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LSM 710 and ConfoCor 3
Operating Manual

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.

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How to make best use of the LSM 710 operating instructions:

This operating manual also includes the instructions for the ConfoCor 3 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 reminds 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

1 Notes on Device Safety

This section contains general notes on device safety, safe operation, and possible hazards caused by failure to observe the instructions.

2 Setup Requirements

The Setup Requirements section outlines the installation and supply requirements of the LSM 710 and ConfoCor 3 Microscope Systems, together with the relevant specifications.

3 Introduction to Laser Scanning Microscopy

This section contains the "LSM 710 and LSM 710 NLO Quick Guide", the "Confocal Laser Scanning Microcopy Principles", and the "Confocal Laser Scanning Microcopy Methods" brochures.

Here you will find an introduction to Laser Scanning Microscopy, with an explanation of the principles of confocal imaging. The section also outlines the ways to present LSM image series in three dimensions, and introduces you to the performance features of your LSM 710.

4 System Operation

In this section you will find the most important steps and procedures of the LSM menu structure. The step-by-step description how to get an image will be shown by typical application examples including the WINDOWS VISTA graphic user environment.

5 Macros and Visual Basic

This section contains a description of the use of additional functions, e.g. maintenance, macros.

6 Tools, Additional Software

This section contains a description of the use of optional software packages and the tools for setting the microscope, e.g. "3D for LSM".

7 Annex

The annex contains the Application-specific Configurations, special notes and information for using the LSM microscope.

8 Multiphoton Laser Scanning Microscopy - Using the LSM 710 NLO

This section provides detailed back ground information on Multiphoton Microscopy including trouble shooting procedures.

9 ConfoCor 3

This section provides background information on Fluorescence Correlation Spectroscopy (FCS) and Photon Counting Imaging

10 Certification

11 Laser Safety Warning Labels

CHAPTER 1 NOTES ON DEVICE SAFETY

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1 NOTES ON DEVICE SAFETY

1.1 General

The LSM 710 laser scanning microscope as well as the ConfoCor 3, including their original accessories and compatible accessories from other manufacturers, may only be used for the purpose of microscopic and spectroscopic 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.

The ConfoCor 3 spectroscope is used for fluorescence correlation spectroscopy, whereas the beam is parked in a sample, which might consist of a solution or a cell, fluorescence fluctuations recorded and analyzed by the so-called correlation function.

 Installation and commissioning of the LSM 710 and ConfoCor 3 systems 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 710 or ConfoCor 3 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 Axio Observer.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 WINDOWS operating system and its graphical user interface. The respective manuals are supplied together with the programs.



The LSM 710 and ConfoCor 3 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 710 and ConfoCor 3 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.

The LSM 710 and ConfoCor 3 meets the EMC requirements for EN 55011 Class A (intended use in industrial environment). If the LSM is operated in a residential area or in a small trade area other devices may be influenced by conducted or radiated disturbance. In this case special EMC measures are required.

1.2 Regulations

Extensive knowledge of the hardware/the system is indispensable for safe operation of the LSM 710 and ConfoCor 3.

 Read these operating instructions and all device publications belonging to the system conscientiously **before** operating the LSM 710 or the ConfoCor 3! 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 710 and ConfoCor 3 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", taking relevant CSA and UL specifications into account.
- DIN EN 60825-1 (IEC publication 60825-1) "Safety of laser equipment".
- 21 CFR §1040.10: "Performance Standards for light emitting products – laser products".
- DIN EN 61326: "Electrical equipment for control technology and laboratory use – EMC-requirements",
- Low voltage directive: 2006/95/EG,
- EMC directive: 2004/108/EG.

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 710 and ConfoCor 3 come 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.

The mains socket outlet must be equipped with a fuse having minimum tripping characteristic C according to IEC/EN 60898.

Line voltage	3/N/PE 400/230 V AC ($\pm 10\%$)	2/N/Ground 240/120 V AC ($\pm 10\%$)
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 2 = 1.5 kVA max. Phase 3 = 2.6 kVA max.	Phase 1 = 3.2 kVA max. Phase 2 = 2.8 kVA max.
Power consumption	4000 VA max.	4000 VA max.
Class of protection	I	I
Type of protection	IP 20	IP 20
Overvoltage category	II	II
Pollution degree	2	2

- EMC test according to DIN EN 61326-1 (10/2006)
 1. Noise emission according to CISPR 11 / DIN EN 55011 (11/2007)
 2. Noise immunity according to table 2 (industrial sector)

In the rare case of a voltage surge on the power supply line (e.g. from indirect lightning stroke), a momentary interruption of the real time system's functionality is possible. This is no defect. A possible protective means to further minimize the probability of a functional disruption is a transient voltage protector in your facility's power system.

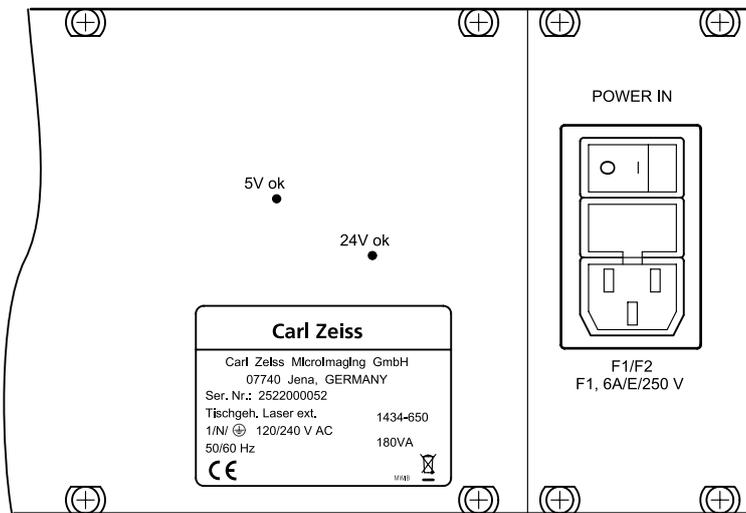


Fig. 1-1 Fuses F1/F2 of the external laser module

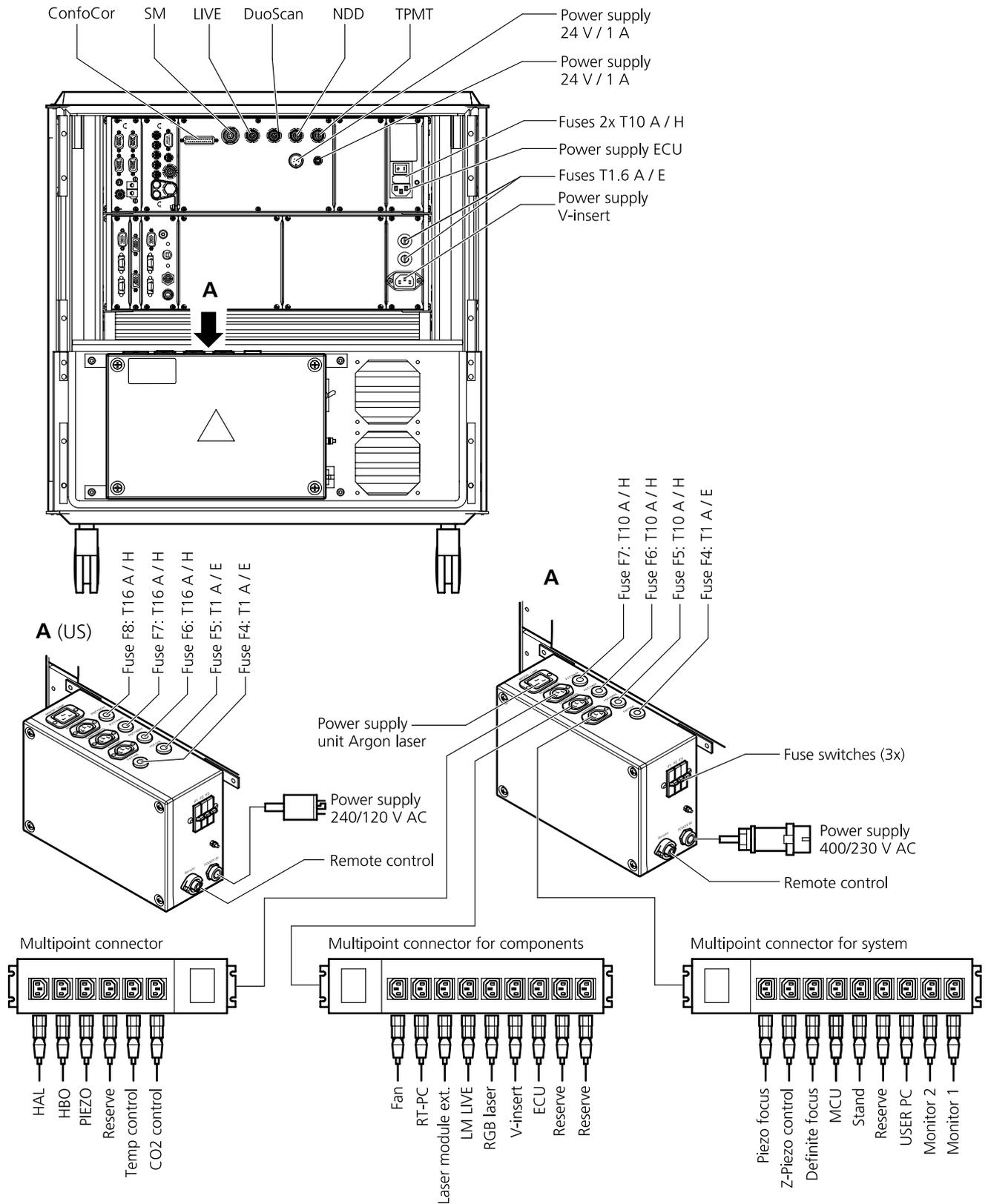


Fig. 1-2 Power connector for LSM 710 and components. Free/reserve outlets may be used to supply power to additional equipment. No more than 1 A can be provided by each outlet.

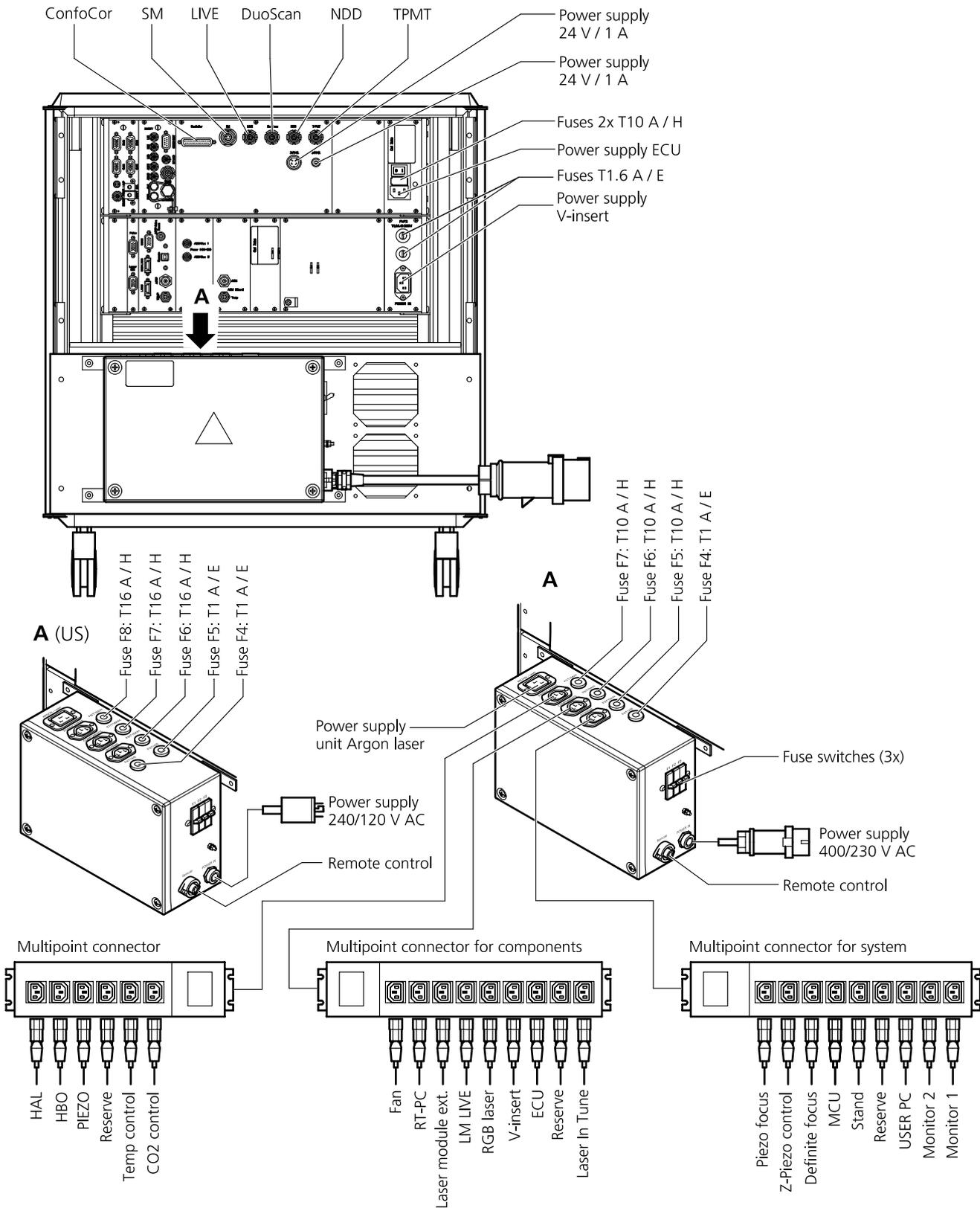


Fig. 1-3 Power connector for LSM 710 with Laser In Tune and components. Free/reserve outlets may be used to supply power to additional equipment. No more than 1 A can be provided by each outlet.

The door interlock interface (Fig. 1-4/1) 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.

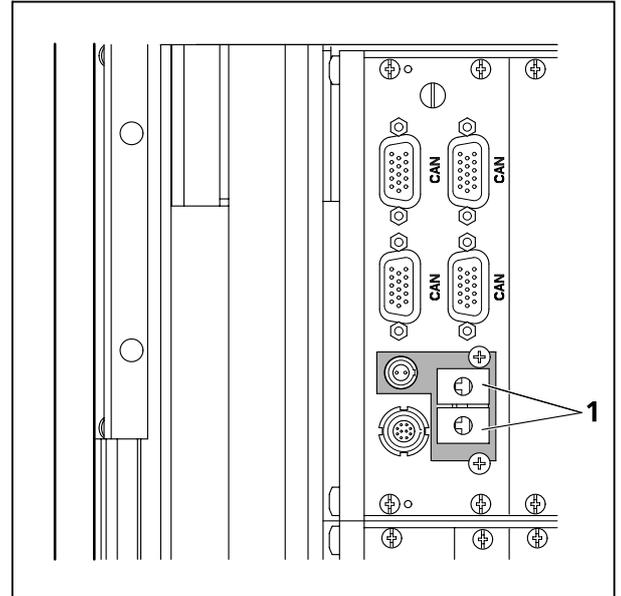
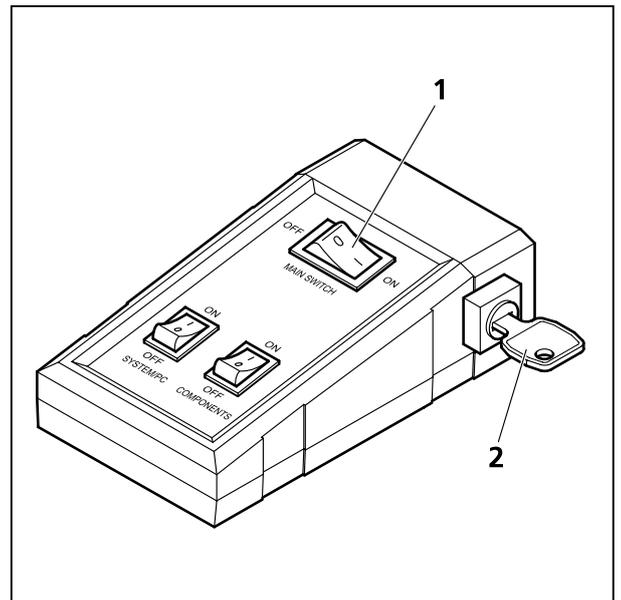


Fig. 1-4 Door interlock interface (1) on the back of the electronic rack (see Fig. 1-2 and Fig. 1-3, top)

The LSM 710 is controlled by a remote control. This remote control contains the main switch for the system and the key switch for the laser.

- To start the system switch the main switch (Fig. 1-5/1) to ON.
- To activate the laser turn the key switch (Fig. 1-5/2) to ON position.



- 1 Main switch ON/OFF
- 2 Laser key switch

Fig. 1-5 LSM 710 Remote control

1.4 Physical Dimensions

	Length (cm)	Width (cm)	Height (cm)	Weight (kg)
Small passively damped system table	90	75	77	80
Small actively damped system table	90	75	77	90
Large actively damped system table	120	90	77	120
Active anti-vibration table (NLO) for Mai Tai Laser or Chameleon	180	150	75	200
Active anti-vibration table (NLO) for two-microscope configuration	250	150	75	400
Scanning Module LSM 710	50	45	22	27
Scanning Module LSM <i>DuoScan</i>	40	15	13	8
Module ConfoCor 3	49	27	18	25
Microscope	50	35	50	20
Electronic Rack with laser units 710	80	60	65	80
Plug-in unit external laser	70	55	25	10
Electronic Rack 5 <i>LIVE</i> / 7 <i>DUO</i>	70	60	65	20
Laser Module <i>LIVE</i>	66	52	22	58
Laser Rack <i>In Tune</i>	80	60	45	40

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 = 15 °C to 35 °C, any conditions different from item 1. and 5.
3. Storage, less than 16 h	T = -20 °C to 55 °C
4. Storage, less than 6 h	T = -20 °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 710 and the ConfoCor 3 systems 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 710 laser scanning microscope and the ConfoCor 3 spectroscope are delivered in several crates.



The LSM 710 and ConfoCor 3 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 electrical connection box.



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 710 and the ConfoCor 3 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.



The HBO 50 and 100 W, XBO 75 W and X-cite 120 lamps used on the light microscopes incident light path emit UV light which is harmful to the human eye and skin when observed without appropriate filters. Never remove the lamp and look direct into the emitted light.

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 710 and ConfoCor 3 should only be operated in conjunction with the system table (vibration damping).



Please note the following for the ConfoCor 3:

- The ConfoCor 3 requires an Axio Observer.Z1 microscope and is attached to channel 4 of the LSM 710. Note that only system tables with sufficient anti-vibration functionality should be used.
- A scanning stage is recommended for automatic positioning for solution measurements. For cell measurements the scanning mirrors are recommended.

1.7 Warning and Information Labels



The warning and information labels attached on the LSM 710 and ConfoCor 3 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 radiation hazards possible when operating the system.



Attention:
High voltage.



Pull the mains plug before opening the device housing.



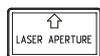
Caution:
Hot surface.



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.

