecules (such as melanocytes or condensed particles such as calcium carbonate crystals) can create dramatic local heating effects within the sample. This effect increases as the overall power from the laser is increased at the sample. It is very important to use minimized power levels to reduce the effects of local heating. For live samples, power levels above 6 mW may disturb cell replication (König et al. 1996) or even cause cells to explode, as in the case of melanocytes in zebrafish embryos. Effects of local heating can not only damage cells, but can also contribute to image artifacts (see Section 8.3.3.5. Samples your mother should have warned you about). As you will see in the next section, optimizing the pulse length at the specimen can improve multiphoton excitation without raising the average power.

8.3.2.4   Achieving Efficient Multiphoton Excitation using Ultrafast lasers

In order to achieve efficient two- or three-photon excitation, the photons must collide with the molecule simultaneously. For single-photon excitation, a continuous wave laser with a continuous photon flux can be used because the probability of excitation is directly proportional to the photon flux or average power of the source. Increasing the laser intensity (turning up the power) increases the photons delivered to the sample, increasing excitation until all of the molecules are saturated. To deliver enough photons to achieve simultaneous absorption of two NIR photons using a continuous wave laser would require enormous power. Ultrafast lasers improve the efficiency of multiphoton excitation by delivering photons in pulsed "wave packets". The high peak intensity needed for multiphoton excitation is created by concentrating photons into very brief pulses which are delivered to the sample over and over again at a rapid rate, about every 13 ns, to ensure efficient dye excitation. Instead of a steady flux of photons bombarding the fluorochrome one after another, multiple photons collide with the molecule simultaneously. This process has the advantage of delivering a high peak intensity, to satisfy the I^2 or I^3 requirement for two- or three-photon excitation, without using enormous amounts of average power.

Many lasers are now available that can produce ultrashort pulses at high repetition rates. Titanium-sapphire lasers, for instance, are capable of producing ~100 fsec pulses over a broad tunable wavelength range (690 nm-1064 nm) with a high repetition rate, ~80 MHz. Similarly, solid state, doubled neodymium doped yttrium lithium fluoride (Nd: YLF) lasers, emitting 1047 nm, 175 femtosecond pulses at 120 MHz have also be used for multiphoton excitation.

Ti: Sapphire lasers are probably the most popular lasers because of the wavelength range that is available. These lasers can operate in both a continuous wave (CW) mode or in a mode that emits pulsed light. Lasers operating in this latter mode are said to be mode-locked (ML), which refers to the fact that the laser is locking in different frequencies together to form a pulse of a particular bandwidth.

The use of ultrafast mode-locked lasers for multiphoton excitation requires to consider several additional factors which are not necessary for continuous-wave lasers used for single-photon excitation. The length of the laser pulse (referred to as the pulse length or pulse width) (τ), the peak intensity produced at the focal plane (I_{peak}), the average power of the laser at the specimen (P_{avg}), the cross-sectional area of the beam (A), and the pulse frequency (F_p) or repetition rate are all important factors for achieving and maintaining efficient multiphoton excitation in the sample.

Raising the average power of the laser, P_{avg} (controlled by the Acousto-Optic-Modulator, AOM), will raise the peak intensity. However, raising the average power will also increase the amount of heat generated in the sample, which may damage vital processes or disrupt cellular structures.

The pulse frequency, F_p, is determined by the design of the laser and is not easily manipulated. For the Coherent Chamelon Ti: Sapphire laser, Fp is equal to ~90 MHz.

The cross-sectional area of the beam, A, is an important term to consider when dealing with light focused through an objective. Simply put, reducing the cross-sectional area of the beam, for instance by focusing the beam through an objective, increases the intensity at the point of focus or in other words increases the amount of photons per area as we saw above.

The exact dimensions of A will vary greatly depending on the properties of the objective. In general, higher magnification, higher NA objectives will reduce A, similar to the way such objectives increase resolution. However, other considerations such as the transmission efficiency of the objective and the amount of dispersion produced by the objectives must also be considered as this will reduce P_{avg} or increase respectively. In addition, spherical aberration caused by the objective and sample and the diameter of the beam at the back aperture of the objective can also affect A. Thus, empirical determination of the best objective is often relied upon. (see Section 8.3.3.3 Objectives recommended for multiphoton excitation).

The pulse length, τ, is a measure of the duration (length) of the pulse of photons being delivered to the sample, measured at the full width, half maximum. Increasing the pulse length, decreases I_{peak}. As mentioned above, ultrafast lasers are able to supply short pulses with a high duty cycle. Unfortunately, the pulse length is very difficult to measure, especially through a microscope objective. However, it is possible to make such measurements and users should refer to Wolleschensky et al. (1998) for details. Several factors that effect the pulse length are discussed below.

8.3.2.5   Optimizing the Peak Intensity without Frying the Samples

Although raising the average overall power is the easiest way to increase the peak intensity, excessive heat can destroy both live and fixed samples, as well as produce unwanted imaging artifacts. Assuming that one is using a laser with a fixed pulse frequency and a high magnification, high NA objective optimized for multiphoton microscopy (see Section 8.3.3.3 Objectives recommended for multiphoton excitation), the pulse length becomes the most important term to minimize.

The Spectral Bandwidth refers to the spectral frequencies of the pulse. Each short pulse produced by the laser has a broad spectral band centered around the selected wavelength. Spectral bandwidth and pulse length are inversely related. The wider the bandwidth, the shorter the pulse.

The Coherent Chamelon produces pulses around 140 fsec.