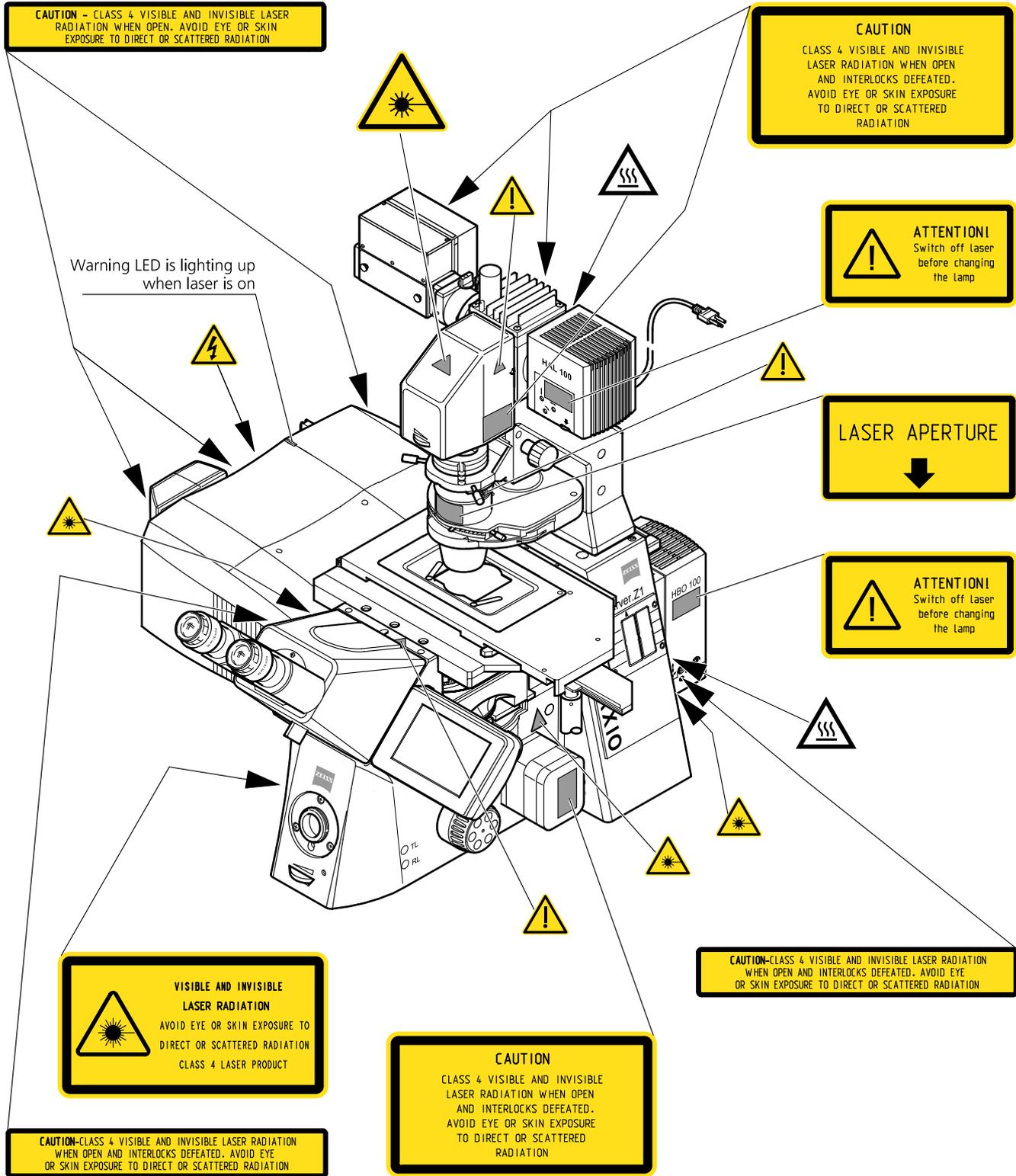


Fig. 1-6 Warning and information labels on the Axio Observer.Z1 microscope with the LSM 710 scanning module

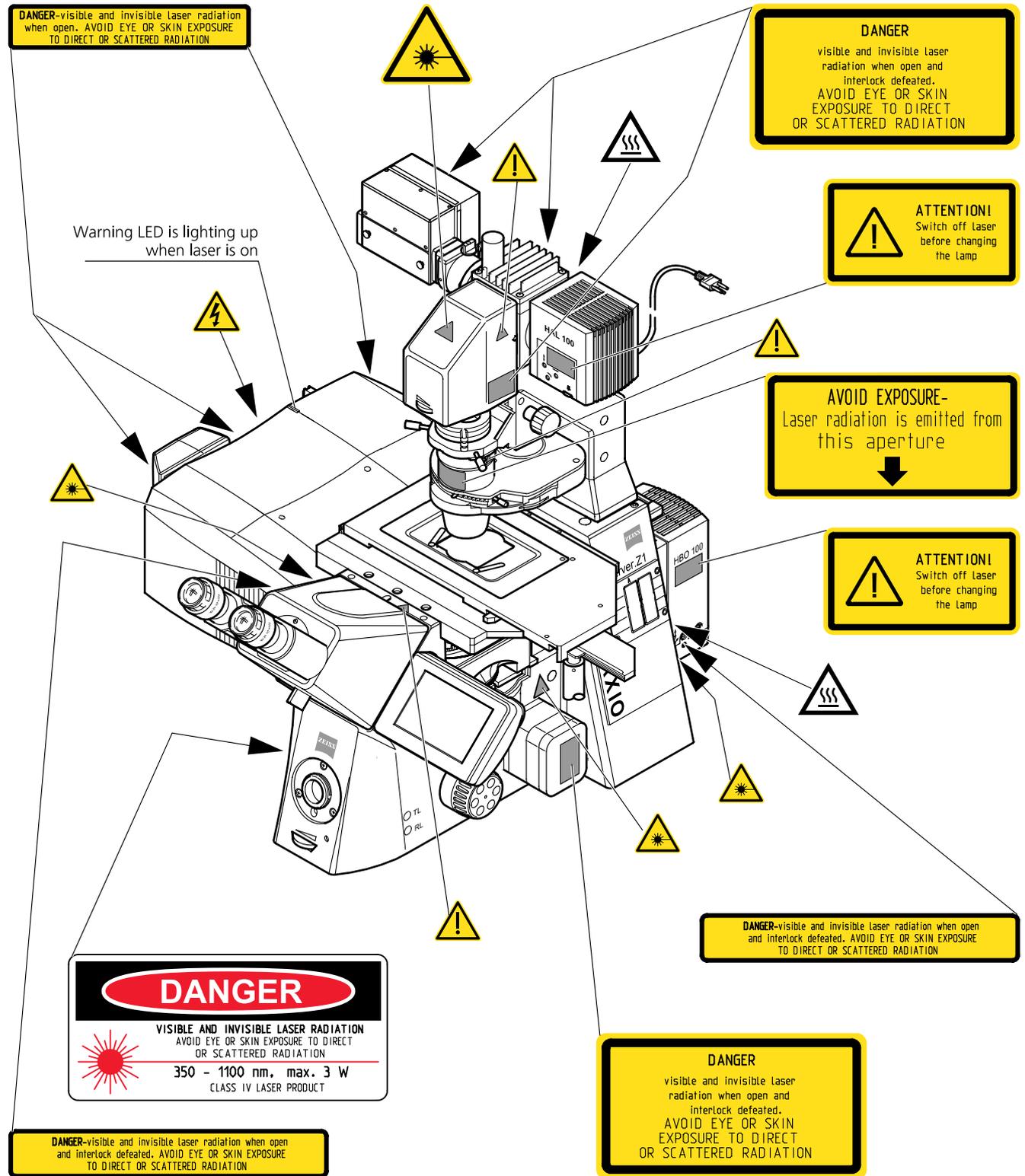


Fig. 1-7 Warning and information labels on the Axio Observer.Z1 microscope with the LSM 710 scanning module (US version only)

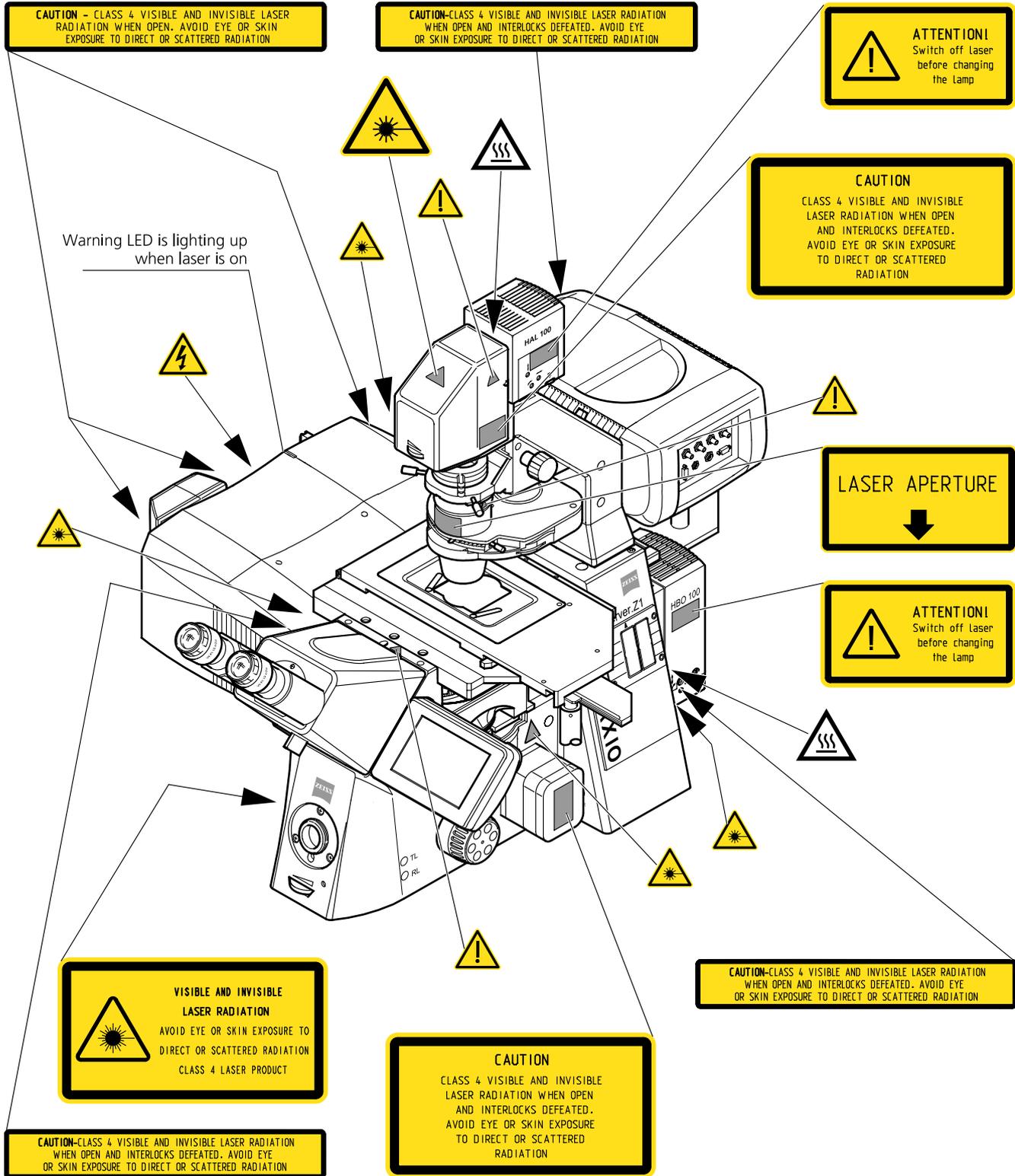


Fig. 1-8 Warning and information labels on the Axio Observer.Z1 microscope with the LSM 710 scanning module and the ConfoCor 3 detection module

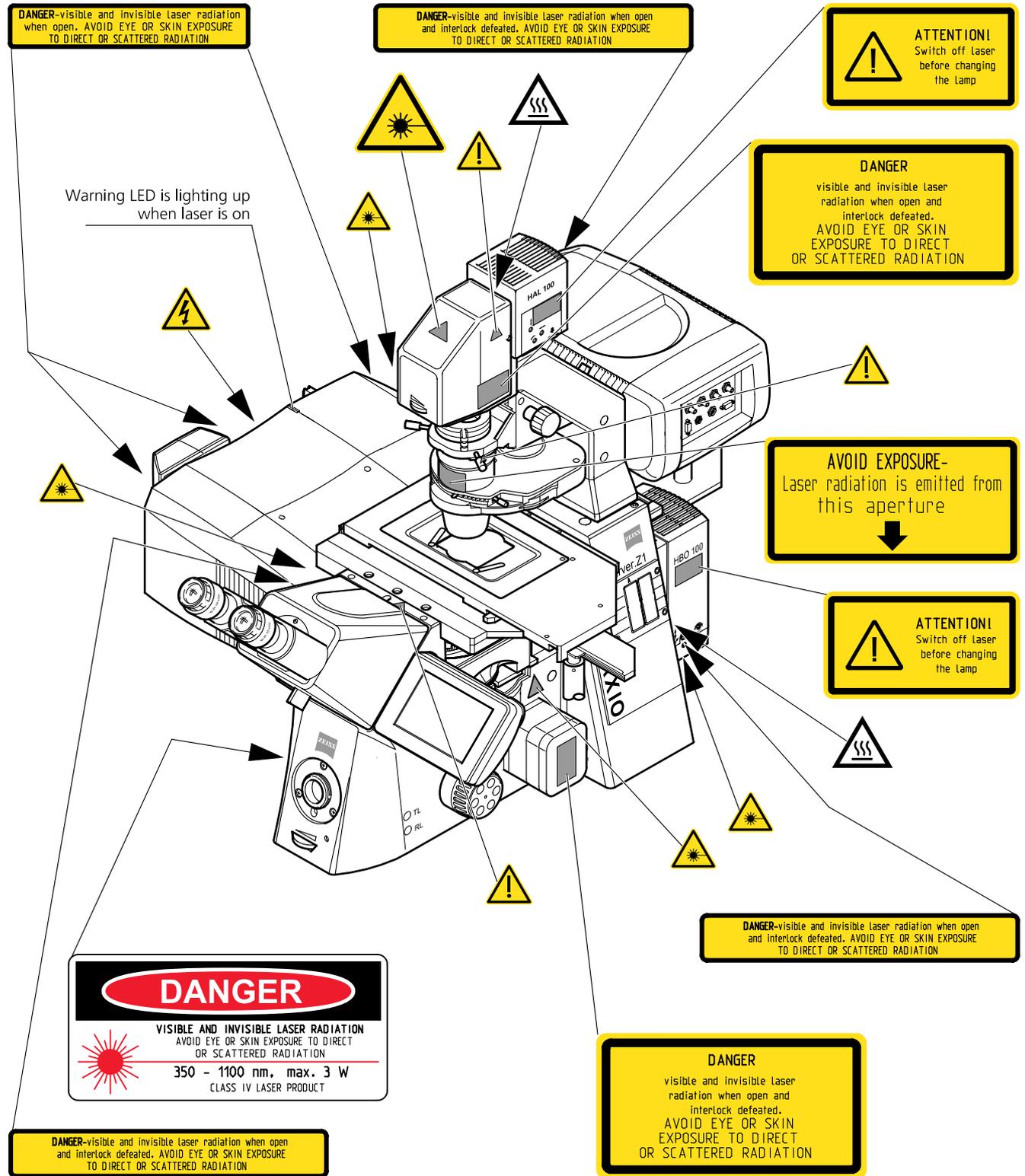


Fig. 1-9 Warning and information labels on the Axio Observer.Z1 microscope with the LSM 710 scanning module and the ConfoCor 3 detection module (US version only)

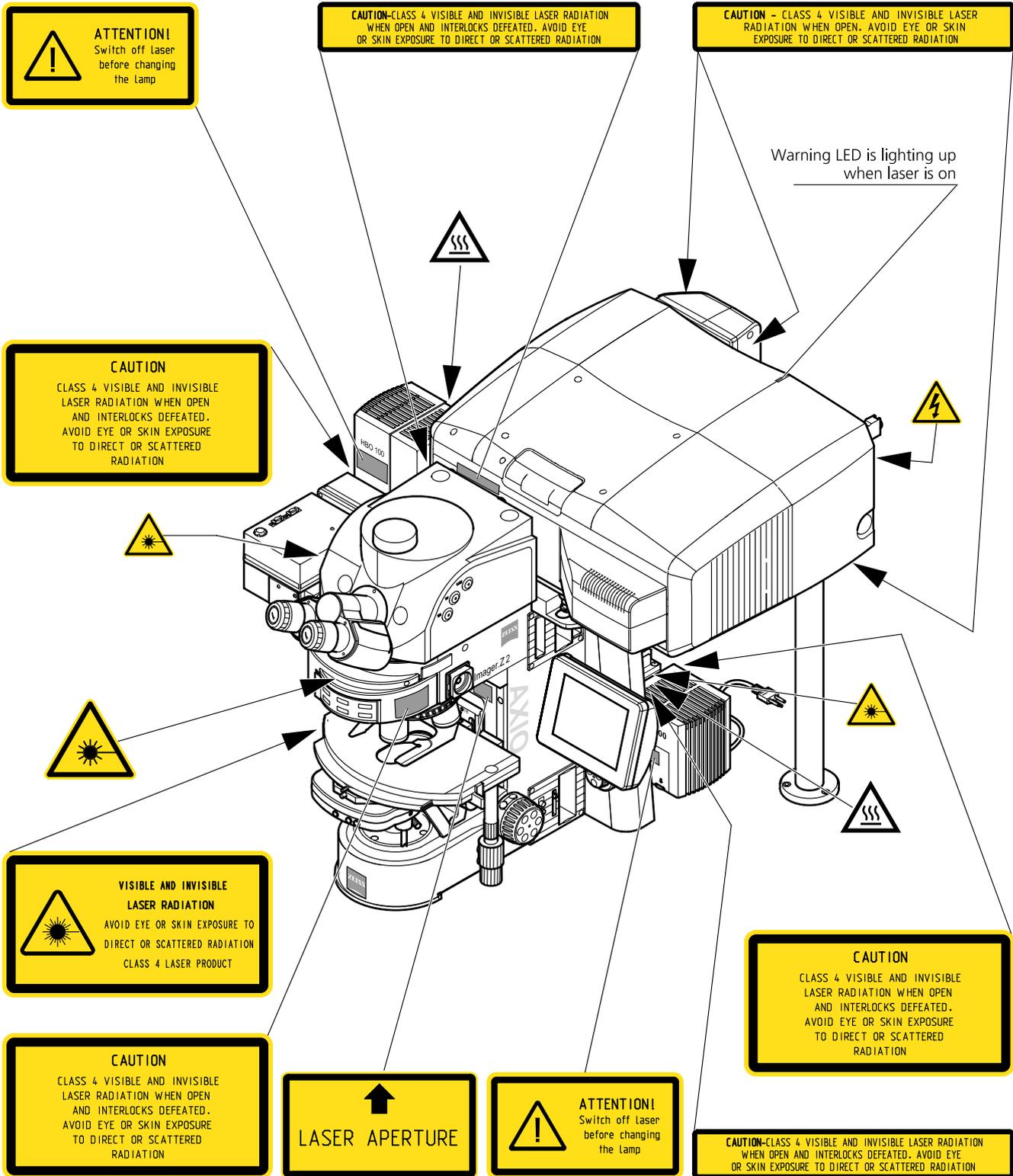


Fig. 1-10 Warning and information labels on the Axio Imager.Z2 microscope with the LSM 710 scanning module

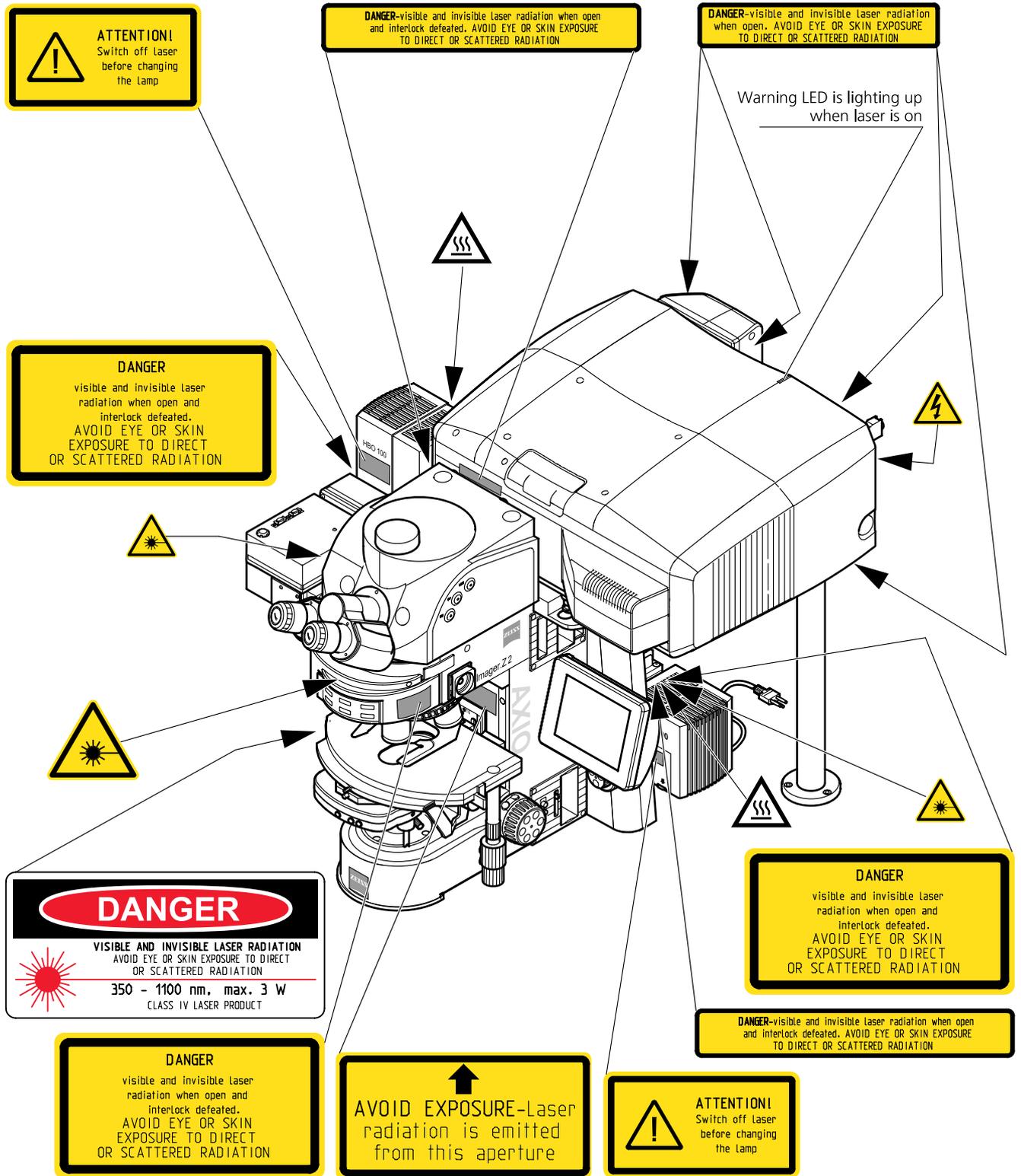


Fig. 1-11 Warning and information labels on the Axio Imager.Z2 microscope with the LSM 710 scanning module (US version only)

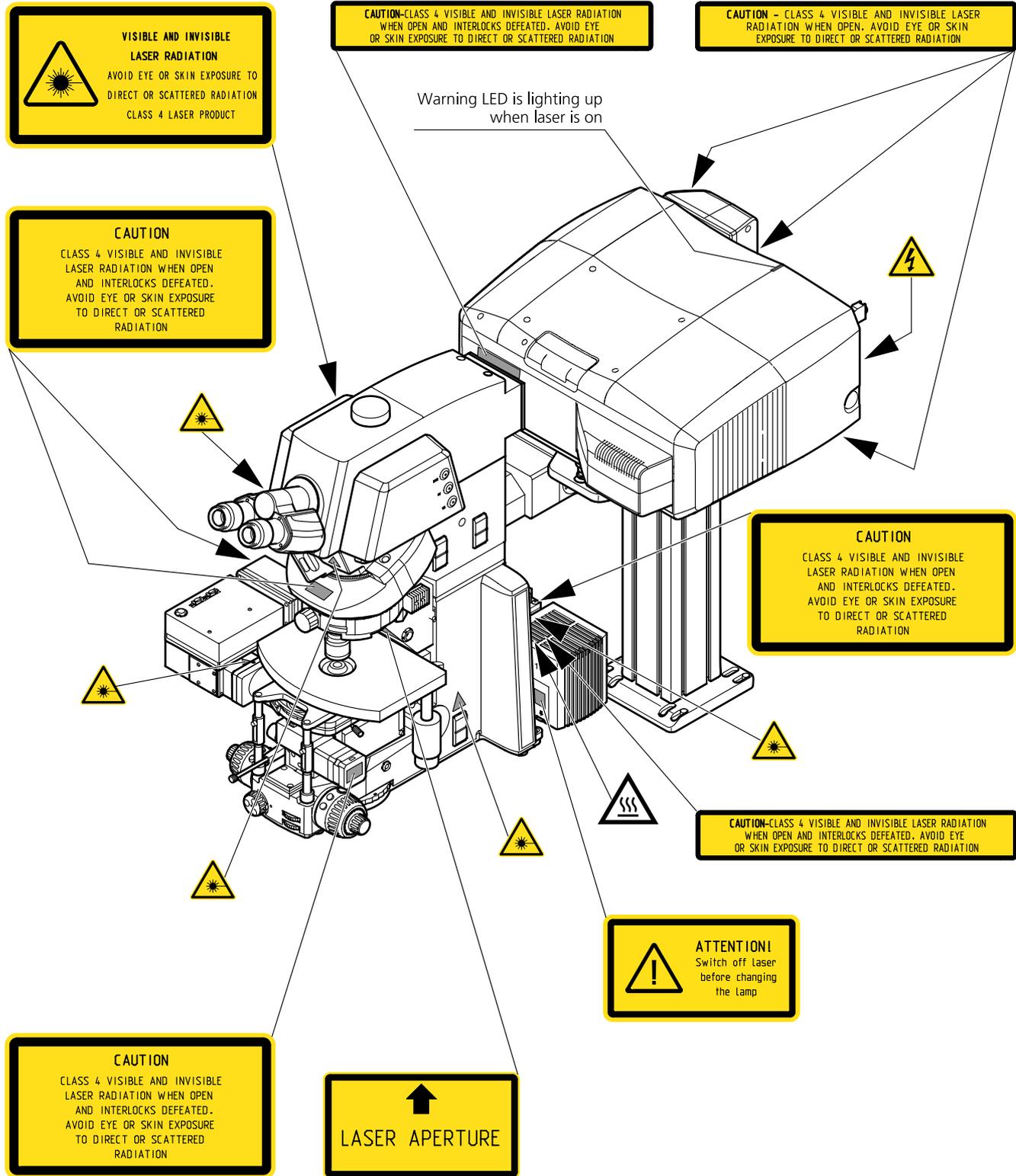


Fig. 1-12 Warning and information labels on the Axio Examiner microscope with the LSM 710 scanning module