GVD is a temporal broadening of the pulse length as the pulse travels through normal dispersive media such as glass (see Diels and Rudolph, 1994 for more discussion). The pulse length becomes broader in dispersive media because the red shifted frequency components, with respect to the center wavelength, travel faster than the blue shifted frequency components. Pulses with a broader spectral bandwidth are more susceptible to the effects of GVD, as there is a greater difference in wavelength, and thus, velocities within the pulse (Fig. 8-7). In addition, the amount of dispersion is related to the thickness of the dispersive media (see Wolleschensky et al. 2001 for review). When possible, thinner glass optics and lenses are used along the routing path.

Within the laser, a prism pair is used to adjust the spectral bandwidth of the laser pulses. However, outside the laser, glass elements within the microscope and scan module, within the objective, and along the routing path, can lengthen the pulse. In this case, the broader the spectral bandwidth, the more broadening of the pulses will be caused by GVD. Thus, for direct-coupled systems without the use of a prechirping unit, it is advantageous to adjust the laser so that the bandwidth of the pulse is at a minimum. For instance, if the bandwidth is less than 7 nm, the pulse length at the sample will be approximately 300 fsec. If the bandwidth is 12 nm, the pulse becomes substantially broadened by the same dispersive elements so that the pulse length is about 700 fsec at the specimen.

6 nm bandwidth   10 nm bandwidth

Fig. 8-7   **Effect of GVD on short pulses with a broad bandwidth. The broader the bandwidth , the more the pulse is stretched. Longer wavelengths travel faster through the glass than shorted wavelengths in the pulse. Thus, a positively chirped pulse is broadened.**

It is possible to compensate for GVD along the routing path and in the microscope to preserve the pulse length to the sample by using a prechirp unit (available from other manufactureres).

8.3.3   Using the LSM 510 NLO direct coupled system

Practical considerations for optimal imaging:

8.3.3.1   Coupling an ultrafast laser to the LSM 510 NLO

Coupling an ultrafast laser to a laser scanning microscope is a fairly simple matter, if several key considerations are understood. Typically, the routing kit for coupling the LSM 510 NLO to the laser will consist of the following components: an Acousto-Optic Modulator (AOM), used to control the intensity of the beam and for beam blanking (allowing for region of interest (ROI) and bi-directional scanning and for performing Auto-Z-Brightness correction); two 45° mirrors for directing the beam to the microscope on the table; and a 1 meter lens. In the case of an upright system, the beam is brought up into the scan head via a periscope, whereas for an inverted microscope, the height of the beam will be adjusted by a smaller periscope positioned in place of the second turning mirror. These devices ensure that the beam remains parallel to the table when horizontal and enters the scan head at the proper height (see diagram). The 45° routing or turning mirrors are adjustable and will be used routinely to peak the coupling of the beam into the scan head. The 1 m lens is used to expand the beam to fill the back aperture of the objective. It is mounted in a fixed position by a trained service engineer during installation and should not be adjusted by the user (Fig. 8-8).
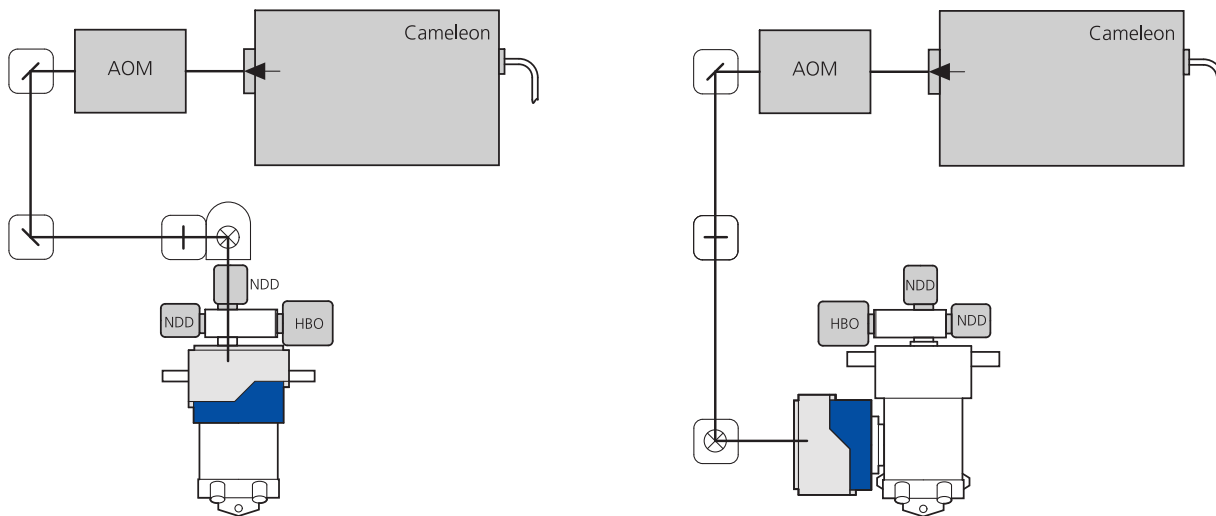
Fig. 8-8   Typical alignment scheme for the LSM 510 NLO direct-coupled system using and upright(left) or inverted(right) microscope.

8.3.3.2   Alignment of the ultrafast laser into the LSM 510 NLO scan head

The alignment of the laser is done by a Zeiss service engineer. However it might be needed to slightly adjust the beam coupling from time to time, depending on the room conditions. The protocols mentioned should only be performed if the user is confident with the technique, otherwise the coupling of the laser can be lost and a Zeiss service engineer is needed for realignment.

The alignment of the laser through the AOM and into the scan head is critical for producing high quality images using multiphoton excitation. Below are two protocols that can be used to optimize alignment of the beam into the scan head.

8.3.3.2.1   Quick alignment protocol

After the system is installed and aligned by a qualified service engineer, it is often the case that only small alignment adjustments are necessary. For instance, proper alignment should be verified after the laser is tuned to a new wavelength. Below is a quick and easy protocol that can be used to center the beam into the back aperture of the objective lens for optimum image quality.

Start by scanning a sample that is normally excited by the wavelength of the external laser. For instance, a pollen grain slide (Carolina Biological) or a Fluocells sample (Molecular Probes) can be used. (NOTE: This procedure may cause bleaching of the sample.)

Adjust the laser power and gain so that the sample is visible in the image acquisition window.

Scan the sample using the fast XY scanning mode.

Using the signal intensity as a guide, peak the alignment of the beam. Begin by using the alignment mirror closest to the laser (first turning mirror). Make improvements using both alignment pins on the mirror mount. (NOTE: a small hex wrench (Allen key) may be needed to adjust alignment). When the best signal intensity is obtained by tweaking the mirror closest to the laser, now make improvements by tweaking the mirror closest to the microscope scan head. Go back and forth between these mirrors to walk the center the beam onto the back aperture of the lens.

As a final verification of proper alignment, check the alignment of the beam onto the back aperture directly by viewing the projected beam without the objective in place. To do this, place a white card on the stage (Fig. 8-9/**A**) or lens paper on the specimen holder for an inverted microscope.

Remove one objective, select 50-100 % transmission, HFT KP 680 or 700, and Fast XY scan. When the system is scanning, a round, red spot should appear on the card (Fig. 8-9/**B**). Seeing this beam may be aided by using an IR viewer, especially at longer wavelengths.
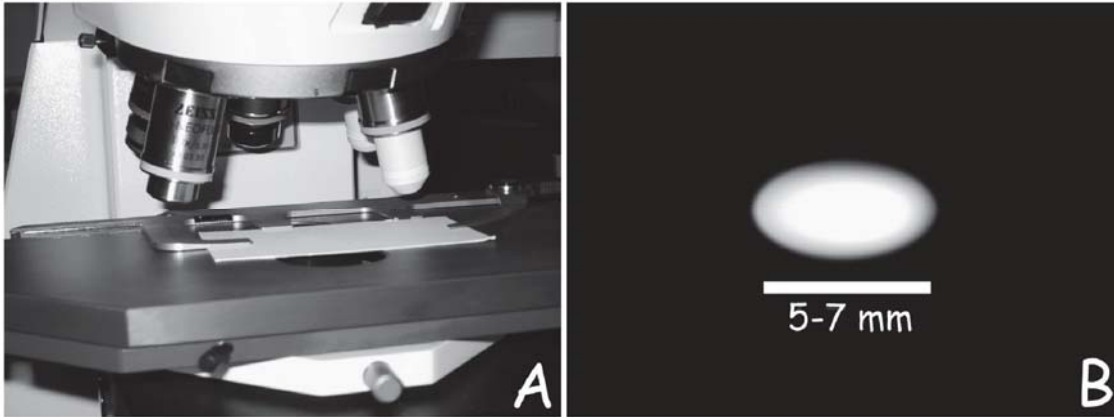
Fig. 8-9   (A) Setup for checking the beam alignment; (B) Optimal shape of the NIR laser spot

Ideally this spot should be 5-7 mm in diameter and should be completely round and uniform. If the beam appears to be clipped (flat on one side), small adjustments in the routing mirrors can improve the alignment (see above). Shadows in the illuminated spot may indicate dust or debris on the routing optics or in the scan head (check all visible elements for debris, then contact a service engineer if the problem persists).

If proper alignment cannot be established using this protocol or if the image quality appears to be poor, use the advanced alignment protocol to peak alignment.

8.3.3.2.2   Advanced alignment protocol

When the system is installed, the service engineer will establish alignment of the laser beam into the scan head by verifying the overlay between the NIR laser and one of the VIS lasers. Although routine use usually does not usually require a complete realignment with the scan head, tuning the laser over large wavelength increments or changing or cleaning the optics in the laser can cause gross changes in beam pointing. If this occurs, it is best to use the following protocol to re-establish proper alignment with the scan head. For this protocol, a partially reflective grid slide is needed (Part number 474028-0000-000, Test Grid Specimen for LSM). Care must be taken to use low amounts of laser power when using this slide or the grid can be easily burned.

Begin by confirming that the correct wavelength for the laser is set in the laser control panel in the software. The wavelength can be changed by selecting the modify option. Refer to the Rees analyzer or the BRF setting to determine the wavelength of the MIRA 900F.

Next, place the partially reflective grid slide on the microscope stage. Bring the grid slide in focus using the 10x Plan-Neofluar objective and transmitted light. Configure the scan head as follows (Fig. 8-10):

This configuration is designed to use the PMTs to detect the reflection of the NIR and VIS laser light off of the grid slide so an image of the grid is made on the screen. For this protocol, the 543 nm line has been chosen. The HFT KP 700/488 beam splitter is used to avoid sending too much 543 nm laser light to the reflective slide. A KP 700/543 beam splitter can be used, but care must be taken not to burn the slide. Alignment using this procedure will ensure that the NIR and VIS lasers overlay on the combining mirror when they enter the scan head.

To perform the alignment, set the laser power for both lasers at 3 % (1 % for a MIRA 900F with a 10W pump laser) and adjust only the gain to improve the intensity of the signal.