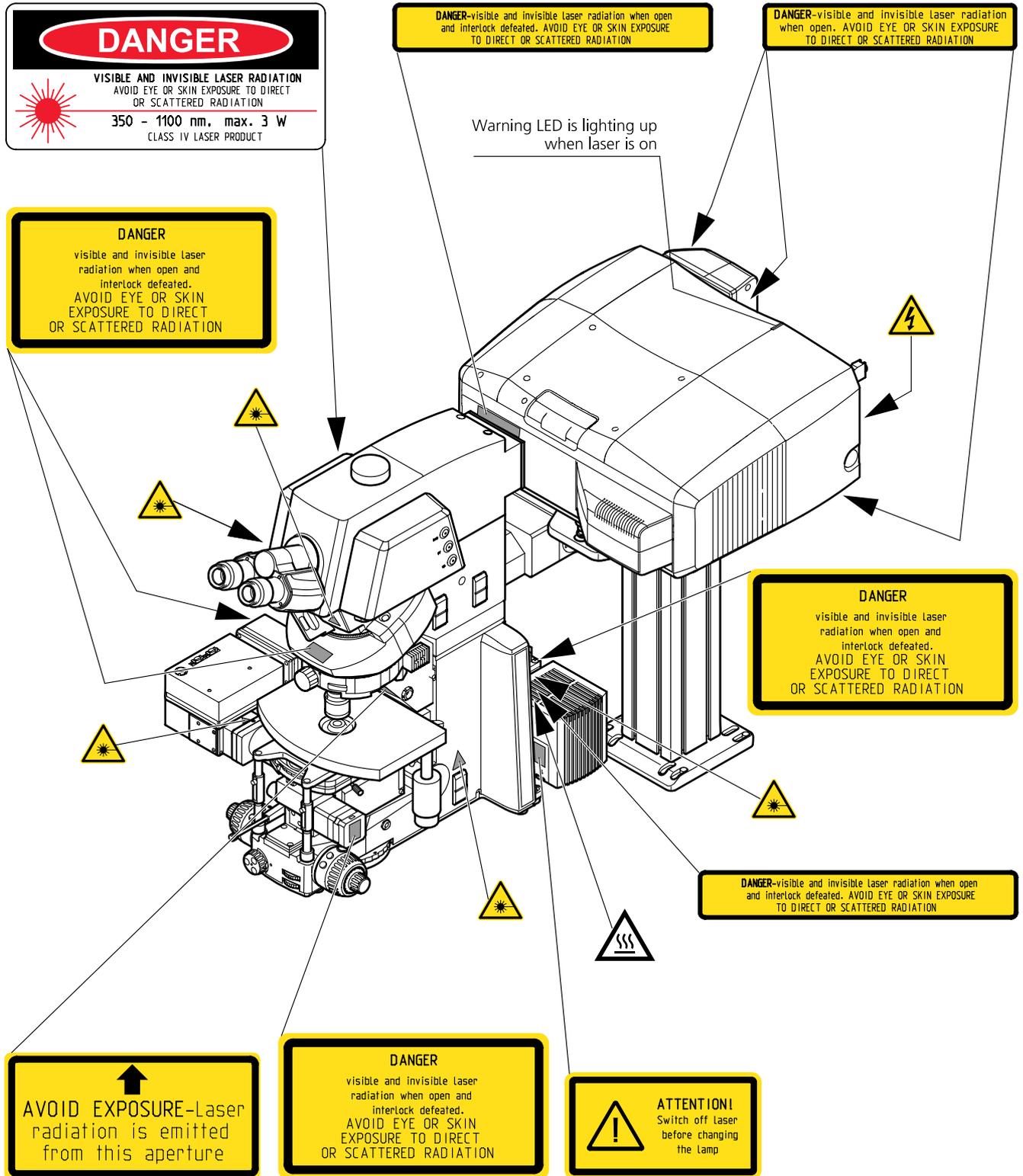


Fig. 1-13 Warning and information labels on the Axio Examiner microscope with the LSM 710 scanning module (US version only)

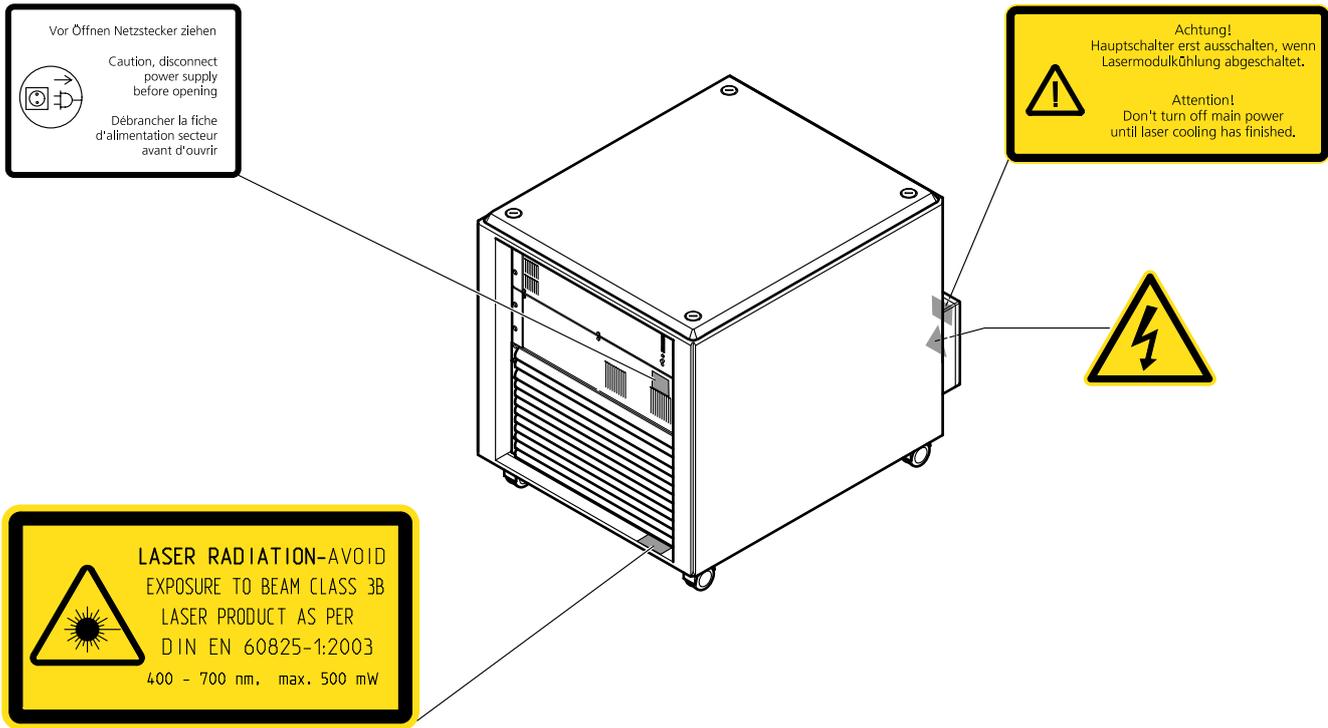


Fig. 1-14 Warning and information labels on the laser and electronic rack

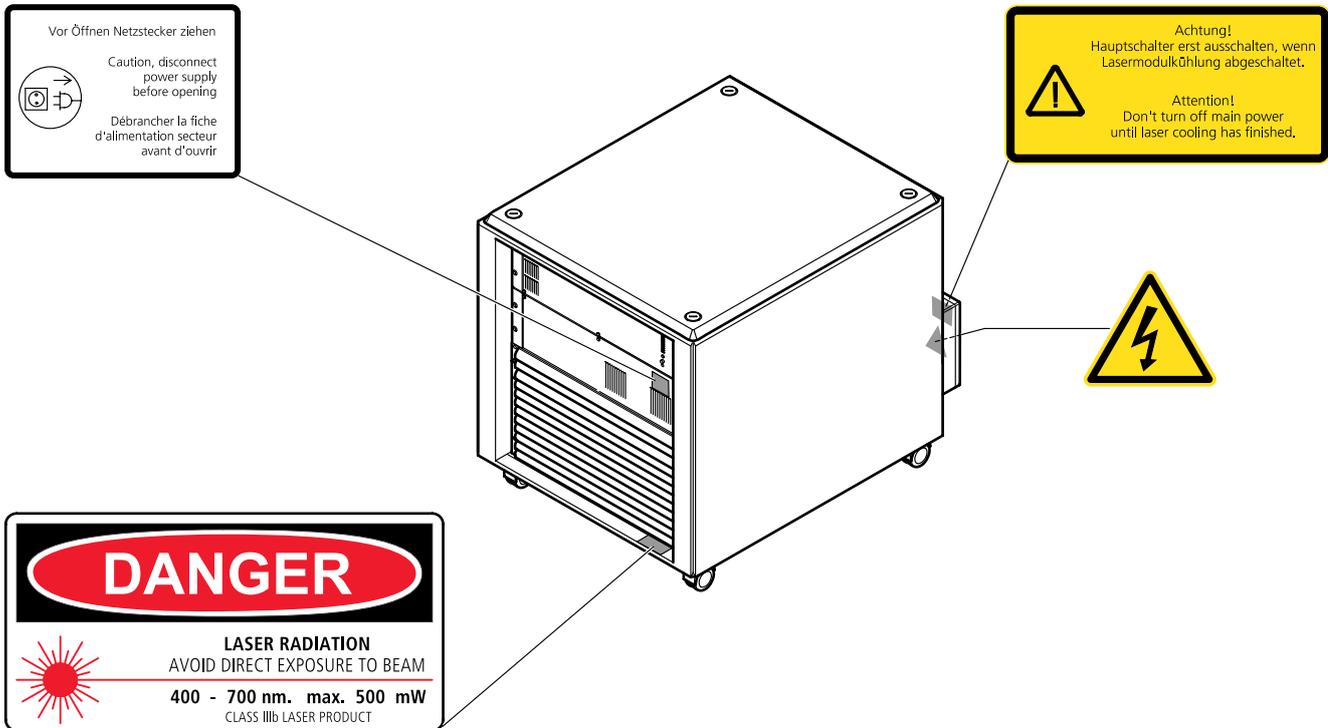


Fig. 1-15 Warning and information labels on the laser and electronic rack (US version only)

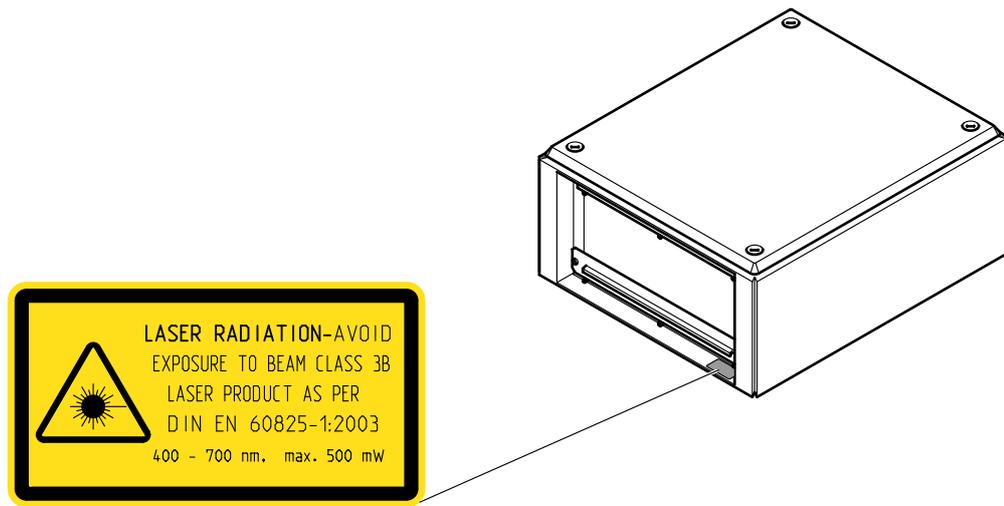


Fig. 1-16 Warning and information labels on the Laser Rack In *Tune*

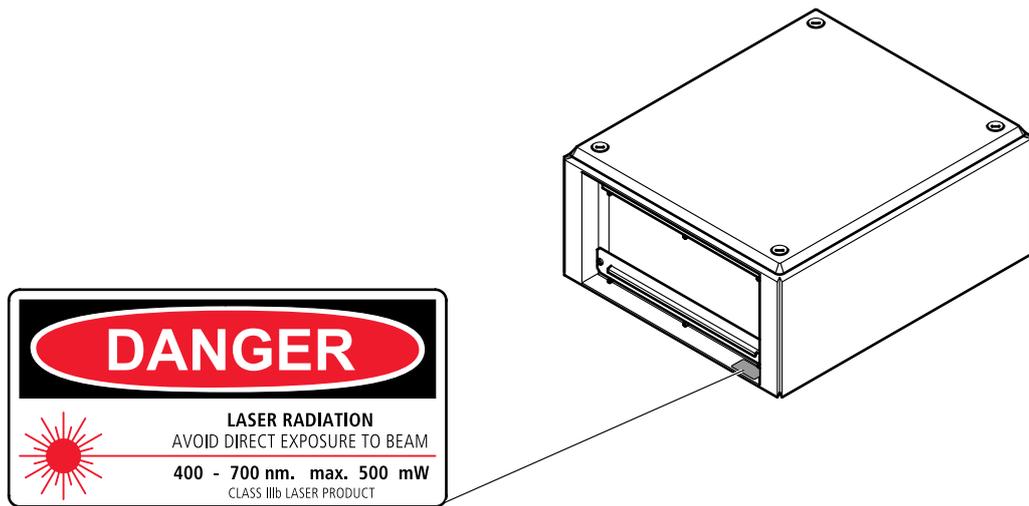


Fig. 1-17 Warning and information labels on the Laser Rack In *Tune* (US version only)

1.8 Notes on Handling the Laser Components and Illumination Systems



The LSM 710 and ConfoCor 3 are laser hazard class 3B instruments. If equipped with a Ti:Sa Laser, the LSM 710 and ConfoCor 3 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.



Caution: Use of controls or adjustments or performance of procedures other than those specified herein may result in hazardous radiation exposure.

The following laser types are currently intended for use in the LSM 710. The use of any other lasers as the ones listed below is not authorized.

Laser	Class	Nominal Power*
1 Diode laser 405 nm	3B	30 mW
2 Diode laser ps/cw 405 nm	3B	30 mW
3 Diode laser ps/cw 440 nm	3B	25 mW
4 Ar/ML 458/488/514 nm	3B	25/35 mW
5 HeNe 543 nm	3B	1 mW
6 DPSS 561 nm	3B	20 mW
7 HeNe 594 nm	3B	2 mW
8 HeNe 633 nm	3B	5 mW
9 Ti:Sa Laser Mai Tai (Spectra Physics) 690-1040 nm (depending on the model)	4	min. 3 W
10 Ti:Sa Laser Chameleon (Coherent) 690-1064 nm (depending on the model)	4	min. 3 W
11 Diode laser 405 nm	3B	50 mW
12 Diode laser 440 nm	3B	20 mW
13 OPSS laser 488 nm	3B	100 mW
14 OPSS laser 532 nm	3B	75 mW
15 DPSS laser 561 nm	3B	40 mW
16 Diode laser 635 nm	3B	35 mW
17 Tunable Laser <i>In Tune</i>	3B	min. 1.5 mW*

* Post-fiber average power

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

The following table shows the laser life times and the corresponding warranty:

Laser wavelength (nm)	Type	Min. power specification for end of life time	Expected life time (in hours)
458/488/514	Laser module LGK 7812 ML5	11.5 mW ¹⁾	> 5000
458/488/514	Laser module LGK 7872 ML8	19.5 mW ¹⁾	> 3000
543	LGK 7786 P with fibre coupling	0.7 mW	> 7000
594	LGK 7512 PF with fibre coupling	1.3 mW	> 10000
633	LGK 7628-1F with fibre coupling	3.1 mW	> 10000
561	FKL module YLK 6120 T	13 mW	> 5000
405	Laser cassette 405 cw	11.5 mW	> 5000
405	Laser cassette 405 cw/ps	11.5 mW ²⁾	> 5000
445	Laser cassette 445 cw/ps	11.5 mW ²⁾	> 5000
488-640	Tunable Laser <i>In Tune</i>	1.35 mW (average power)	> 3000

1) Power specified for single laser lines, life time specified for total power

2) Average power for cw operation

If used properly, the LSM 710 and ConfoCor 3 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 safety officer before commissioning the laser.
- The LSM system and the Ar laser 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 laser ON LED on the scan head lights up when laser radiation is emitted.
- 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-Touch screen 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.

1.9 Notes on Handling the Computer and Data Media

The computer used as standard in your LSM system is a high-end workstation Pentium/Xeon computer with WINDOWS VISTA operating system (depending on availability).

 Make sure, though, that you receive your LSM/ConfoCor 3 system with the operating system installed, with initialization and start files set up and with the LSM/ConfoCor 3 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 VISTA 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 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, slot fuse carriers or screw type fuse carriers. The fuse type is given on the hardware itself and also in Fig. 1-2.

For exchanging fuses proceed as follows:

- Open the fuse carrier by turning 90° clockwise.
- Pull out the fuse carrier and replace the fuse by a new one of the same type.
- Insert the fuse carrier, push it in and lock it by turning 90° anti-clockwise.

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 exchanged 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.

For exchanging the halogen lamp on stands with HAL 35 W proceed as follows:



Before exchanging the halogen lamp switch off all laser units.



Caution:

Hot surfaces on lamp housing (Fig. 1-18/1), heat sink (Fig. 1-18/2) and halogen lamp:
Make sure to let them cool down sufficiently!



Caution:

Do not touch the lamp with your bare hands; if required, clean the lamp using pure alcohol **before** switching the lamp on for the first time in order to prevent contaminations from burning in.

- Switch off the laser and let the halogen lamp cool down for about 15 minutes.

- Unlock the lamp housing (Fig. 1-18/1) by slightly turning it anticlockwise.
- Remove the halogen lamp with alignment base (Fig. 1-18/3).
- Remove new 12 V 35 W halogen lamp with adjustment base from the packing box. Insert it in the mount taking care that the tip of the mount engages in the centering notch of carrier plate.
- After replacing the halogen lamp, attach the lamp housing and lock it.

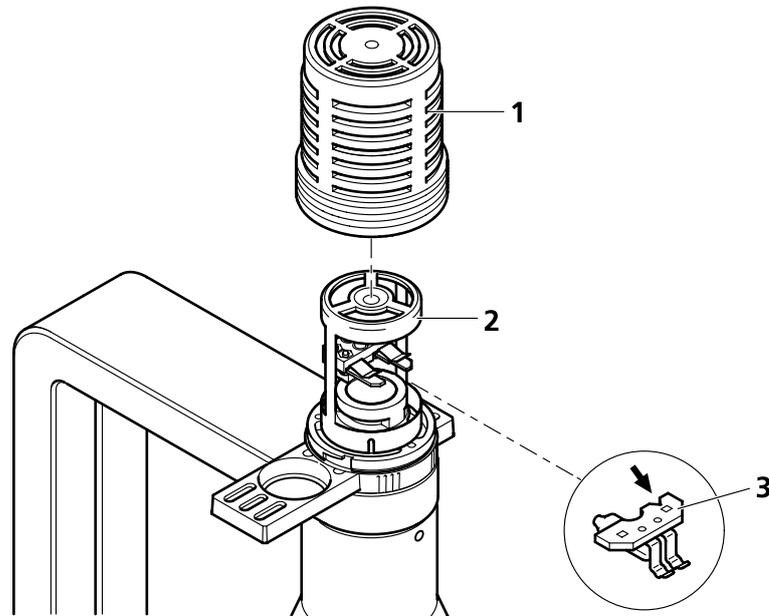


Fig. 1-18 Replacing the halogen lamp

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.

We strongly recommend to get the calibration objective for the objective turret, since the calibration and test tasks in the system maintenance tool (see CHAPTER 6: "TOOLS, ADDITIONAL SOFTWARE") require this tool.

Especially the scanner calibration is recommended every 1-2 years, when the high speeds (13-15) are used frequently.

1.11 User Interface



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 dismantled by the user or are accessible by the user:

- Halogen lamp,
- Transmission PMT,
- Switching Mirror,
- Scan heads,
- NDD's (see CHAPTER 8: "MULTIPHOTON LASER SCANNING MICROSCOPY ..." for details),
- ConfoCor 3 filter wheels (exempt the block filter wheel),
- ConfoCor 3 external connectors,
- LSM 710 filter wheel

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 which have to be operated in the following way:

Interlock with sensor ring and contact ring:

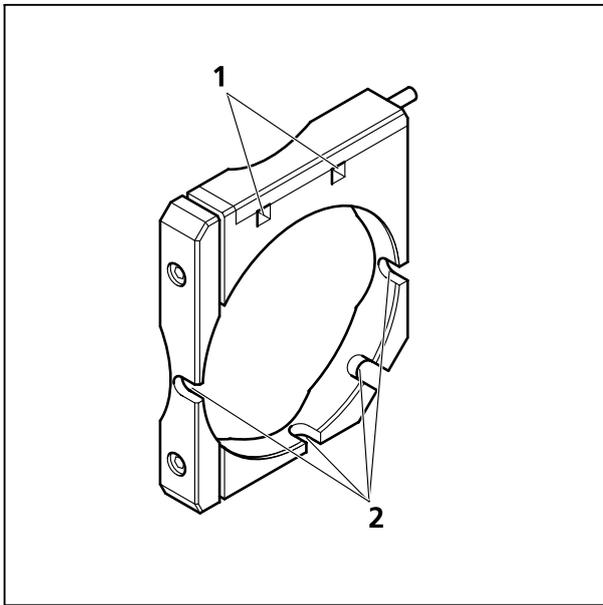


Fig. 1-19 Sensor ring mounted to the interface ports on the microscope side

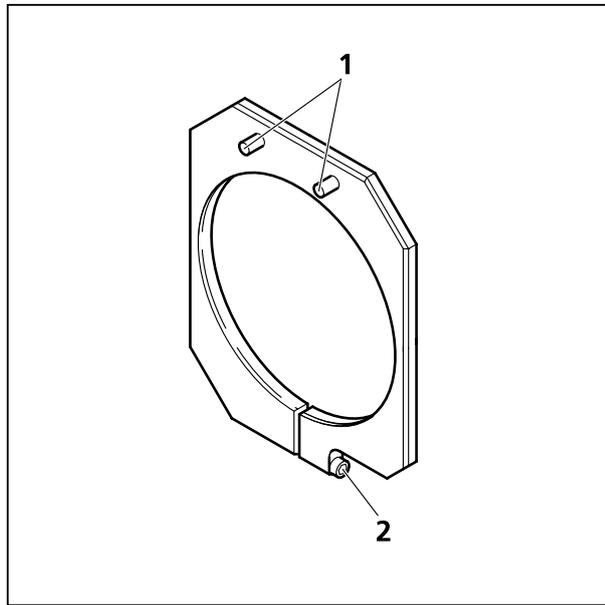


Fig. 1-20 Contact ring mounted to the lamp, TPMT or switching mirror

The interlock is working when the sensors of the sensor ring (Fig. 1-19/1) are depressed by the pins on the contact ring (Fig. 1-20/1). 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.

For dismounting the lamps, TPMT or switching mirror slightly unscrew the contact ring first (Fig. 1-20/2) so it can be moved away from the sensor ring (Fig. 1-19). Then unscrew the lamp, TPMT or switching mirror turning the main screw which is in one of the recesses of the sensor ring (Fig. 1-19). Hold the device to be dismantled with one hand while unscrewing to keep it from dropping. The now empty port has to be closed with the blind cap (Fig. 1-21) 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.

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.

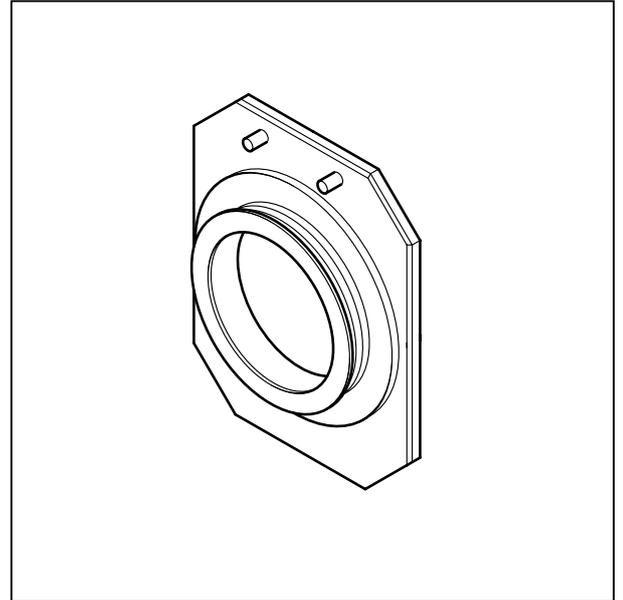


Fig. 1-21 Blind cap for closing any port equipped with an interlock device

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 28 kg.

- Depending on the microscope stand, it might be necessary to remove the HBO lamp house first before removing the scanhead.
- In case of the Axio Imager stand with an angled adapter on the HBO lamp house, the adapter will remain on the stand while the lamp house is removed!

Moving the scan heads between Axio Observer.Z1, Axio Examiner and Axio Imager.Z2:

- Loosen the three fastening screws of the LSM 710 (Fig. 1-22/1 and Fig. 1-23/1).
- Slowly pull the scanning module (Fig. 1-23/2) away from the microscope port or the tube (Fig. 1-23/3).
- For mounting the scan head onto a microscope, make sure the pins and the electronic connections of the safety interface match closely.
- Push the scan head to the microscope or the tube.
- Fasten the three fastening screws of the LSM 710 (Fig. 1-22/1 and Fig. 1-23/1).

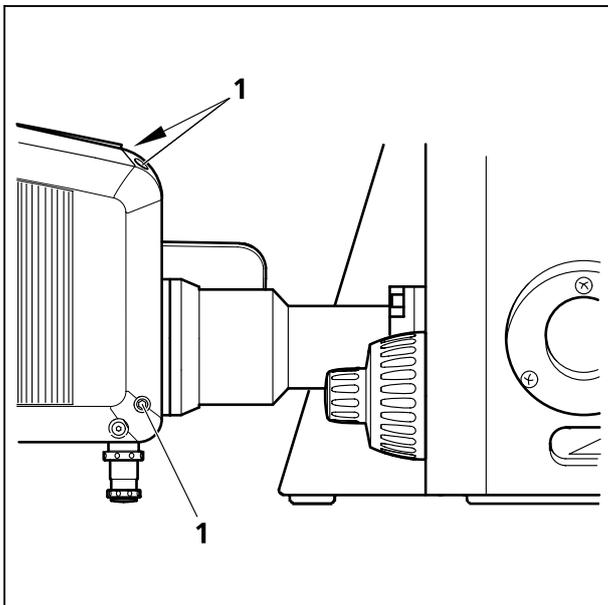


Fig. 1-22 Port connection between LSM and Axio Observer.Z1

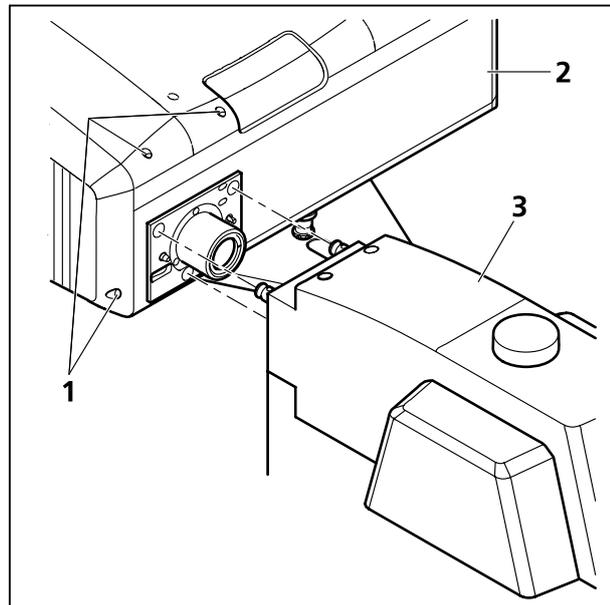


Fig. 1-23 Port connection between LSM and Axio Examiner or Axio Imager.Z2

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. 1-25/5) by sliding the inner tube into the outer tube (Fig. 1-25/4).
- Turn the tube assembly counter clockwise to unscrew the tube (Fig. 1-25/4) from the small black tube (Fig. 1-25/2) which is fixed to the scan head (Fig. 1-25/3).
- 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. 1-25/2) at the back of the scan head (Fig. 1-25/3). 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. 1-25/5).
- Open the shutter in the periscope using an Allen key (Fig. 1-24/1) and slide in the tube completely, this will hold the shutter open.

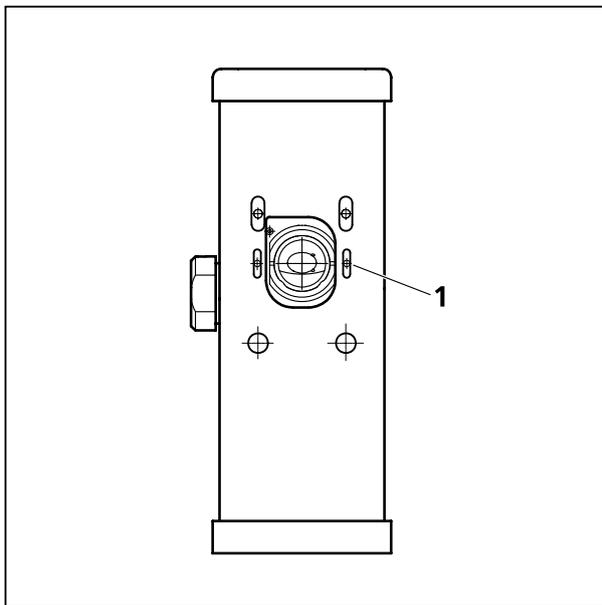


Fig. 1-24 Shutter opening on the periscope. Also applies for the large periscope for upright microscopes.

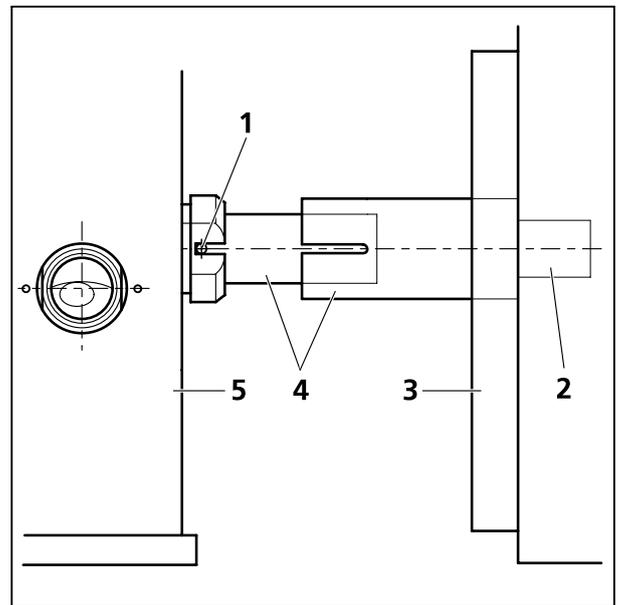


Fig. 1-25 Tube connection from the periscope housing (5) to the scan head (3). Applies for upright and inverted microscopes.

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 (Axio Examiner, Fig. 1-26/1) or on the rear side of the microscope (Axio Imager.Z2 or Axio Observer.Z1; Fig. 1-27/1 and Fig. 1-28/1). 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.

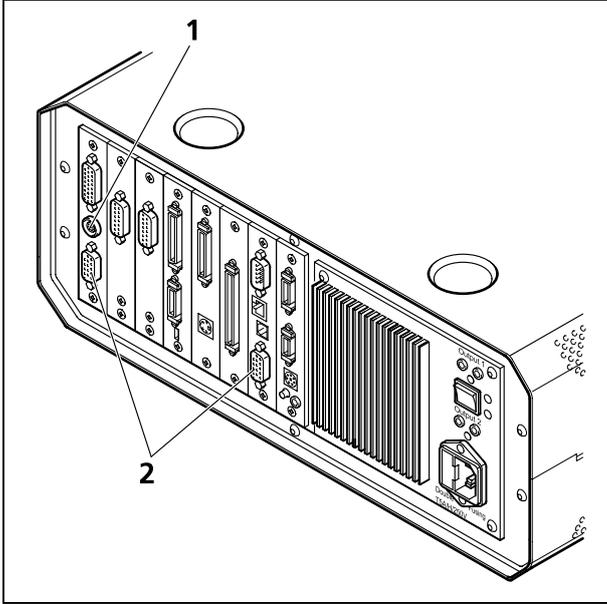


Fig. 1-26 Electronics Box of Axio Examiner with main connection to safety interface (1) and CAN-connectors (2)

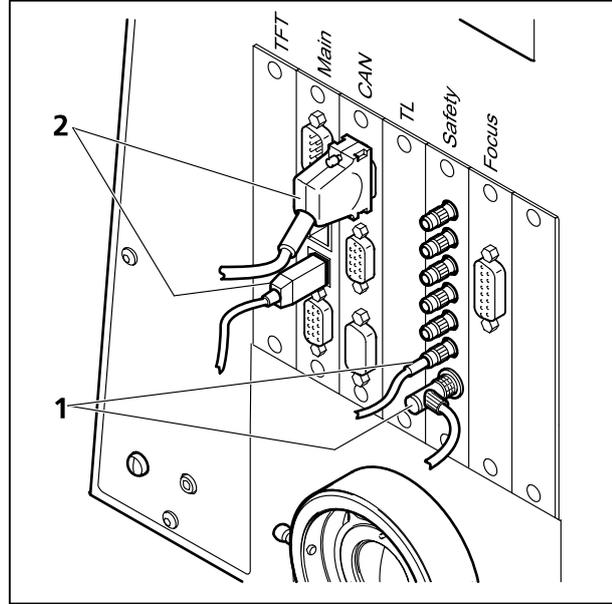


Fig. 1-27 Connection of Axio Imager.Z2 to safety interface (1) and electronics (2)

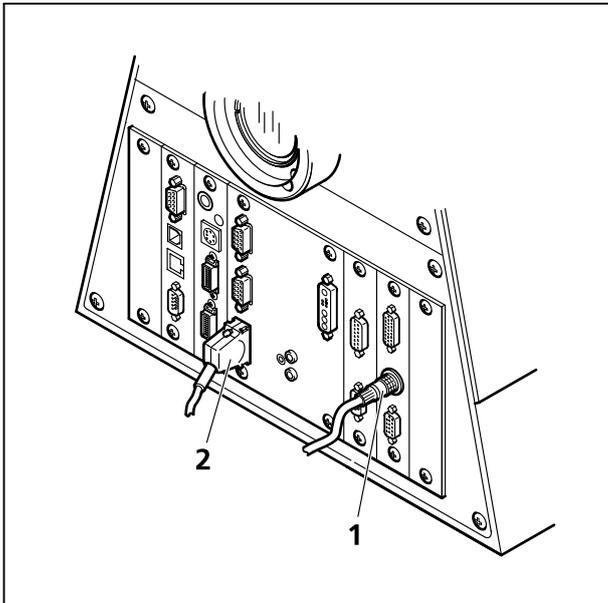


Fig. 1-28 Connection of Axio Observer.Z1 to safety interface (1) and electronics (2)

2. The CAN connections on the microscopes are shown in Fig. 1-26/2, Fig. 1-27/2 and Fig. 1-28/2. 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 microscope 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**.

1.11.3 Changing the Filter Wheel in the LSM 710

You can remove and replace filter wheels by push and click.

 When opening the cover, the safety control will switch off the laser light.

To exchange the filter wheel of the LSM 710 proceed as follows:

- Loosen the four fastening screws (Fig. 1-29/1).
- Remove the cover (Fig. 1-29/2) of the scanning module.
- Turn the filter wheel (Fig. 1-30/1) until the free position points upwards. Do not touch the filters!
- Grab the filter wheel on its free position and pull it out upwards.
- Grab the new filter wheel on its free position. Do not touch the filters!
- Insert the new filter wheel and snap it into the holder.
- Close the cover and fasten the four screws.

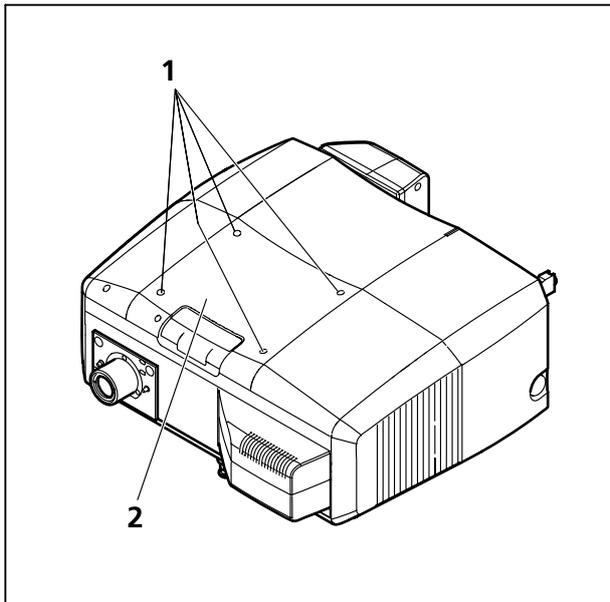


Fig. 1-29 Removing the cover

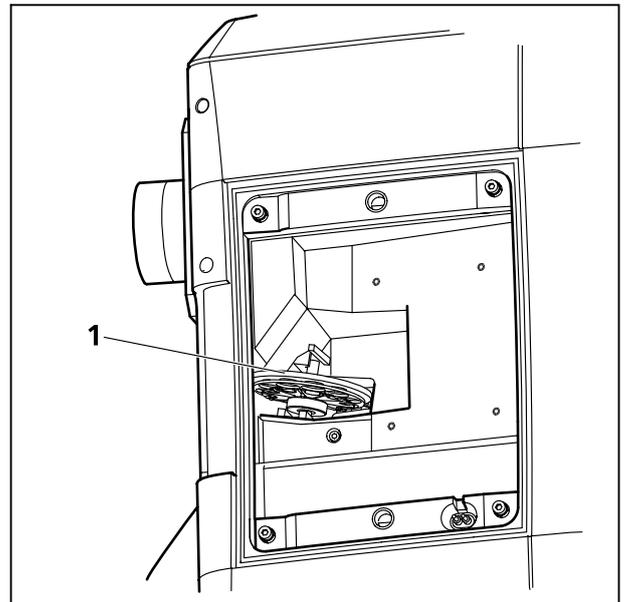


Fig. 1-30 LSM 710 filter wheel

1.11.4 Changing Filter Wheels in the ConfoCor 3

By opening the lid (see Fig. 1-31) you have access to all the filter wheels (exempt the Blocking filter wheel) in the ConfoCor 3.

You can remove and replace filter wheels by push and click.



Be careful not to disrupt any connectors in doing this.



Do not remove the ConfoCor 3 detection head from its attachment to the LSM 710. Laser light can escape the system through the external port used as the attachment site for the ConfoCor 3 that can lead to bodily damage.

-  Please note that the data base has to be exchanged if filter wheels are replaced with another filter set.
-  When opening the lid, the safety control will switch off the laser light.
-  The BF = block filter can only be exchanged by service.

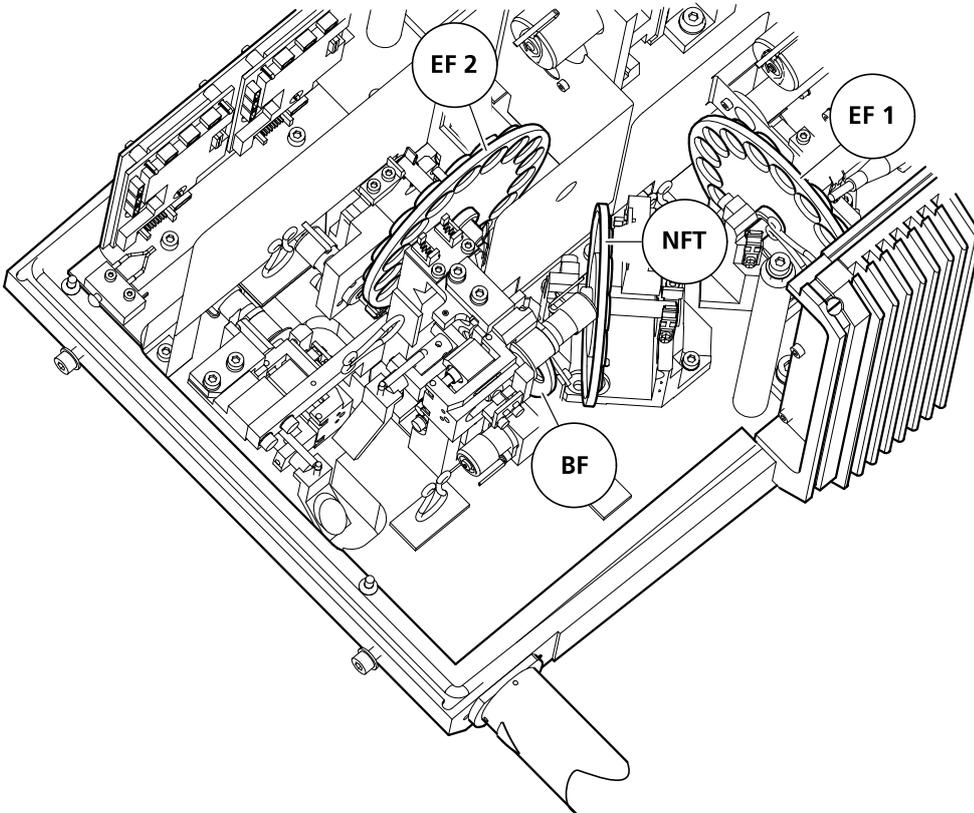


Fig. 1-31 Top view APD module. The locations of exchangeable filter wheels are indicated (NFT = secondary beam splitter; EF 1 = emission filter 1; EF 2 = emission filter 2).

1.11.5 Using External Connectors

In the rear of the APD module, you have external access to the TTL pulses of both channels delivered at the APD 1 and APD 2 plugs (see Fig. 1-32).

 Please note that the plug is of BNT type and BNT triax cables should be used. If using BNC connectors and coax cables the signal quality can suffer dramatically.