⚠ WARNING: Too much laser power can cause damage to the grid slide! Be sure attenuation is set before scanning.
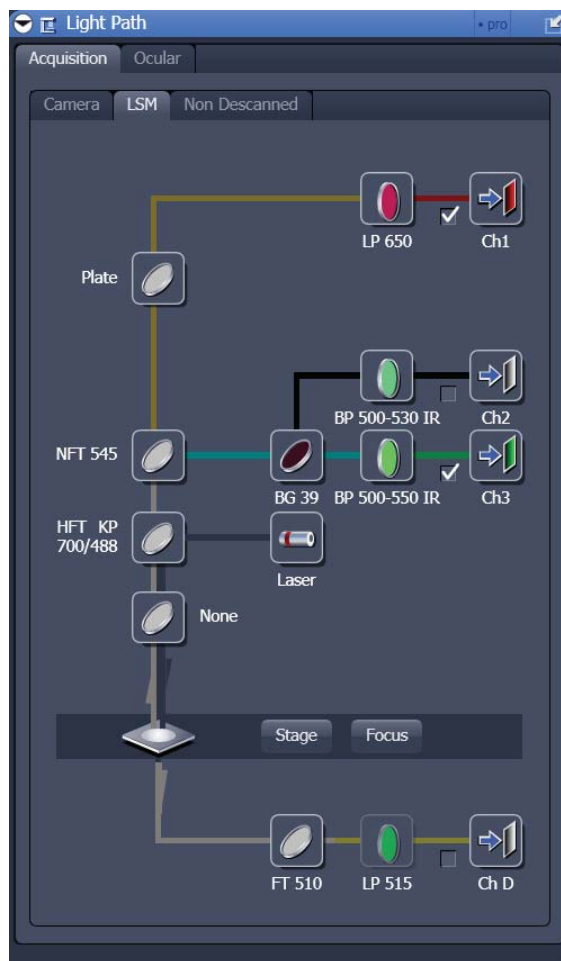

Fig. 8-10   **Configuration for testing the overlay of the grid reflection images gained with the vis (543 nm) laser and the NIR laser**

Begin scanning using the FAST XY mode. An image of the grid should appear on the screen. If not, adjust the gain for each channel so that the output from both channels is visible. The two colors represent the reflection of the 543 nm line (green) and the reflection of the NIR laser (red) on the metallic grid surface. The focus may also need to be adjusted in order to get the optimal output from both channels. If the red image is out-of-focus when the green image is in focus, the collimating lens should be adjusted so that both beams are focused on the same spot. To adjust the collimator, open the Maintain panel in the LSM 510 software. While the laser is scanning, click on the pinhole button. At the bottom of this panel, there is a slider to adjust the NIR collimator.

☞ DO NOT ADJUST THE VIS COLLIMATOR.

Adjustments in the NIR collimator should improve the focus of the NIR beam. If no signal at all is detected in the red channel, turn up the laser transmission in small increments and begin peaking alignment.

☞ Note that once alignment is improved, the laser intensity will need to be attenuated to avoid burning the slide. If no red signal at all can be found, contact a service engineer.

Once both beams are in focus, begin optimizing the overlay of the grids by first adjusting the routing mirror that is closest to the laser. Make small adjustments using both alignment screws while watching the effect on the monitor screen. Adjustments of this mirror will increase the intensity of the red signal. Readjust the gain as needed. After optimizing the alignment using this mirror, begin adjusting the mirror that is closest to the scan head (NOTE: A small hex wrench [Allen key] may be needed to adjust alignment). Adjustments in this mirror will improve the alignment of the NIR overlay on the VIS overlay. Go back and forth between these mirrors to walk the center the beam onto the back aperture of the lens.

8.3.3.2.3   Tips on maintaining alignment with the scan head

Since the NIR beam is aligned into the scan head through mirrors and free space, it is possible that any alteration in these routing components can effect proper alignment. Below are some tips to maintaining good alignment:

- Do not move the position of the 1m lens. Even small adjustments of these components may cause significant changes in the alignment of the routing optics.

- If recurring alignment drifts or power fluctuations in the laser are observed, consider floating the optical table and/or regulating the room temperature more closely. Changes in the temperature of the table or optical components can often cause drift in alignment of the laser and routing optics.

- Avoid tuning the laser in increments larger than 50 nm without peaking the alignment.

8.3.3.3 Objectives recommended for Multiphoton Excitation

For nonlinear microscopy, objectives should be optimized for the following parameters: high transmission in the NIR and the VIS-wavelength range, long working distance with a high numerical aperture, limited pulse broadening due to GVD, a uniform GVD across the pupil of the objective for the excitation wavelength range and a small propagation time difference (PTD). As we have already discussed, low transmission in the NIR can lower the average power at the sample, thus lowering the peak intensity.

Fig. 8-11   Schematic pulse transformation by a singlet lens showing the influence of chromatic aberrations on the peak intensity due to Propagation Time Difference (PTD).

Long working distance objectives with a high numerical aperture are clearly favored for deep imaging so that the beam can be focused deep into the tissue and the emission photons can be collected efficiently. Objectives should have limited GVD to reduce the chance that short pulses will be lengthened en route to the sample, which will again reduce the peak intensity.

In addition to GVD, chromatic aberration of objectives leads to pulse distortions. Specifically a radius-dependent group delay is introduced (Kempe et al., 1993; Netz et al., 2000). Therefore, different radial portions of the beam across the pupil of the objective arrive at different times at the focal region and cause a temporal broadening of the pulse. This results in lower peak intensity in the focal region (Fig. 8-11). This effect is also referred to as PTD.

Table 1 summarizes the important characteristic parameters for objectives recommended for nonlinear microscopy and for the optics inside the LSM 510 NLO including the microscope stand. The dispersion parameters were calculated based on the material data and the thickness of each optical element on axis. For comparison, the dispersion parameter for the optics within the LSM 510 NLO including the microscope stand and the AOM is 7500 fsec^2.