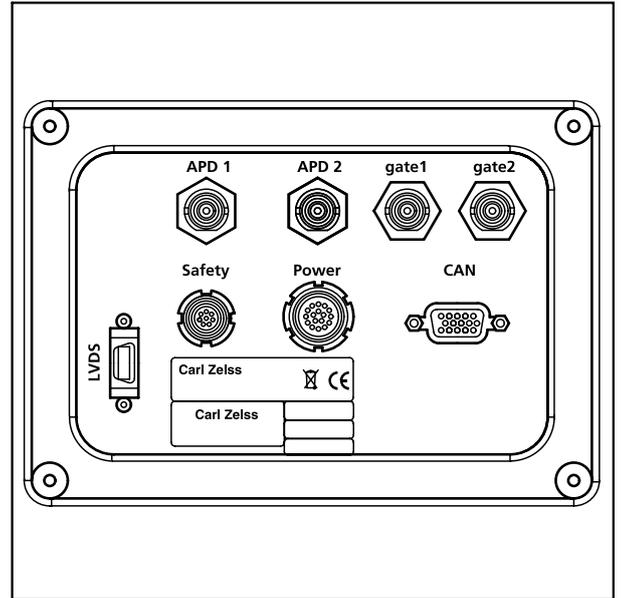


Fig. 1-32 Rear view APD module

The assignment of the connectors is as follows:

Name	APD	GaAsP (no serial offer)	Meaning
LVDS	14-pin MDR	14-pin MDR	Main communication link
Sub-LVDS		14-pin MDR	Communication to optional module
Safety	8-pin LEMO EXJ-1B	8-pin LEMO EXJ-1B	Laser safety
Power	16-pin LEMO ECJ-2B	16-pin LEMO ECJ-2B	Power consumption: less than 20 W
CAN	15-pin HD Sub-D female	15-pin HD Sub-D female	Optional CAN Port / Service Port
RS232		9-pin Sub-D male	Service Port
APD 1 / 2	Triax BNT		Photon pulse- level: 2.5 V at 50 Ω, 30 nsek long
Gate 1 / 2	Triax BNT		Gate APD- TTL level

To connect the ConfoCor 3 to an optional correlator card, use the connectors APD 1 / 2. As long as the maximum count rate is not reached, each TTL Pulse corresponds to one photon. Take care to use 50 Ω termination to avoid reflections and use double shielded 'triax' cable to avoid heavy distortions of the signal. To connect to BNC equipment, connect inner shield to ground and leave outer shield open.

For gating the APD use TTL signals.

To connect the ConfoCor 3 to an optional FLIM card, use the connectors APD 1 / 2. APD 1 / 2 delivers directly the APD signal (jitter 350 psec) according to the manufacturer's data sheet.

Quick Guide

LSM 710 / LSM 710 NLO and ConfoCor 3 Laser Scanning Microscopes



LSM Software ZEN 2009

April 2009



We make it visible.

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Introduction

This LSM 710 / LSM 710 NLO and ConfoCor 3 Quick Guide describes the basic operation of the LSM 710 / LSM 710 NLO and ConfoCor 3 Laser Scanning microscopes 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, Axio Examiner).

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 710 / LSM 710 NLO and ConfoCor 3 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

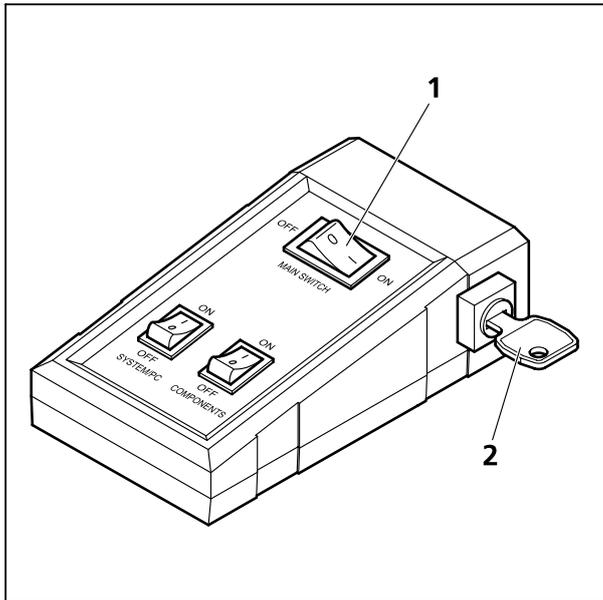


Fig. 1 Power remote switch

Switching on the LSM system

- Switch on the main switch (Fig. 1/1) and the safety lock (Fig. 1/2).
- When set to **ON** the power remote switch labeled **System/PC** provides power to the computer. This allows use of the computer and ZEN software offline
- 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.

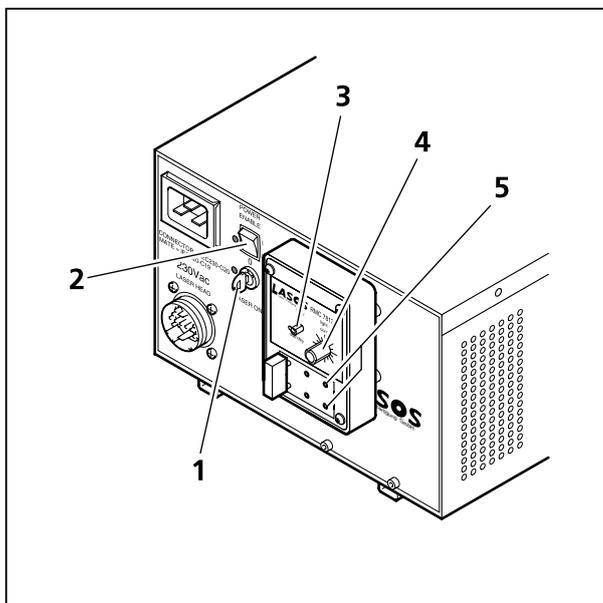


Fig. 2 Power supply of Ar-ML laser

Switching on the Ar-ML Laser

- If the Ar-ML laser is required, switch it on via the toggle switch (Fig. 2/2) on the power supply and turn the key (Fig. 2/1). The laser is automatically kept in standby mode for 5 minutes to warm up.
- Set the idle-run-switch (Fig. 2/3) to run. It takes about 50s until the laser has reached the set output power (green LED) provided the warm up time of 5 minutes is already completed.
- Adjust the required power level with the control knob (Fig. 2/4) (default position should be 11 o'clock).

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 710 Startup** window appear on the screen (Fig. 3)

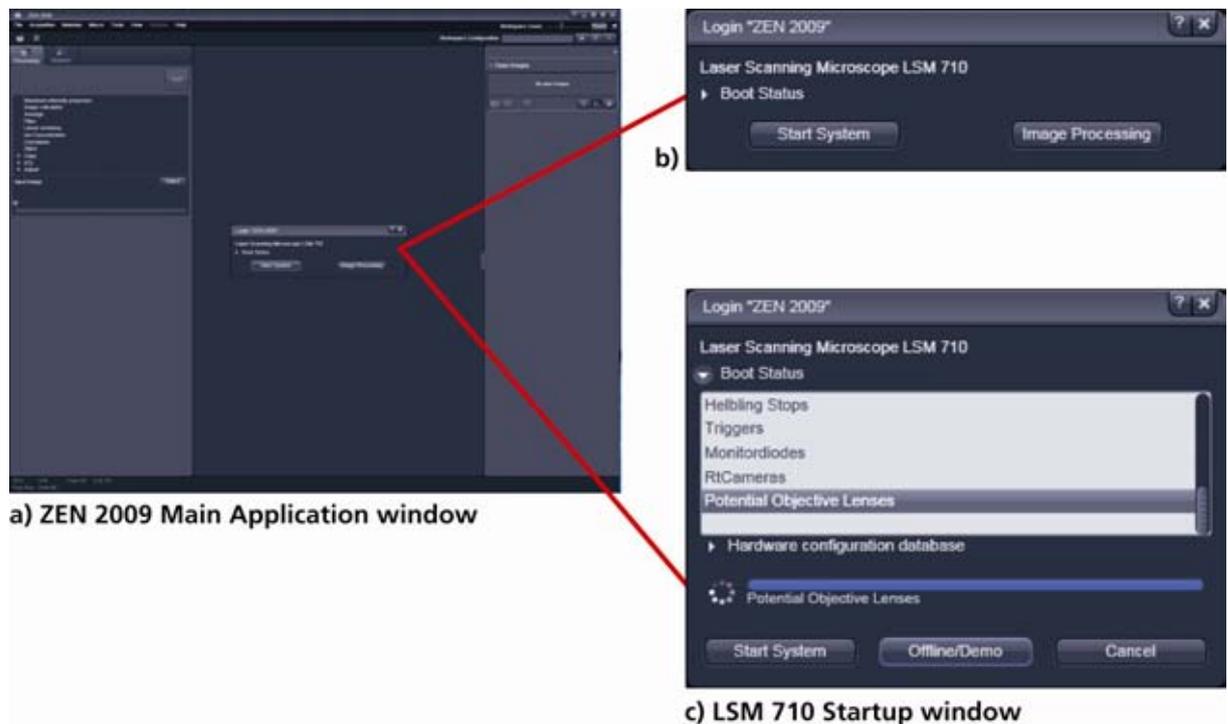


Fig. 3 ZEN Main Application window at Startup (a) and the LSM 710 Startup window (b and c)

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.

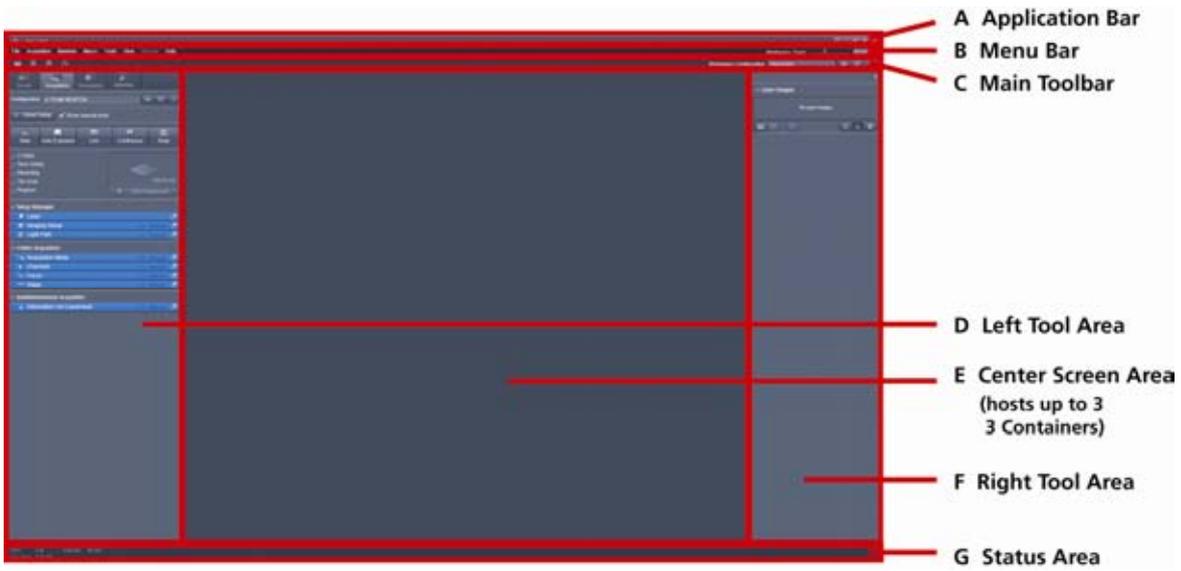


Fig. 4 ZEN Main Application Window after Startup with empty image container

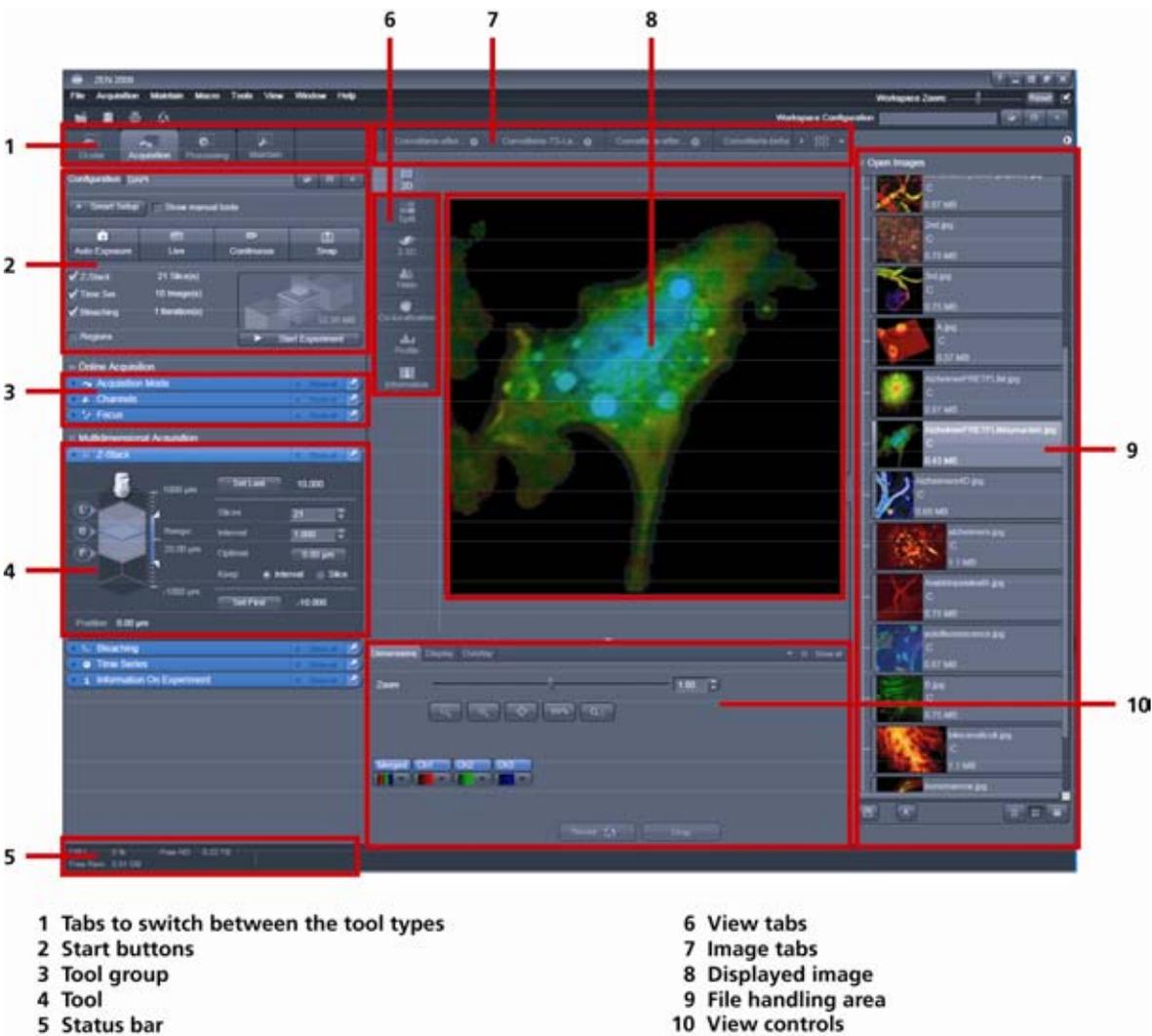


Fig. 5 ZEN Main Application Window after Startup with several images loaded

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.

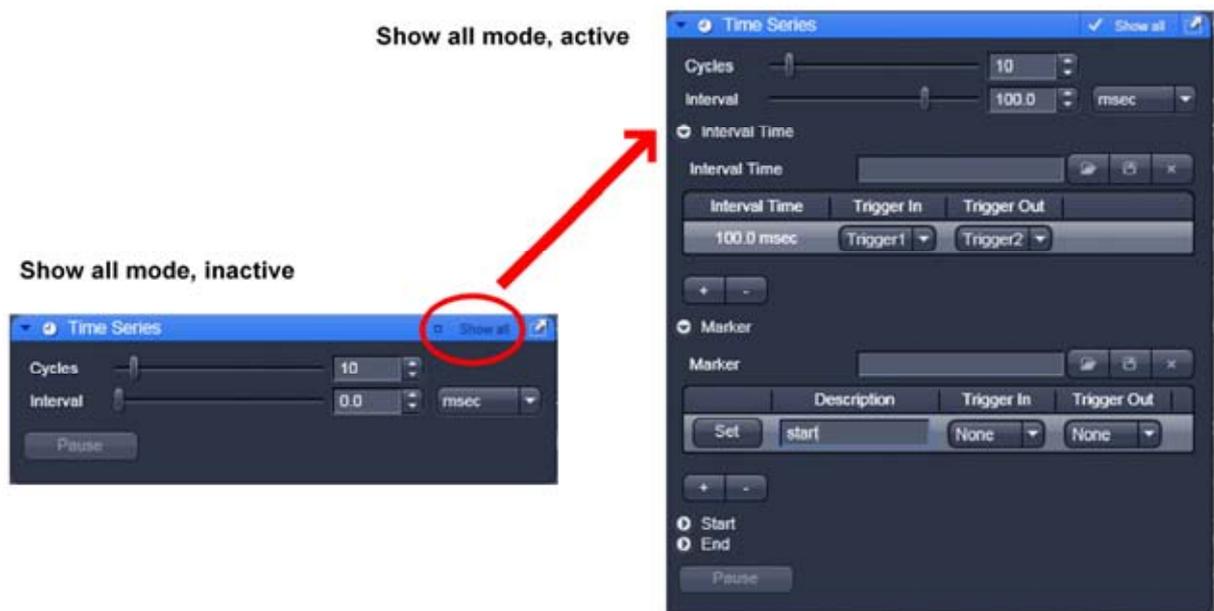


Fig. 6 Show all mode

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** deactivated, 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).

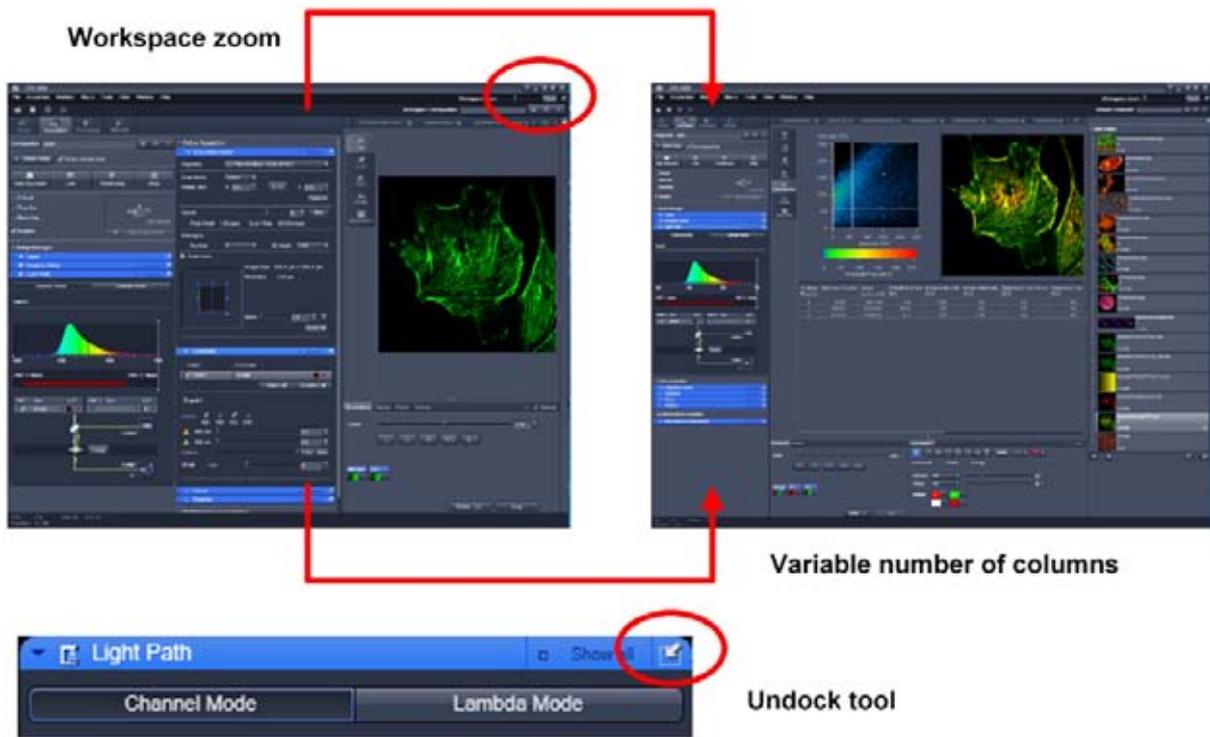


Fig. 7 ZEN Window Layout configuration

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**  or the **Auto Exposure**  button. For an empty image document press the **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.