The variation of the dispersion parameter for different beams across the pupil of the objective is listed in the forth column. It can be seen from both parameters that the pulse broadening is nearly independent of the particular objective used and the position of the beam across the pupil of the objective. The pulse broadening due to the GVD of the different objectives and the LSM 510 NLO including the microscope stand and the AOM was calculated for 100 fs pulses at a wavelength of 800 nm and is listed in column 5.

The pulse broadening due to PTD is listed in the last column. The PTD was calculated for the whole optical setup, since it depends on the chromatic aberrations of the complete system including the LSM 510 NLO and the microscope stand for a wavelength of 800 nm. The PTD effect is negligible if the pulse length is not much shorter than 100 fsec. It can be seen that the special IR corrected objectives show a smaller PTD in comparison to the standard UV/VIS corrected objectives.
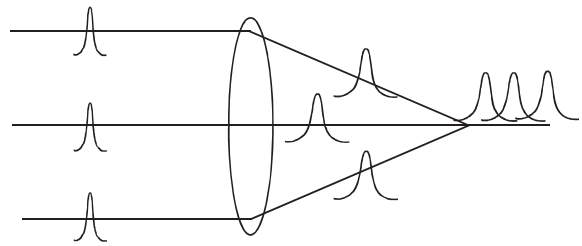
Objective	Working Distance [mm]	Dispersion Parameter [fsec]^2		Pulse broadening factor of 100 fsec pulse	Max. PTD [fsec] (*)
		On axis	Variation		
IR-Achroplan 40x/0.8 W	3.61	1714	± 20	1.14	-3
IR-Achroplan 63x/0.9 W	2.00	1494	± 15	1.11	-9
Plan Neofluar 40x/1.3 oil	0.20	2328	± 30	1.23	9
Plan Apochromat 20x/0.75	0.61	1531	± 10	1.12	10

Table 1   **Summary of specific parameters for objectives recommended for 2-Photon applications. All data were measured at 800 nm. (*) Propagation time difference (PTD) is calculated for the whole optical setup including the LSM 510 NLO and the microscope stand.**

Negative values indicate that pulses at the edge of the pupil are delayed with respect to pulses traveling on axis.

With regard to transmission, objectives for biomedical applications traditionally have been optimized for UV and visible range imaging. Thus, many are not corrected for transmission of NIR or IR light. The transmission of an objective depends strongly on the design of the antireflection coating. Typical transmission curves for two different objectives are shown below. The Achroplan series is coated with UV/VIS-antireflection coating giving optimal performance from 350 nm to approximately 800 nm. In contrast, the IR-Achroplan series has a VIS/IR coating that enables high transmission from 420 nm to 1400 nm. The Achroplan objectives are best for multiphoton applications, where excitation is below 850 nm and the fluorescence from the specimen is in the ultraviolet or lower visible wavelength range.

These criteria describe the imaging parameters for many ion indicators, nuclear dyes such as DAPI or Hoechst, Blue Fluorescent Protein, and Fluorescein or Alexa 488. The IR-Achroplan objectives are best for multiphoton applications requiring an excitation wavelength greater than 850 nm and where the fluorescence emission is above 420 nm (Fig. 8-12). These latter criteria apply to many dyes, such as DiI, and many of the fluorescent proteins, such as CFP, GFP, YFP, and dsRed. However, some important fluorochromes can be imaged well with either objectives.

Rhodamine, for example, has a multiphoton absorption peak at 840 nm, and like Alexa 568 it can be excited well at 800 nm. Thus, the examples are given to indicate ideal imaging criteria; however, non-optimized parameters can often be used to produce high-quality images.

Fig. 8-12   **Typical transmission curves of an IR-Achroplan and an Achroplan.**

While the Achroplan objectives clearly provide the best transmission curves with the longest working distance, other objectives have proven to be useful for multiphoton imaging. For instance, the Plan-Apochromat objectives have favorable properties for multiphoton imaging as Table 1 indicates. In addition, the C-Apochromat 40x W/NA 1.2 and its 63x counterpart have proven to be very effective objectives, perhaps due to their high NA and collection efficiency. Moreover, these objectives have a 0.23 and 0.25 mm working distance, respectively, and can be very useful for collecting large 3-D data sets. Unfortunately, these objectives begin to decline in transmission at around 840 nm, so they are not optimal for dyes with more red-shifted excitation wavelength absorption maxima, but can often be used without concern for the lost photons

8.3.3.4   Choosing Fluorescent Probes for MPLSM

Presently, there are thousands of different fluorochrome derivatives and conjugates that can be used to study a variety of biological events. Many of these fluorochromes have been optimized for single-photon excitation and scientists are just beginning to concentrate on developing optimized fluorochromes for multiphoton imaging applications (see Albota et al, 1999). Furthermore, it is difficult to predict whether or not a particular dye will be useful for multiphoton imaging and many of us have relied on trial and error while a greater understanding of the physical principles of multiphoton excitation is evolving. That being said, there are some important parameters that one can use to help predict which dyes may be best for a particular application.

Similar to the way one would choose a fluorescent probe for conventional fluorescence microscopy, the best fluorochromes for multiphoton imaging are the ones that efficiently absorb light, reliably emit fluorescence, and are photostable. Values such as the multiphoton cross-section and the fluorescence quantum yield are important to consider when choosing a fluorochrome.

The multiphoton cross-section is a measure of how strongly a fluorochrome absorbs photons at a given wavelength and is expressed in units of 10^{-50} cm^4 sec/photon or 1 GM (Goeppert-Mayer unit, after the scientist who first predicted multiphoton absorption, Maria Goeppert-Mayer). A list of cross-sections can be found in Xu (2000) or at

www.uga.edu/caur/dyechart.pdf

The web-site shows a collection of excitation cross sections for various dyes. Currently available dyes have multiphoton cross-section values ranging from 40-200 GM (see Xu, 2000).