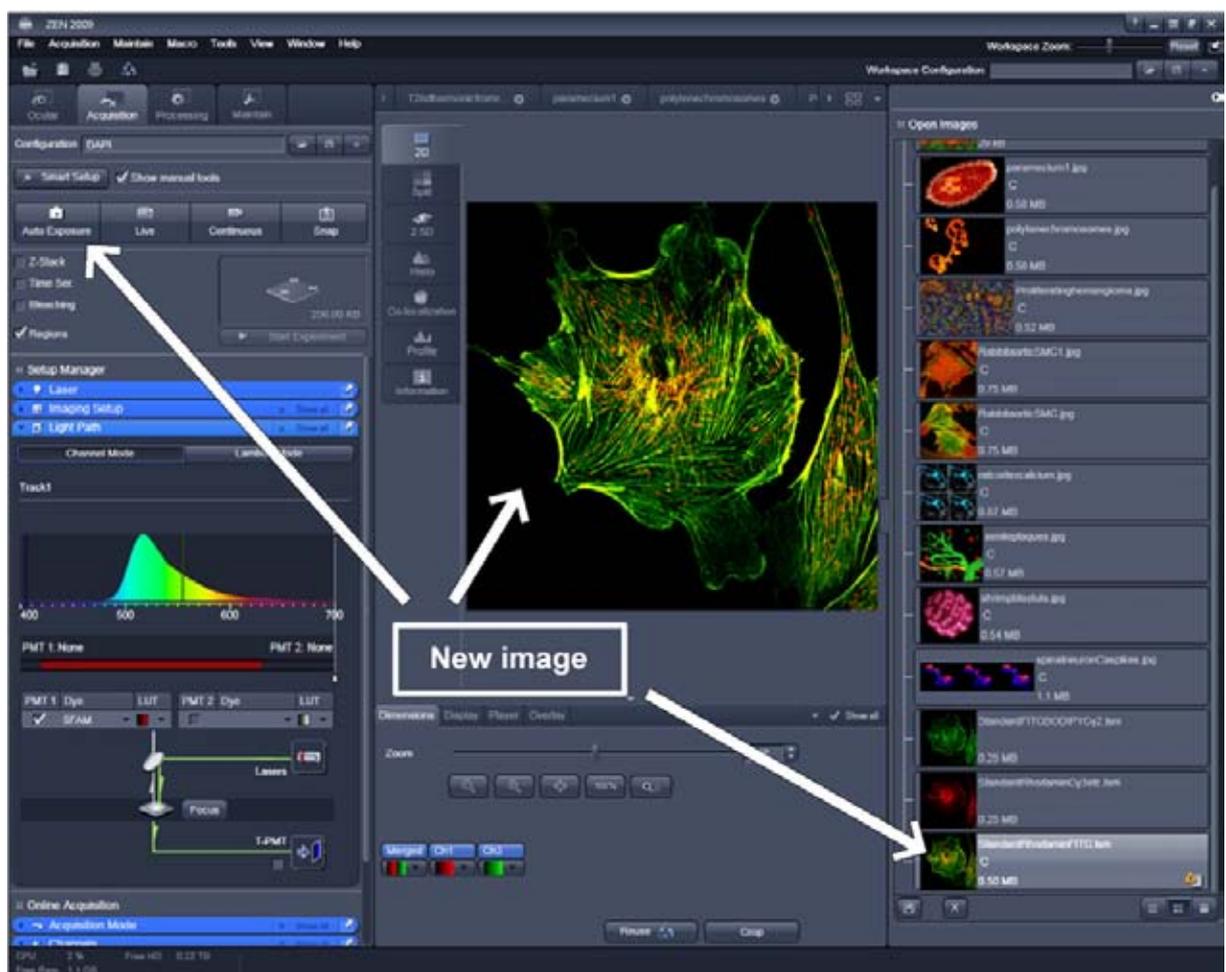


Fig. 8 New image document in the Open Images Areas

Advanced data browsing is available through the **ZEN File Browser** (Ctrl + F or from the **File Menu**). The ZEN File Browser can be used like the WINDOWS program file browser. Images can be opened by 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.

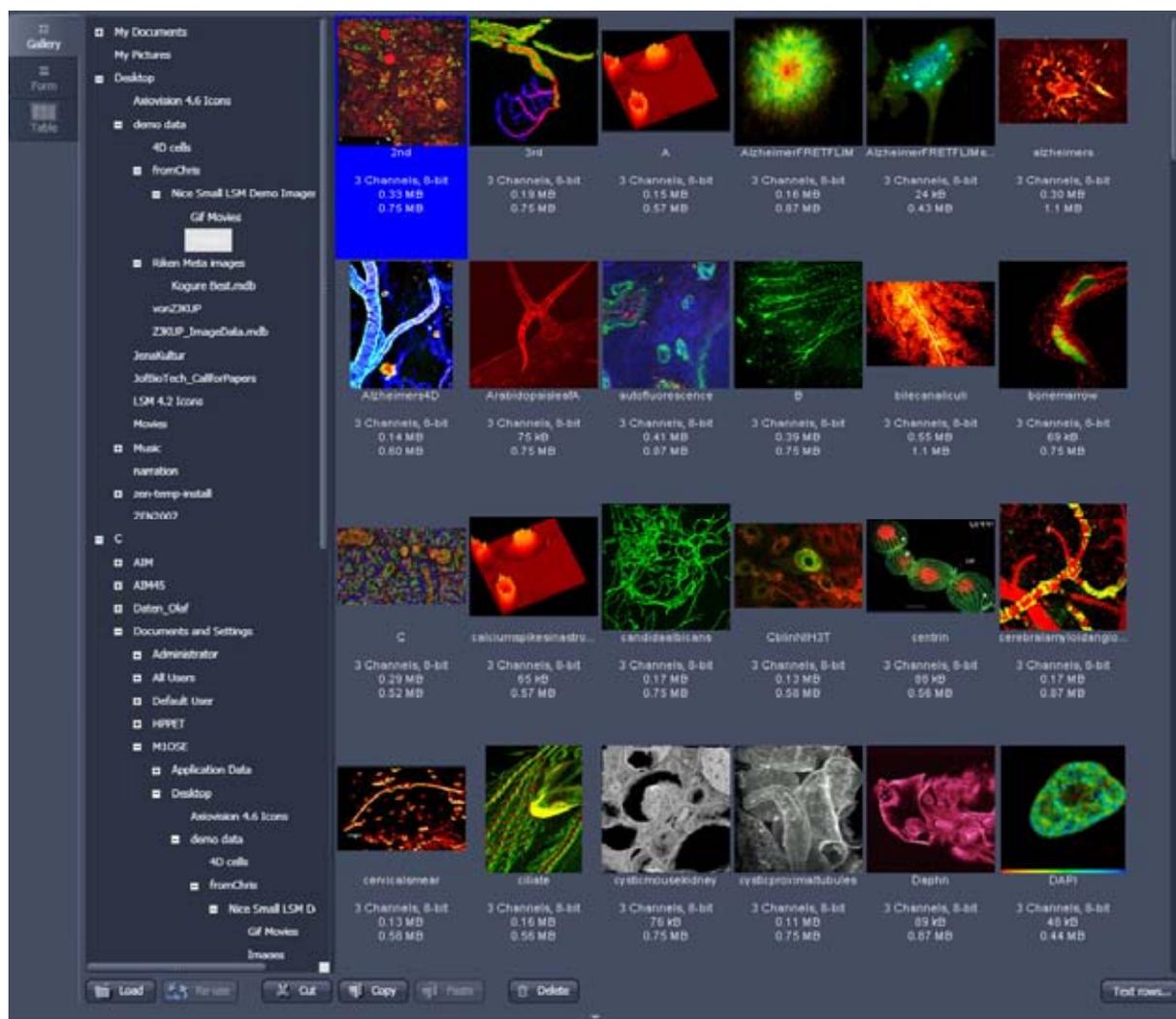


Fig. 9 File Browser

Turning on the lasers

ZEN 2009 operates all lasers automatically. Whenever they are used (manually or by the **Smart Setup** function) the lasers are turned on automatically. The **Laser Life Extender** function of the software shuts all lasers off if ZEN is not used for more than 15 minutes.

To manually switch lasers on or off:

- Click the **show manual tools** tickbox and open the **Laser** tool. All available lasers can be operated within this tool (Fig. 10).

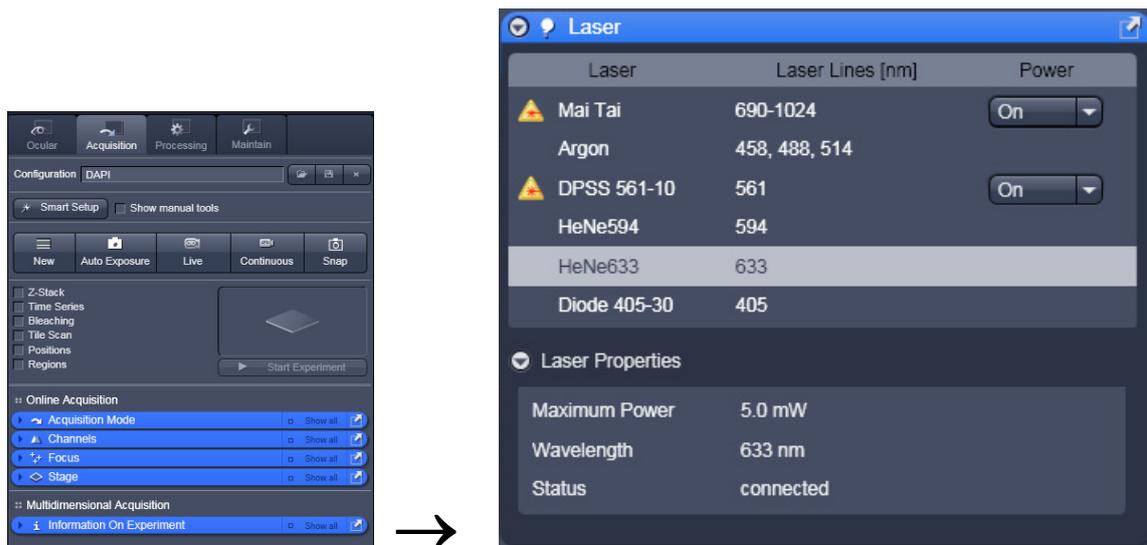


Fig. 10 Laser Control tool

Setting up the microscope

Changing between direct observation and laser scanning mode

The **Ocular** and **Acquisition** buttons switch between the use of the LSM and the microscope:



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

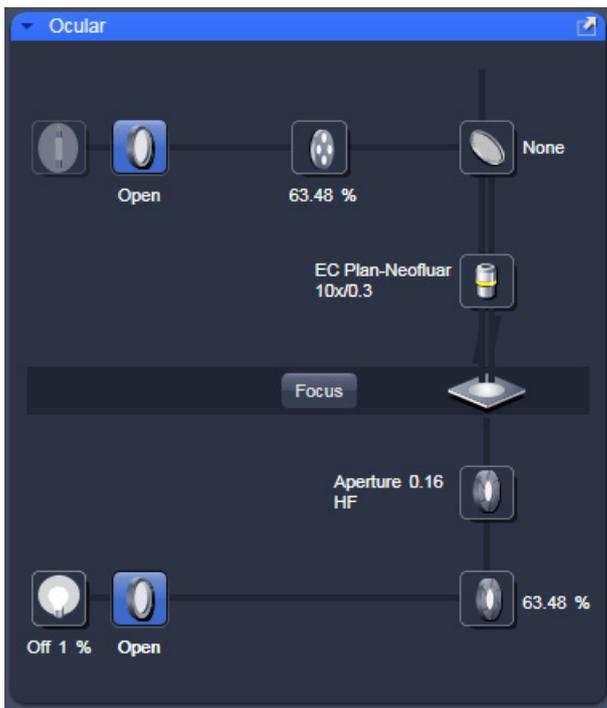


Fig. 11 Microscope Control window, e.g.: Axio Imager.Z2

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.

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

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

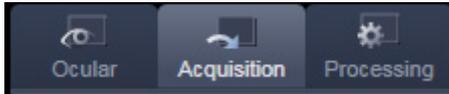


Fig. 12 Microscope Control window with Transmitted Light pop-up menu



Fig. 13 Configuration panel

Configuring the beam path and lasers



- Click the **Acquisition** button.

Smart Setup

The tool **Smart Setup** is an intuitive, user-friendly interface which can be used for almost all standard applications. It configures all the system hardware for a chosen set of dyes.

- Click on the **Smart Setup** button  to open the smart setup window. This window can be accessed any time from the software to change dye combinations.
- Click on the arrow in the dye list and simply choose the dye(s) you want to use in your experiment from the list dialogue. In this dialogue, the dyes can be also searched by typing the name in the search field.

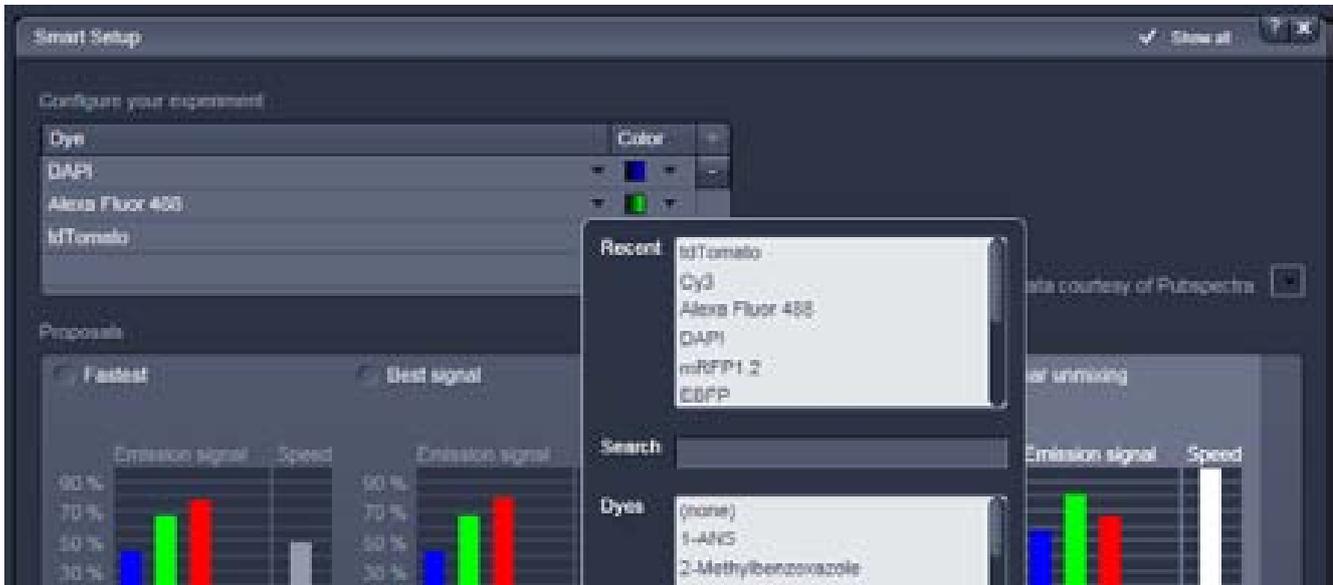


Fig. 14 Smart setup tool

Once finished with the input, **Smart Setup** suggests four alternative considerations (see below): One for **fastest** imaging, one for the **best signal**, **best compromise** between both speed and best signal and the optimal setup for later **linear unmixing** of the dyes.

The graphs display relative values for the expected emission signals and cross-talk. The resulting imaging scheme (single or multitrack) is shown below the graphs.

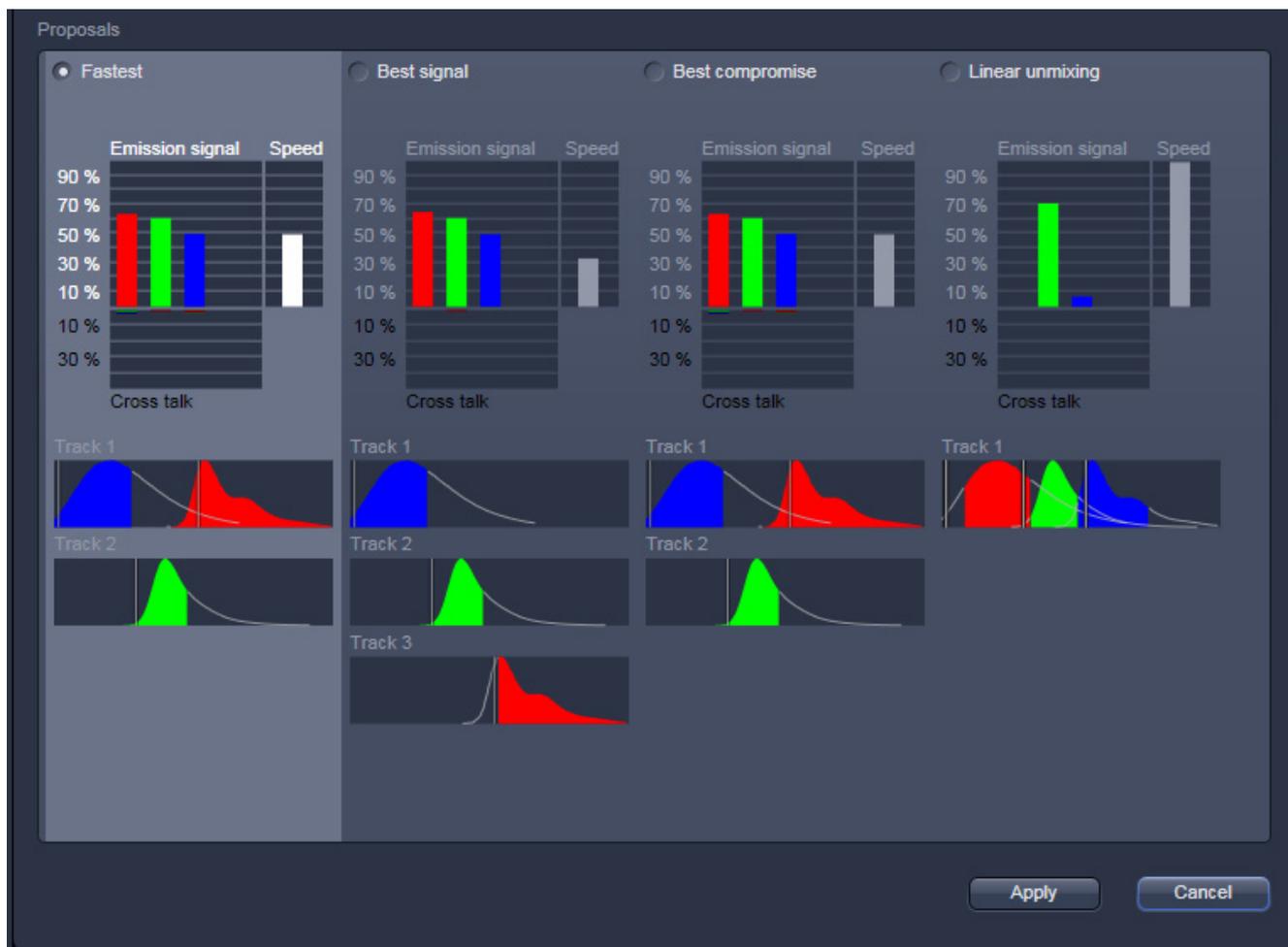
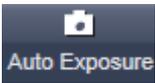


Fig. 15 Proposals panel of the Smart Setup tool

Pressing **Apply**, automatically sets the hardware parameters in the displayed way for the dyes chosen.

If the option **Linear Unmixing** is selected, the system is set in the lambda mode automatically.

Pressing the **Auto Exposure**  button will then optimize the settings of the Gain (Master) and offset for the given laser power and pinhole size. Further image optimisation from this point can be done easily.

Setting up a configuration manually

Simultaneous scanning of single, double and triple labeling:

- Advantage: faster image acquisition
- Disadvantage: potential 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. 16.

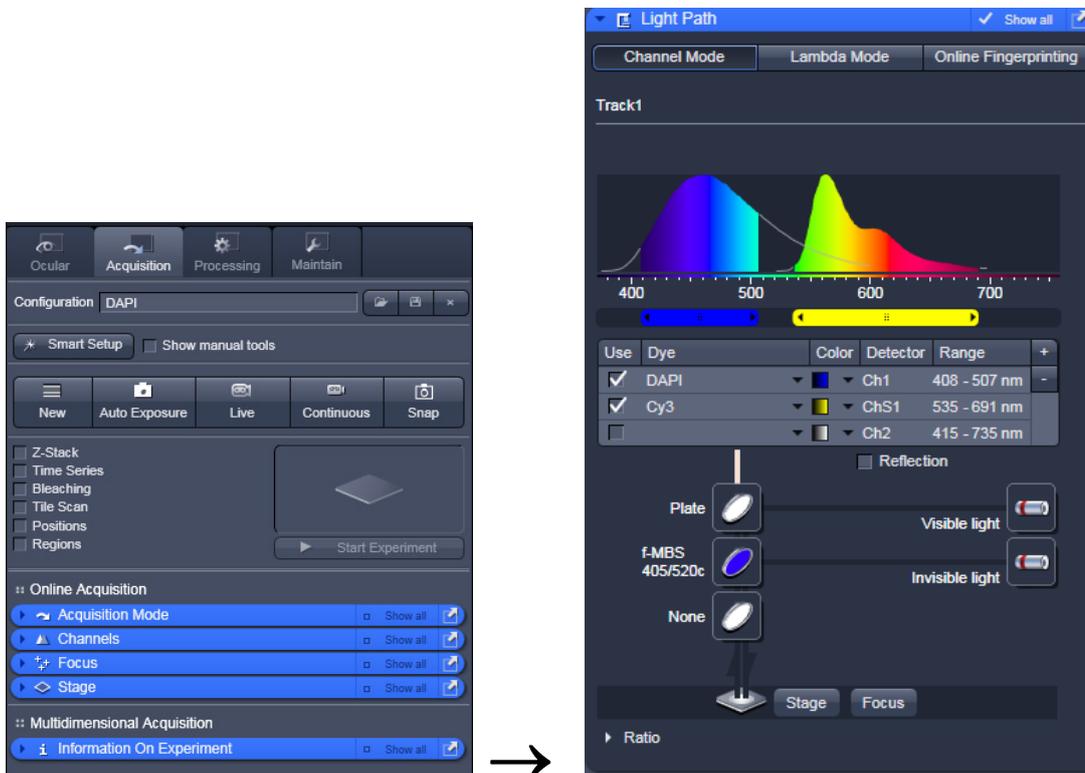


Fig. 16 Light Path tool for a single track (LSM)

Settings for track configuration in Channel Mode

- Select **Channel Mode** if necessary (Fig. 17).

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:

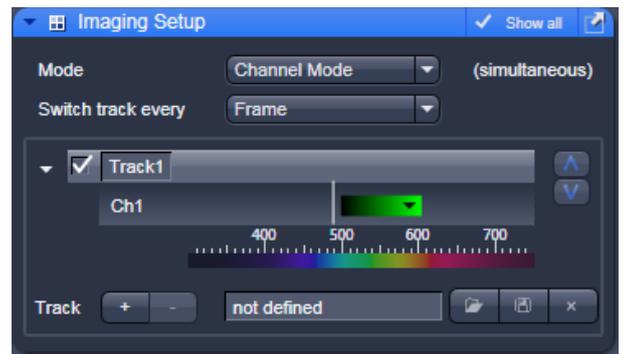


Fig. 17 Imaging Setup tool for a single track (LSM)



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 (MBS) 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, QUASAR 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. 18) to visualize the activated laser lines for excitation (vertical lines) and activated detection channels (colored horizontal bars).



Fig. 18 Detection Bands & Laser Lines display



Fig. 19 Track Configurations window

- For storing a new configuration click  and enter a desired name in the first line of the list box (Fig. 19), 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. 17, Fig. 18 and Fig. 19).

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. 20).
- 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!

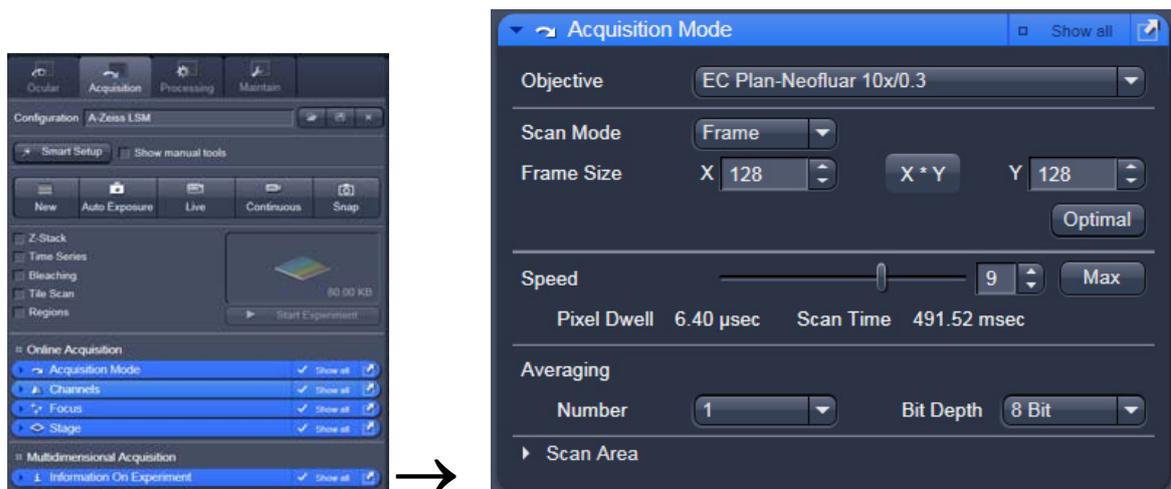


Fig. 20 Acquisition Mode tool

Adjusting scan speed

- Use the **Scan Speed** slider in the **Acquisition Mode** tool (Fig. 20) 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. 20).

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.

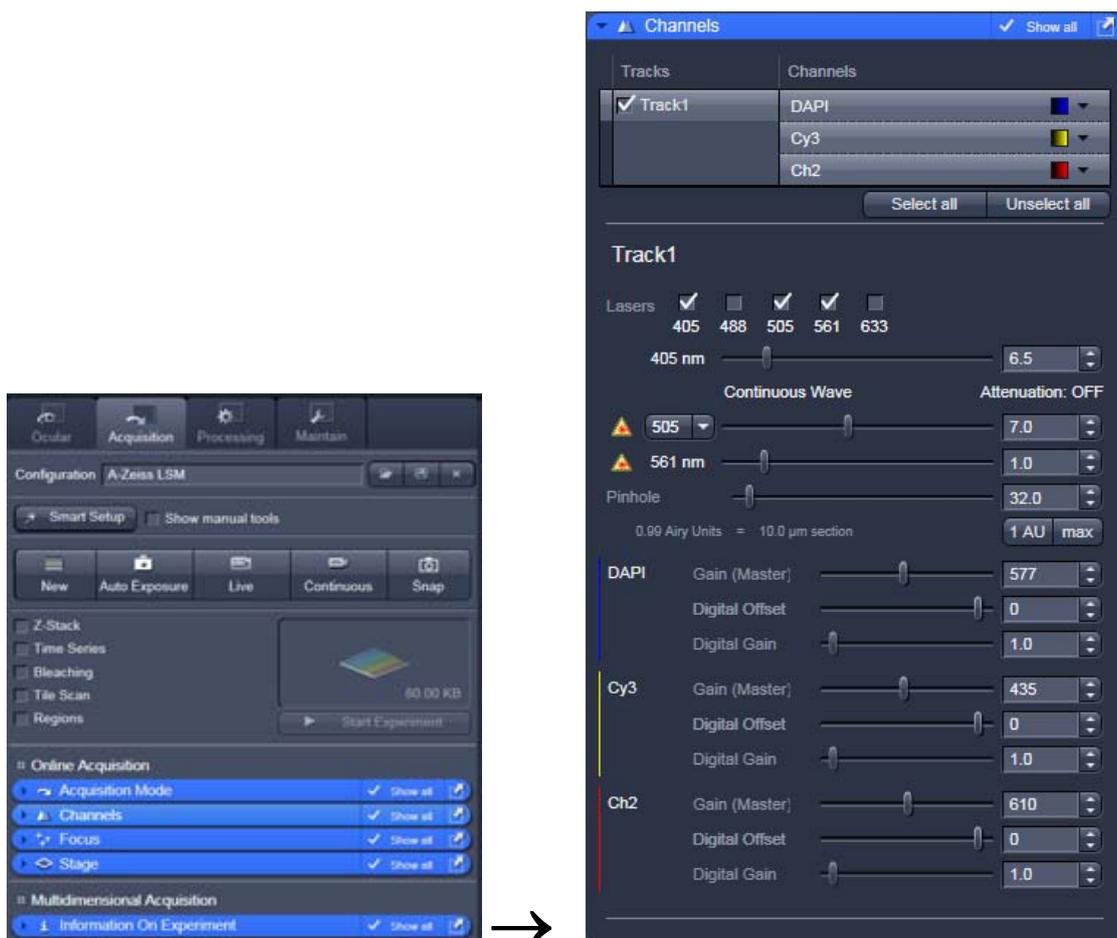
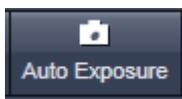


Fig. 21 Channels tool

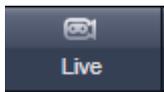
Image acquisition

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

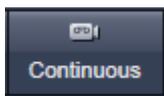
- Use one of the **Auto Exposure**, **Live**, **Continuous** or **Snap** buttons to start the scanning procedure to acquire an image.
- Scanned images are shown in separate windows.
- Click on the **Stop** button to stop the current scan procedure if necessary.



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.

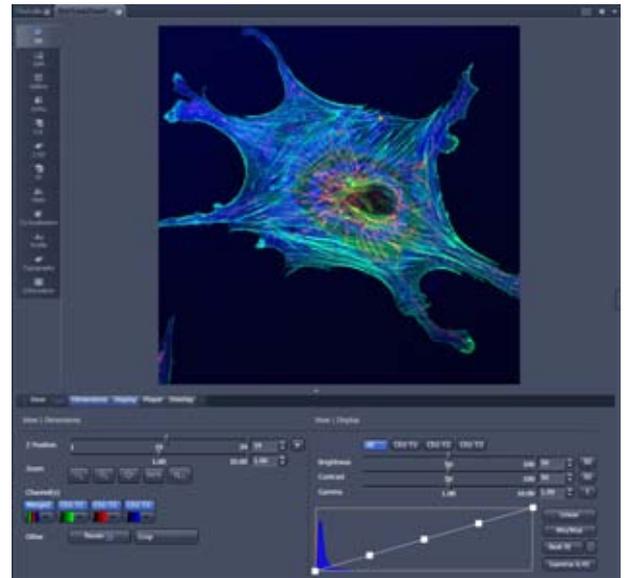


Fig. 22 Image Display

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. 23).

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



Fig. 23 View Dimensions Control Block

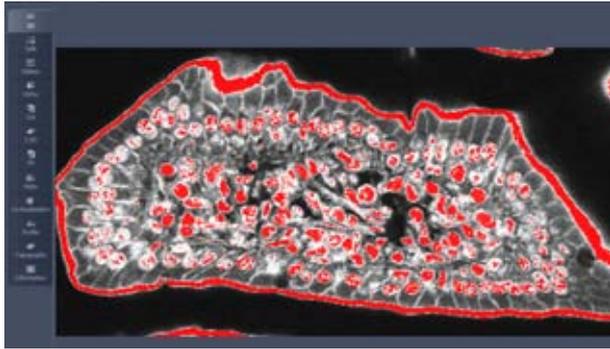


Fig. 24 Image Display

The scanned image appears in a false-color presentation (Fig. 24).

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. 25).
- Set the **Gain (Master)** high.
- When the image is saturated, reduce AOTF transmission in the **Laser** control section of the **Channels Tool** (Fig. 25) using the slider to reduce the intensity of the laser light to the specimen.

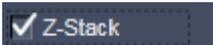
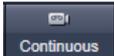
Adjusting gain and offset

- Increase the **Digital Offset** until all blue pixels disappear, and then make it slightly positive (Fig. 25).
- Reduce the **Gain (Master)** until the red pixels only just disappear.

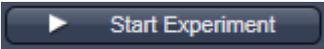
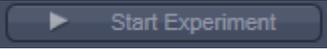


Fig. 25 Channels tool

Scanning a Z stack

- Select **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  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.
 - 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  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** 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** button is grayed out and only single images can be scanned.

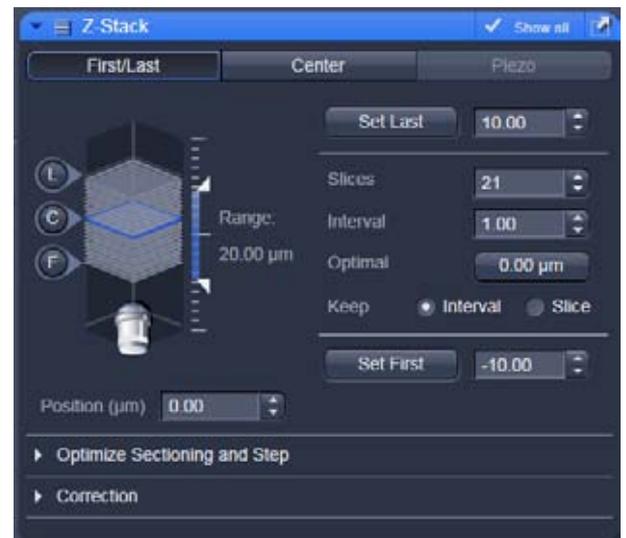


Fig. 26 Z Stack tool

Storing and exporting image data

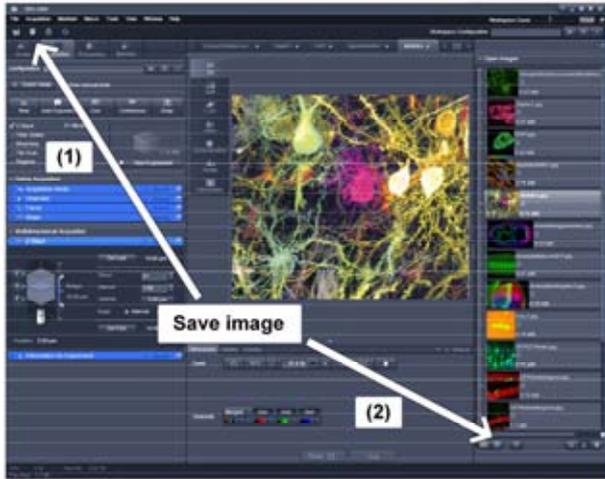


Fig. 27 Save Image buttons in ZEN

- 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. 27/1), or click on the  button at the bottom of the **File Handling Area** (Fig. 27/2).

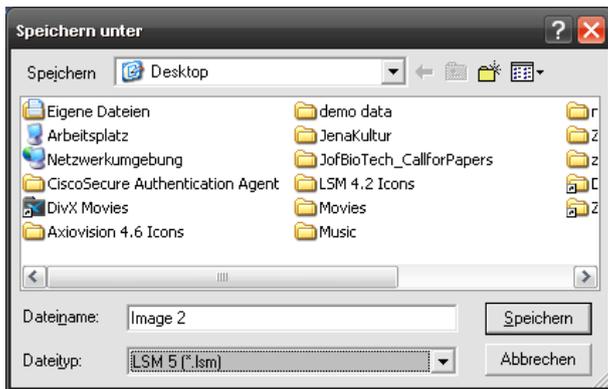


Fig. 28 Save as window

- 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.

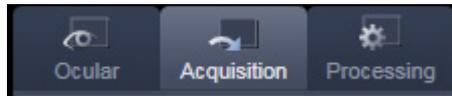
If you close an image which has not been saved, a pop-up window will ask you if you want to save it. Choosing **yes** will lead you to the WINDOWS **Save As** window.



Fig. 29 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.

Using the ConfoCor 3 module



- Click on the **Acquisition** button.
- Use the **ConfoCor** Tool Group in the **Left Tool Area** to acquire and analyze **FCS** data.



Fig. 30 ConfoCor Tool Group

Setting a configuration



System Configuration

- Open the **System Configuration** tool to define the light path, laser lines and pinhole position.

The **Light Path** and **Pinhole** panels of the **System Configuration** window display the selected track configuration which is used for the FCS procedure and the pinhole size (see Fig. 31).

Fig. 31 The ConfoCor 3 Measure Tool: System Configuration

You can change the settings of this panel using the following function elements:



Activation / deactivation of the excitation wavelengths (check box) and setting of excitation intensities (slider). Open the **Laser Control** tool via the **Laser** icon.



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



Selection of a block filter 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.

- Set the Pinhole diameter (via slider or input box).
- Press the **Count rate** button to open the Real Time display window for the detector **Count rate** in all active channels. Adjust the Laser power In the **Laser Control** panel to obtain a satisfactory count rate.
- Press **Adjust Pinhole** to align the pinhole for each newly defined beam path. After adjusting the sample carrier, align the pinhole automatically in **X** and **Y** by first conducting a coarse and then a fine alignment.

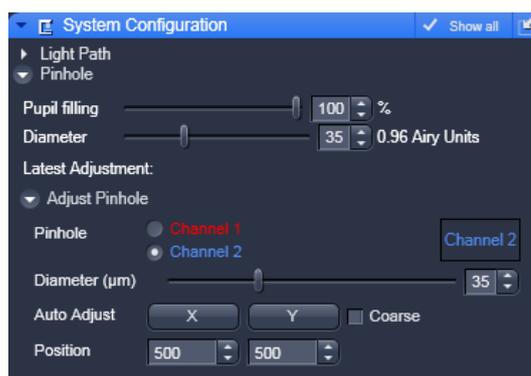


Fig. 32 Pinhole adjustment

Use the **Models** tool to define model equations to which measured data can be fitted.

You have three options to define a model:

- **Correlation:** assemble a correlation model from predefined equations, which will be fitted analytically
- **PCH:** assemble a photon counting histogram model, which will be fitted numerically
- **Formula:** program a user defined model equation, which can be fitted analytically

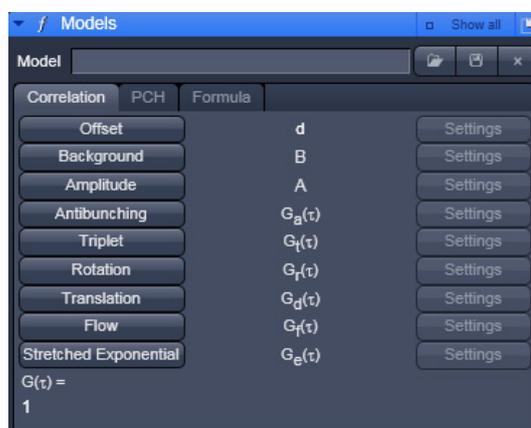


Fig. 33 Models tool

Starting a measurement

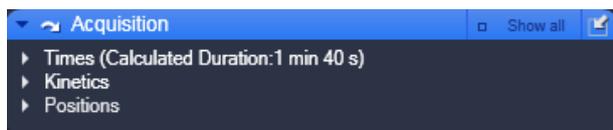


Fig. 34 The ConfoCor 3 Measure Tool: Acquisition

- Select the **Acquisition** toolbar.

The **Times**, **Kinetics** and **Position** panels of the **Acquisition** toolbar display the selected measurement conditions and the positions which are used for the FCS experiment.

You can change the settings of this panel using the following function elements:



Enter the **Bleach Time**, **Measureme Time** and **Repeat Count** into the corresponding input boxes.



Activate / deactivate a kinetic procedure by ticking the **Use Kinetics** check box. Enter the time distance between measurements, the cycle number and the shape in the corresponding input boxes.



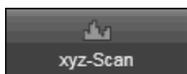
Select the carrier and the sample or laser position by the stage or by the scanners.



Press the **New** button to open a new **FCS diagram** into an image container. If a measurement is triggered, all data are displayed in that window if highlighted.



Press the **Count rate** button to open the Real Time display window for the detector **Count rate** in all active channels. This allows you to optimize your experiment by changing the laser power and the pinhole size while monitoring the count rate.



Press the **xyz-Scan** button to display the current coordinates. You can define boundaries where a scan is performed with simultaneous acquisition of the count rate. This allows you, for example, to identify labeled molecules accumulated in the membrane.



Press the **Snap** button to trigger one measurement at the highlighted or first defined position.



Press the **Start ConfoCor Experiment** button to trigger a measurement. All defined positions will be approached consecutively.



Press the **Stop** button to end a measurement. All data accumulated so far will be available and can be stored.

After measurement completion, the data is displayed in the FCS Correlation diagram within an Image Container (see Fig. 35).

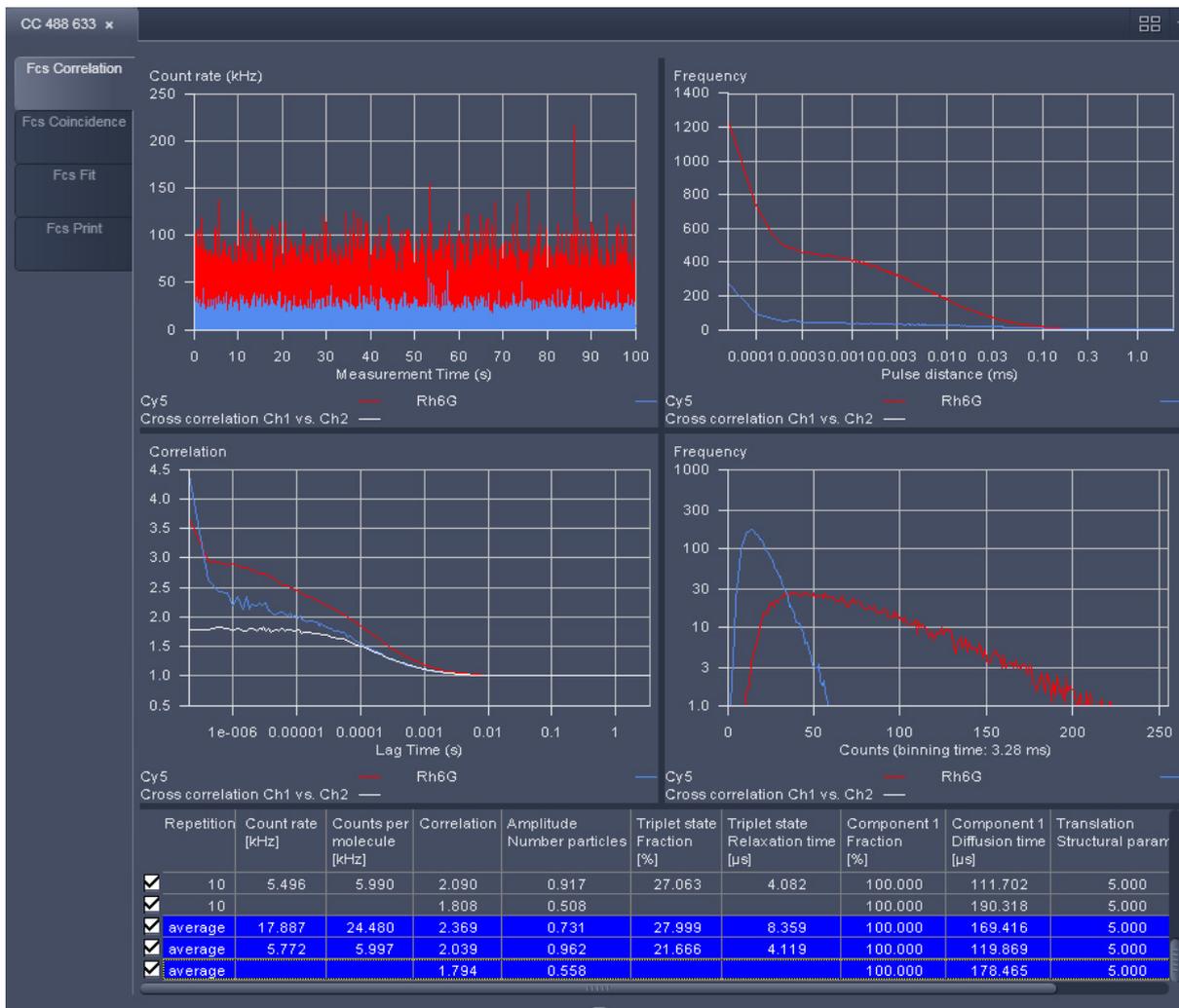
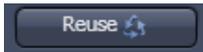


Fig. 35 FCS Correlation diagram

You have the following function elements:

- Fcs Correlation** Activate the **FCS Correlation** panel to display measured data (Fig. 35).
- View Options** Press the **View Options** button to define the graph you want to display.
- Count rate** Press the **Count rate** button to display the count rate trace.
- Correlation** Press the **Correlation** button to display the correlation function.
- Photon counting histogram** Press the **Photon counting histogram** button to display the photon distribution per time unit.
- Data Options** Press the **Data Options** to handle your data.
- Save Data** Press the **Save Data** button to open the **Save** window. You can save the whole data set in an ANSI text format. Optionally you can save the raw data trace if that option was set in the **FCS Options**.



Pressing the **Reuse** button will set the system configuration to exactly the same values, as used in the experiment.



Pressing the **Reload** button will open the current measurement, if stored raw data are available. This allows you to alter the parameters of your mathematical calculations.

Analyzing the data

The acquired FCS data is analyzed in the Fit display of the FCS diagram (see figure Fig. 36).