These values are only an indication of how well a dye will perform in biological imaging using MPLSM. A one to one correlation between the cross-section and dye performance cannot always be drawn. Measurements of multiphoton cross-section are usually performed with the dye dissolved in organic solvents or at non-physiological conditions and the local environment of a dye may influence absorption and emission characteristics. Also, these values are determined at an optimal pulse-width that cannot always be achieved at the level of the specimen. Although the cross-section is a valuable first approximation of how a dye will perform, trial and error provides the truest test for a particular application.

The multiphoton cross-section not only provides information about how well a particular molecule is excited by pulsed NIR light but also indicates the multiphoton absorption peak, a value that has been surprisingly difficult to predict. While it may seem logical that the two-photon absorption peak should be twice the one-photon peak, this has not proven to be the case. An emerging rule of thumb is that the peak is usually shorter (more blue-shifted) than twice the one-photon absorbance peak (Xu, 2000). However, one should also keep in mind that the brightest signal may not be obtained at the predicted excitation peak but at the point where the excitation peak and the power peak of the laser overlap.

Blue/Cyan dyes

Alexa 350 (780 nm-800 nm)

Hoechst (780 nm-800 nm or 900-1100 nm)

DAPI (780-800 nm or 900 nm-1100 nm)

CFP (800 nm-900 nm)

Green dyes

Oregon Green (800 nm-860 nm)

Alexa 488 (800 nm-830 nm)

GFP (840 nm-900 nm)

BODIPY (900-950 nm)

FITC (750 nm-800 nm)

DiO (780 nm-830 nm)

Yellow Dyes

YFP (890 nm-950 nm)

Orange dyes

DiA (800 nm-860 nm)

Red dyes

DiI (830-920 nm)

Rhodamine B (800 nm-860 nm)

Alexa 568 (780 nm-840 nm)

Table 2   Recommended multiphoton excitation wavelengths for common dyes.

For tunable Ti: Sapphire lasers, there is a peak output at or near 800 nm, with less average power produced at lower and higher wavelengths; as a result, it is possible to excite a given dye at a non-optimal wavelength by using more power. However, more power may also mean more heat or bleaching, so one should be careful to optimize the signal-to-power ratio.

In addition to how well a molecule absorbs light, the best molecules to choose are the ones that also efficiently release light as the molecule relaxes back to the ground state. The fluorescence quantum yield is a measure of the proportion of emission photons that are shed per excitation event.

In general, this value is the same for one- or multiphoton excitation and values for common fluorochromes can be found in Haugland (1996).

As mentioned above, photostability is an important factor when choosing a dye. Photobleaching is minimal in out-of-focus regions using MPLSM, but bleaching still occurs at the point of focus. In fact, some dyes that are relatively stable using one-photon excitation, have more rapid bleaching rates using MPLSM (Patterson and Piston, 2000). This phenomenon is not fully understood, so the bleaching rate of dyes of interest should be tested for each application. In cases where rapid bleaching occurs, we have found that ProLong (Molecular Probes, Eugene, OR) has helped stabilize the signal in fixed samples.

Table 2 is a rough guide of fluorochromes recommended by the Biological Imaging Center at Caltech. Currently, there is no comprehensive reference for all dyes excited by two- or three-photon excitation for the user to refer to for information on the peak absorbance wavelength or relative brightness of a given dye.

The information given below is based on empirical observation and does not reflect a serious, scientific investigation of all dyes, nor does it indicate the exact optimal excitation wavelength for each dye in question. Instead, this list is offered as a rough guideline of what is known to work for various imaging applications and the user is encouraged to empirically determine which dyes are most useful for their own applications. Clearly the success of any fluorescence imaging experiment relies on how well the target is labeled. The same is true for MPLSM.

8.3.3.5   Samples your mother should have warned you about

While multiphoton excitation has many advantages, there are also some disadvantages to using high intensity NIR or IR light for fluorochrome excitation. Probably one of the biggest disadvantages is the heat that is generated via the absorption of NIR photons by water. The local heating that is caused can affect vital cellular processes, such as cell replication (König et al., 1996) and other growth abnormalities in sensitive specimens. Thus, great care should be taken to maximize PMT settings so that minimal power levels can be used while imaging. Particular care should be taken when imaging cells or specimens that contain molecules that absorb light such as melanin.

In some cases, the absorption of NIR photons can cause dramatic local heating effects and cause cells to explode. This effect has been seen frequently in zebrafish embryos, but can be avoided by blocking melanin production with chemical agents, such as PTU or by using albino embryos.

Other molecules, such as salt crystals, can also focus light within tissues and intensify heating effects. This can also lead to imaging artifacts, perhaps due to signal produced via second harmonic generation (SHG). In Fig. 8-13 Calcium Carbonate crystals in the inner ear of the frog have produced blotchy artifacts.
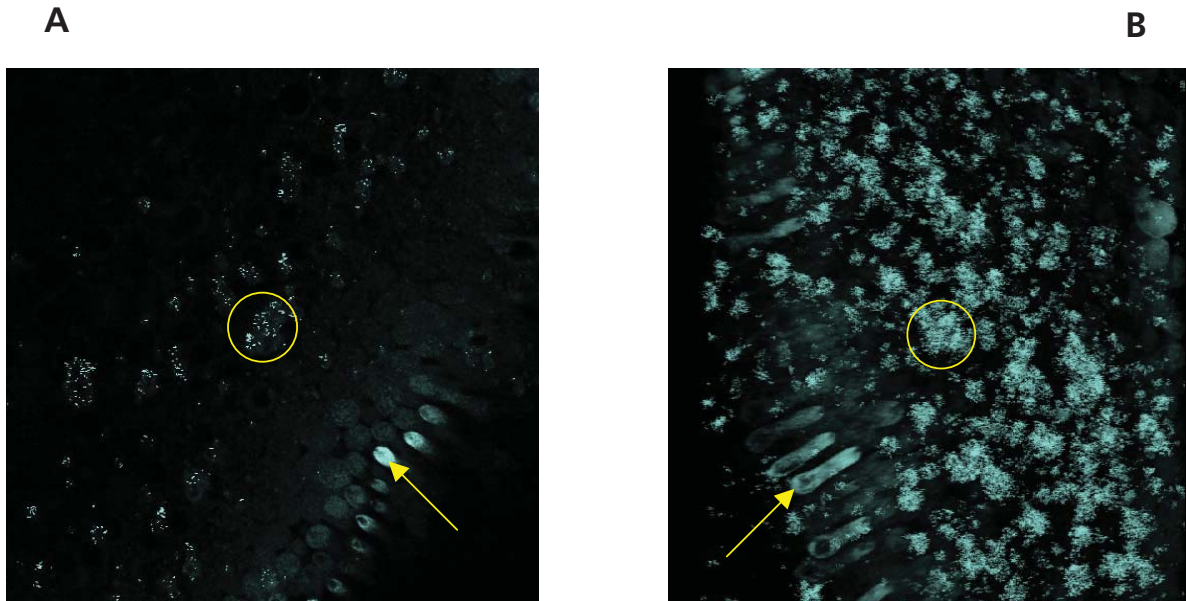
Fig. 8-13   Multiphoton image (A) and stack of images (B) of the frog otolith. Cells have been fluorescently stained by soaking the freshly prepared specimen in FM164 (arrow). Signal produced by the artifact is shown inside the circle. Sample courtesy of Bill Roberts, University of Oregon.

The artifacts completely obscure the three-dimensional reconstruction shown in Fig. 8-12/**B**.

This effect is not specific to Calcium Carbonate and has been seen in other samples, such as those with crystals of salt from evaporated sea water (data not shown).

Artifacts can also occur in fixed tissue. Local heating effects can cause bubbles to form in the mounting media or induce localized photo-chemistry in some mounting medias. It is important to test all mounting medias before use for multiphoton imaging. Fig. 8-14 shows an image where a large artifact is produced in an image using multiphoton excitation. In this example, Epon-araldite resin, generally used for EM embedding, was used. This is particularly bad for multiphoton imaging as it quickly becomes heated using focused NIR light.

Similar effects can been seen if oil and water are mixed, such as on the top of a coverslip when different immersion objectives are used on the same sample. Similar to the way water sizzles in a frying pan, steam will be produced from the focused heating of the oil and image artifacts result. Usually, these types are artifacts are dynamic as the water evaporates or the oil breaks down and the artifact may grow as you image for a longer time. It is important to clean the coverslip of your samples well with ethanol to dry off the water before mounting in oil or to clean off the oil before mounting in water.
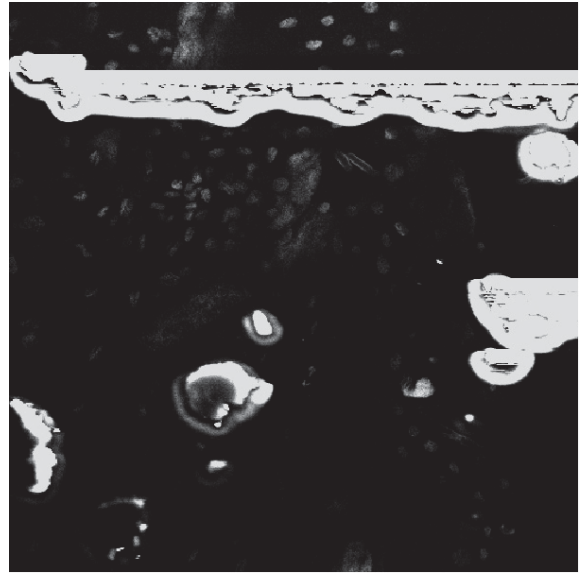

Fig. 8-14   Bubbles have formed in the mounting media of a fixed sample of a moth embryo. The embryo was labeled with DiA, but the specific signal is obscured by the artifact produced. Sample courtesy of Patty Jansma, University of Arizona.

8.3.4   Troubleshooting Checklist

Several key things can go wrong when imaging that will result in poor or no image being produced. Below is a checklist to help the user produce the best images possible.

8.3.4.1   No image being produced

Laser mode-lock:

Check to see if the laser is turned on and that it is mode-locked. For Coherent Chamelon and Spectra Physics Mai Tai lasers this is indicated in the LSM software, Laser control window.

AOM not on or too little laser power:

Check to see if the laser is turned on in the laser control panel and that the box is checked next to the percent transmission slider. If no image is seen and the laser is on, check the gain on the PMT and then increase the laser power. Check to see if increasing the percent transmission increases the power to the sample (use the monitor diode) in a linear way. If not, the AOM settings may need to be reset using the "linearize AOTF" macro.

Inefficient fluorochrome excitation (see 8.3.3.4):

Use a control slide to determine if the problem is in the sample. If excitation is seen with other samples, optimization of the laser wavelength may be necessary.

Laser power to the sample. Verify that the laser is focused through the objective by placing a white card on the microscope stage and removing the objective.

A bright red spot approximately 5-7 mm wide should be seen on the white card.

If no light is seen, then the beam may not be focused into the scan head or the shutter of the AOM may be closed. Check to see if there is a normal level of power going into the scan head. If no beam can be found entering the scan head, the shutter or the AOM may not be working and a service engineer should be called. If there is a beam after the 1 m lens, there may be poor alignment going into the scan head. Report this to a service engineer or attempt realignment under the guidance of a service engineer or expert user. Partial illumination of the card reflects poor alignment and the beam should be realigned into the scan head.

Non-linear power attenuation:

If changing the percent transmission leads to non-linear power attenuation, the AOM can be linearized using the AOTF fit macro. By opening this macro and following the instructions, one can verify the linearization of any of the laser lines and then run a linearization protocol to reoptimize the settings. Be sure the laser is set to the appropriate wavelength before running this macro. Choose only the NIR wavelength to optimize the AOM attenuation for the Ti: Sapphire laser.

8.3.4.2   Poor image quality

Lines or spaces across the image:

This can result from an unstable mode-lock. CW breakthrough appears as blank lines in the image. Q-switching will result in wavy lines in the image. A service engineer from Choherent or Spectra need to check the laser.

Poor fluorescence sensitivity:

This can result from many factors or a combination of factors. These include the following:

Correct wavelength (see 8.3.3.4):

Be sure that the laser is set at the best wavelength for the fluorochrome being examined. Empirical testing may be necessary to determine the best wavelength. Tuning to a different wavelength may also help to reduce autofluorescence from some tissues.

Poor Beam Alignment into the scan head (see 8.3.3.2.2):

Remove the objective and look at the reflection of the beam onto a white card. The beam should be round and even without any flat edges. Peak the alignment of the beam into the scan head.

Poor transmission through the objective (see 8.3.3.3):

Many objectives are corrected for work with UV and visible range excitation and have poor transmission of photons in the near IR.

Sliders/polarizers in the path:

DIC sliders and polarizers in the path can reduce excitation and emission sensitivity. Remove sliders or slide them to an open position.

Poor sensitivity in fixed samples:

Fixatives and mounting medias can sometimes limit the sensitivity of fluorescence detection or increase autofluorescence reducing the signal-to-noise ratio. For multiphoton excitation, the mounting media can also reduce the peak intensity of the laser deep into the sample. Empirical determination of the best fixative and mounting media for a particular application may be necessary.

8.3.5   References

Albota, M., Beljonne, D., Bredas, J.L., Ehrlich, J.E., Fu, J.Y., Heikal, A.A., Hess, S.E., Kogej, T., Levin, M.D., Marder, S.R., McCord-Maughon, D., Perry, J.W., Rockel, H., Rumi, M., Subramaniam, C., Webb, W.W., Wu, X.L., Xu, C. Design of organic molecules with large two-photon absorption cross sections. Science 281, 1653-1656.

Diels, J.C. and Rudolph, W. (1995) Ultrashort Laser Pulse Phenomena: fundamentals, techniques, and applications on a femtosecond time scale, San Diego: Academic Press Inc.

Haugland, R. (1996) Handbook of Fluorescent Probes and Research Chemicals. M.T.Z. Spence, ed., Eugene, OR: Molecular Probes.

Patterson, G. H. and Piston, D. W. (2000) Photobleaching in two-photon excitation microscopy. Biophys. J. 78, 2159-2162.

Kempe, M. (1993) Impact of chromatic and spherical aberration on the focusing of ultrashort light pulses by lenses, Opt. Lett, 18, 137-139.

König, K., Simon, U., Halbhuber, K-J (1996) 3D Resolved Two-Photon Fluorescence Microscopy of Living Cells Using a Modified Confocal Laser Scanning Microscope, Cellular and Molecular Biology 42, 1181-1194.

Netz, R., Feurer, T., Wolleschensky, R. and Sauerbrey, R. (2000) Measurement of the pulse-front distortion in high numerical aperture lenses, Appl. Phys. B-Lasers, 70,833-837.

Rumi, M., Ehrlich, J.E., Heikal, A.A., Perry, J.W., Barlow, S., Hu, Z, McCord-Maughon, D., Parker, T., Röckel, H., Thayumanavan, S., Marder, S., Beljonne, D and Brédas J-L. (2000) Structure-Property Relationships for Two-Photon Absorbing

Chromophores: Bis-Donor Diphenylpolyene and Bis(styryl)benzene Derivatives J. Am. Chem. Soc. 122, 9500-9510.

Squirrell, J.M., Wokosin, D.L., White, J.G. and Bavister, B.D. (1999) Long-term two-photon fluorescence imaging of mammalian embryos without compromising viability. Nature Biotechnology 17, 763-767.

Wolleschensky, R., Feurer T., Sauerbrey R., and Simon I. (1998) Characterization and optimization of a laser-scanning microscope in the femtosecond regime. Applied Physics B-Lasers and Optics 67, 87-94.

Wolleschensky, R., Dickinson, M.E. and Fraser, S.E. (2002) Group-Velocity Dispersion and Fiber Delivery in Multiphoton Laser Scanning Microscopy. In, Confocal and Two-photon Microscopy: Foundations, Applications, and Advances. Ed. A. Diaspro. New York: Wiley-Liss, Inc.

Xu, C. (2000) Two-photon Cross Sections of Indicators. In, Imaging Neurons: A Laboratory Manual. R. Yuste, F. Lanni, and A Konnerth, eds. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Recommended Books and Reviews:

Handbook of Biological Confocal Microscopy (1995) ed. J. Pawley, New York: Plenum Press.

Imaging Neurons: A Laboratory Manual. R. Yuste, F. Lanni, and A Konnerth, eds. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Methods in Cellular Imaging, A. Periasamy, ed., Oxford, UK: Oxford University Press

Denk, W. and Svoboda, K. (1997) Photon Upmanship: Why Multiphoton Imaging Is More than a Gimmick. Neuron, 18,351-357.