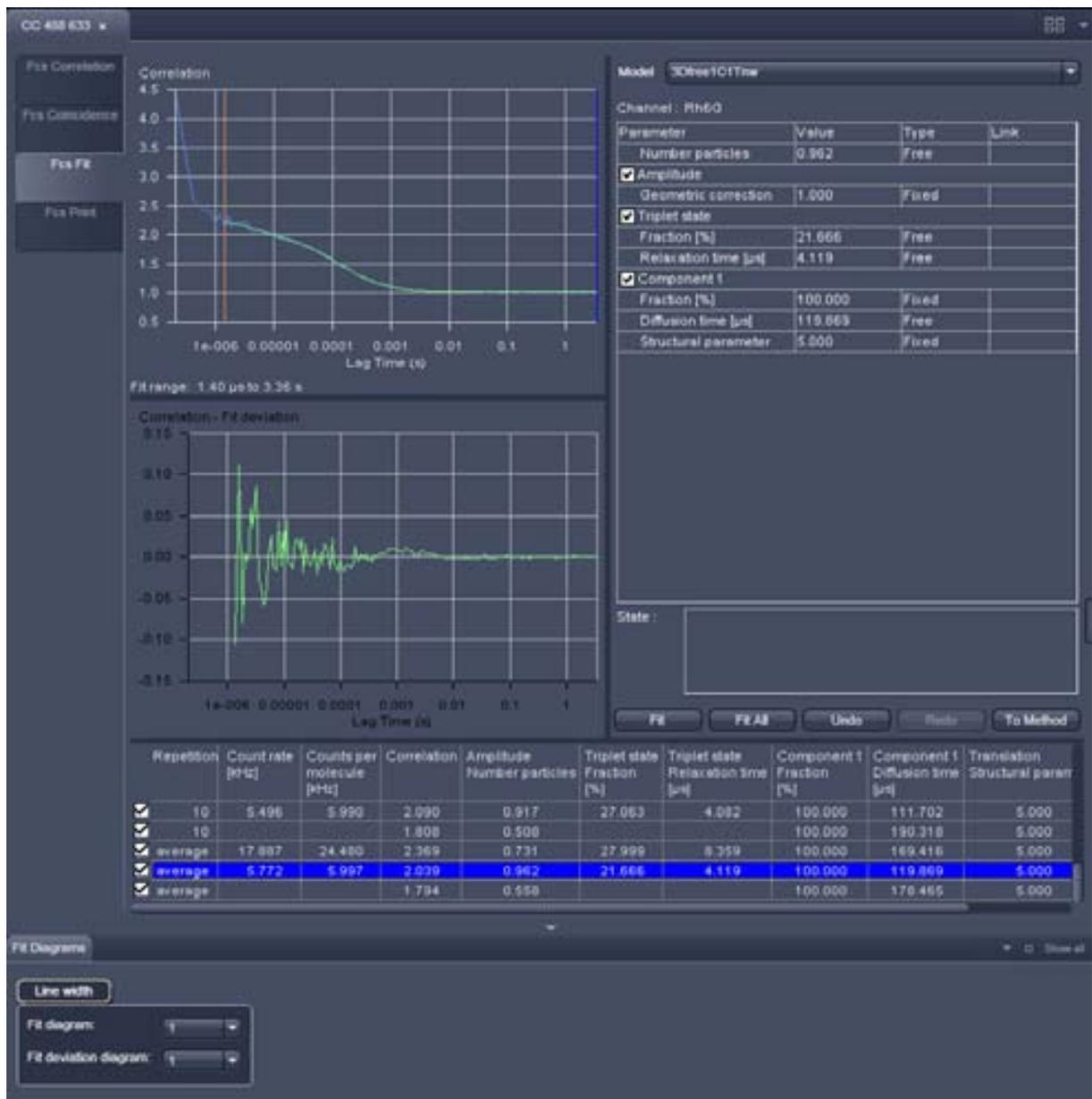
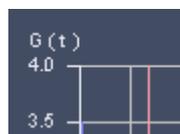


Fig. 36 FCS Fit diagram

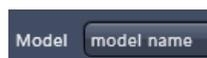
You have the following options:



Activate the **FCS Fit** panel to display fitted data (Fig. 36).



Set the red and blue bars to define the start and end points of the curve fit window.



Load a predefined model from the Model drop-down menu. You can assemble a model by pressing the **Model** tool in the **ConfoCor** tool group.



Define the conditions of the fit by activating / deactivating terms, setting the type of a parameter (fixed, free, or start value), defining limits and globally link parameters in the **Model** table.



Pressing the **Fit** button will fit the current loaded correlation functions to the defined model. The fitted data will be displayed in the **Model** and **Result** tables.



Pressing the **Fit all** button will take all ticked channels and fit them according to the chosen model.



Pressing the **Undo** button will cancel the last operation, or previous ones as well, if the button is pressed repeatedly.



Pressing **Redo** will redo the last cancelled operation, or previous ones, if the button is pressed repeatedly.

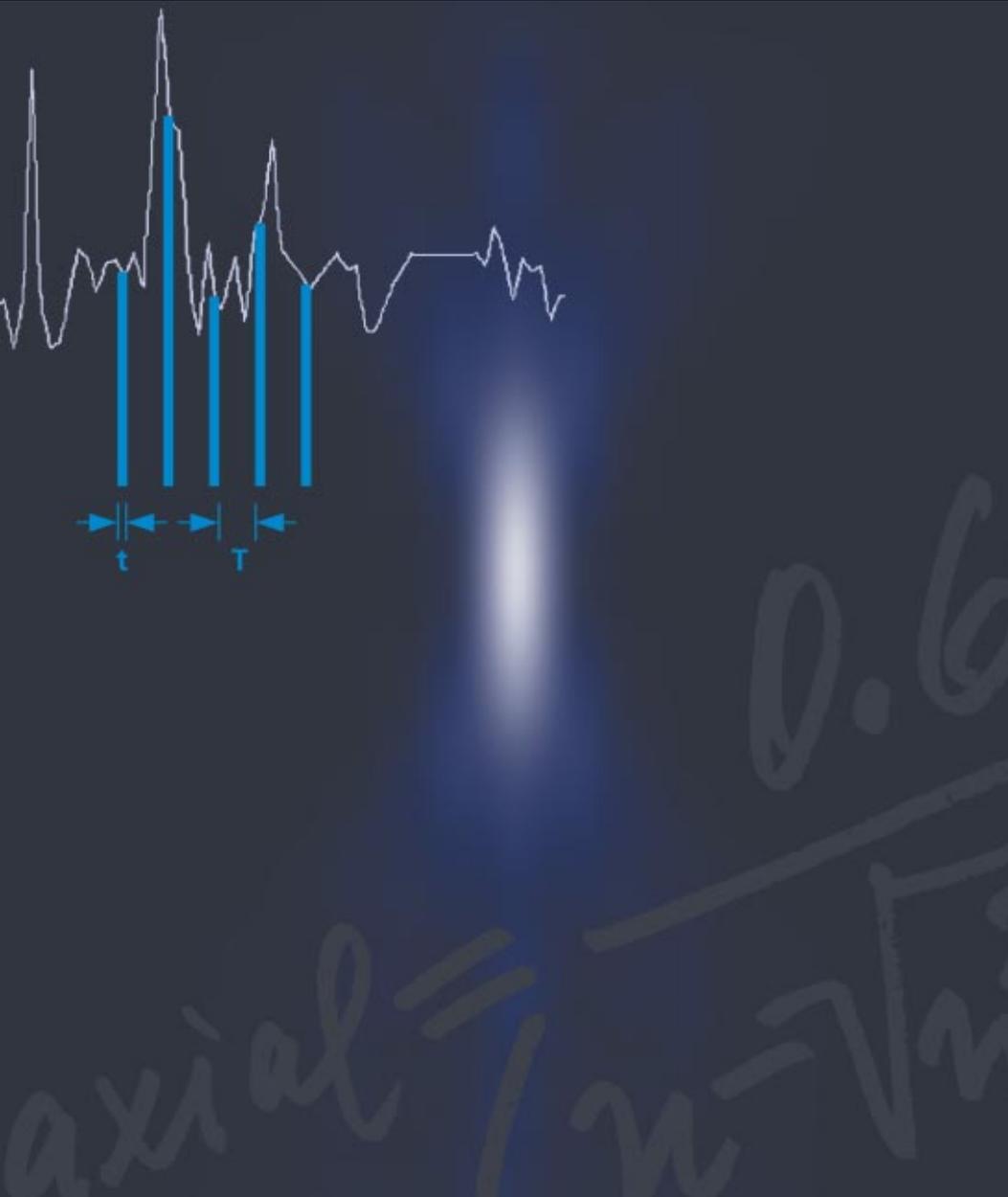


Pressing the **Write to Method** button will write back the settings to the method. If the method is stored, the settings will be active when the method is selected the next time.

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.
- Switch off the Ar-ML laser with first the idle-run-switch (Fig. 2/3) and second the key switch (Fig. 2/1), then wait until the fan of the Argon laser has switched off. Don't switch off the main power yet.
- On the power remote switch 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 Ar-ML laser of by the main power switch on the power supply (Fig. 2/2).

Confocal Laser Scanning Microscopy



Optical Image Formation
Electronic Signal Processing



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.

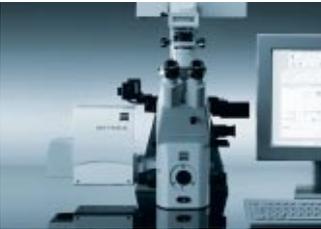


1992

The LSM 410 is the first inverted microscope 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 multiphoton microscopy.

1999

The LSM 5 PASCAL – the personal confocal microscope.



2000

The LSM is combined with the ConfoCor 2 Fluorescence Correlation Spectroscopy.

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.

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Fluorescence	III
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Literature

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.

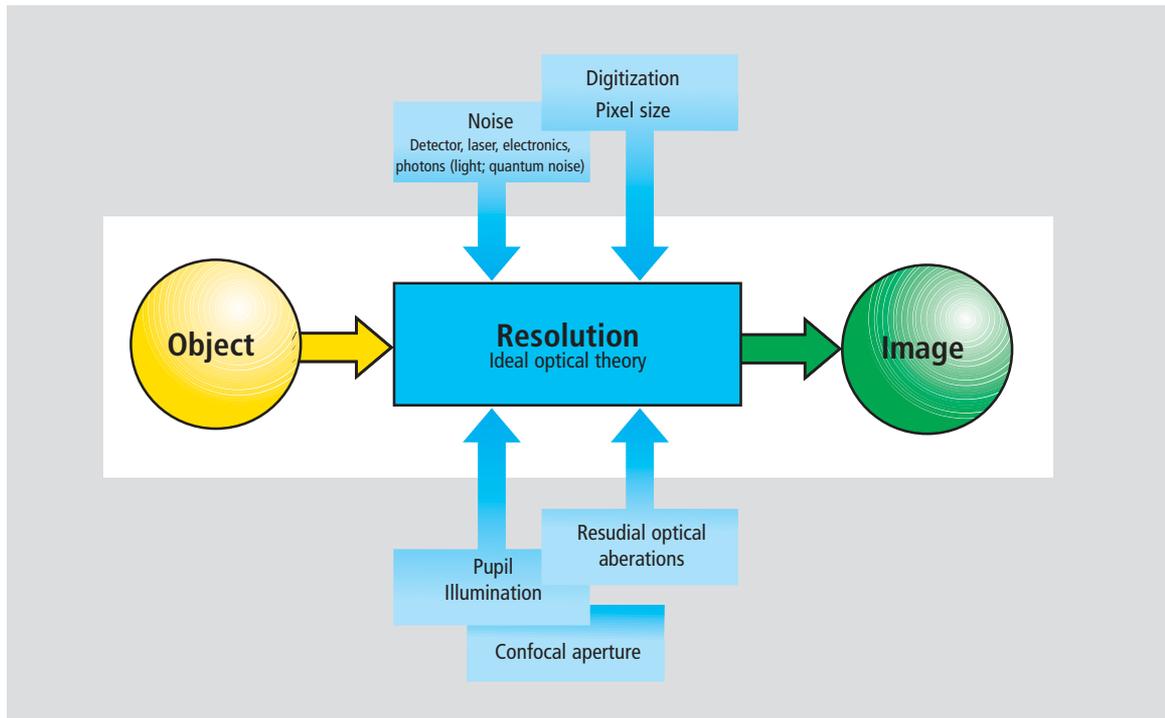
The conditions in fluorescence applications will be given priority treatment throughout.

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).

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.



Introduction

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).

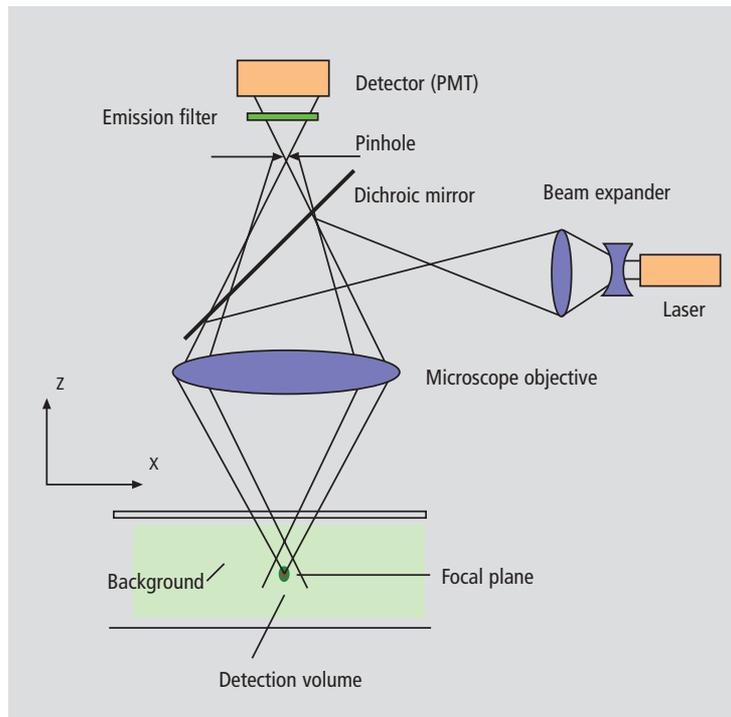
As the laser beam is focused to a diffraction-limited spot, which illuminates only a point of the object at a time, the point illuminated and the point observed (i.e. image and object points) are situated in conjugate planes, i.e. they are focused onto each other. The result is what is called a confocal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

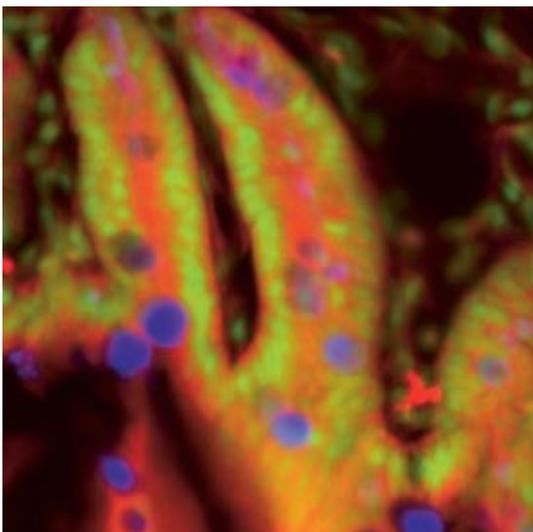


Optical slices

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).

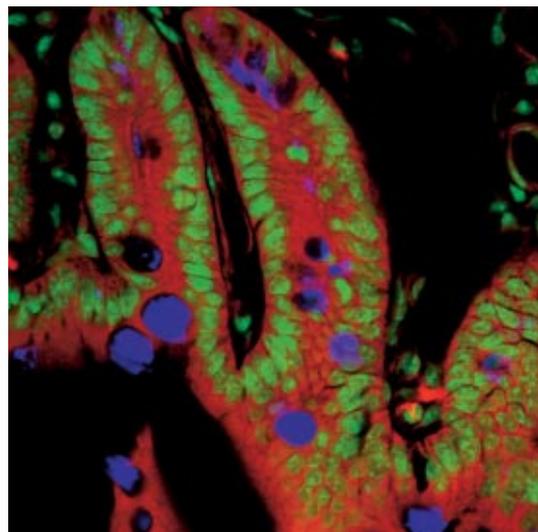


A confocal LSM can therefore be used to advantage especially where thick specimens (such as biological cells in tissue) have to be examined by fluorescence. The possibility of optical sectioning eliminates the drawbacks attached to the observation of such specimens by conventional fluorescence microscopy. 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.

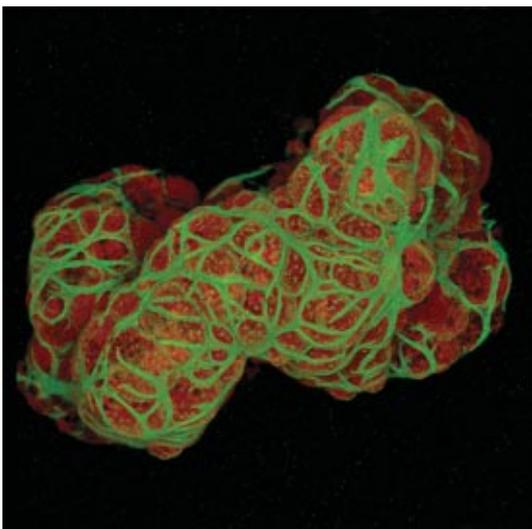


Introduction

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.

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 phalloidin (green), cytoplasm and nuclei of acinar cells with ethidium homodimer-1 (red).



Time series

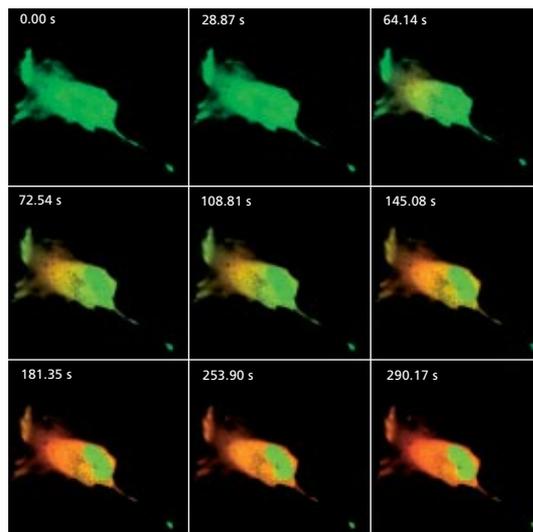
A field of growing importance is the investigation of living specimens that show dynamic changes even in the range of microseconds. Here, the acquisition of time-resolved confocal image series (known as time series) provides a possibility of visualizing and quantifying the changes.

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. 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.

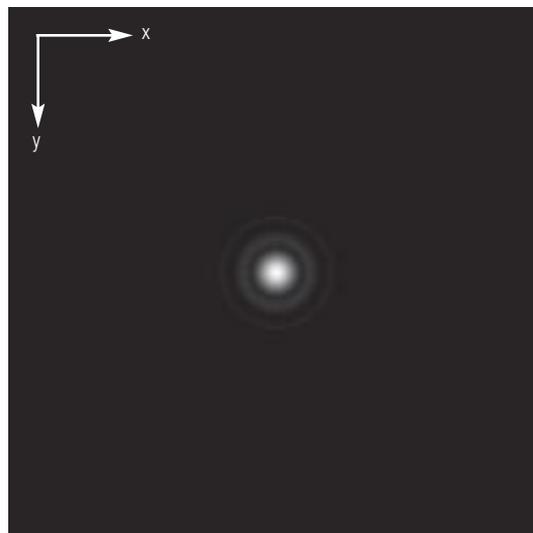
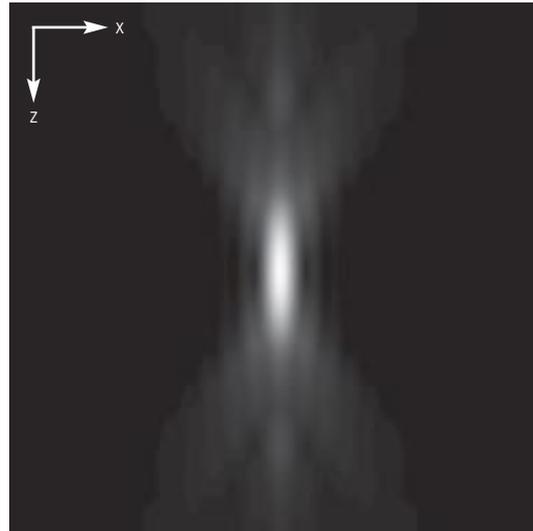


Fig. 6 Section through the 3D-PSF in Z direction – top, and in XY-direction – bottom (computed; dimensionless representation); the central, elliptical maximum is distinctly visible. The central maximum in the bottom illustration is called Airy disk and is contained in the 3D-PSF as the greatest core 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:

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

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

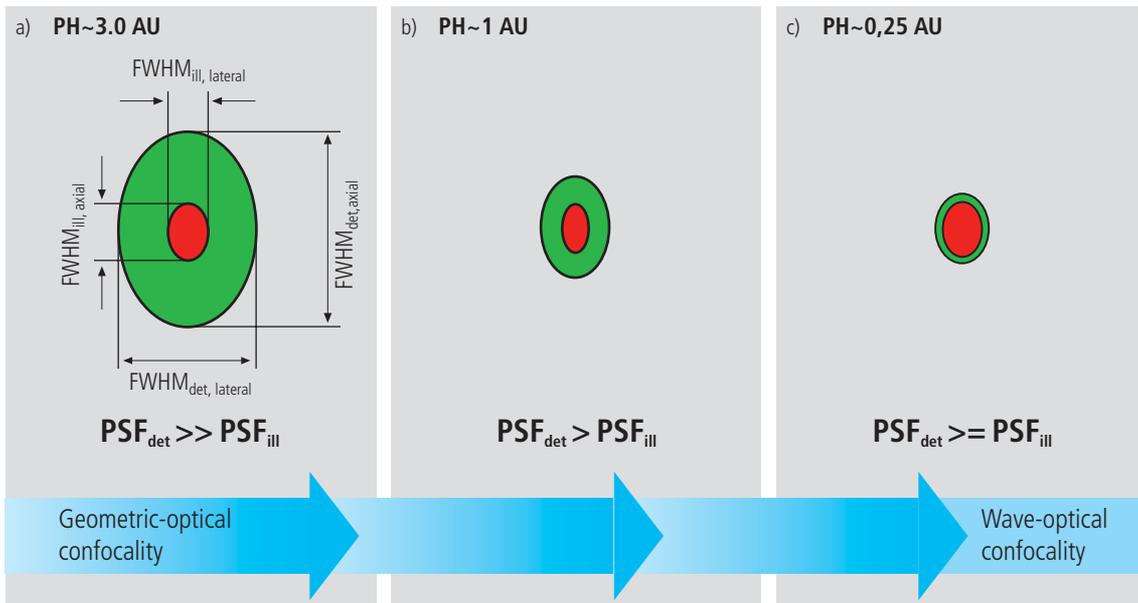
Wherever quantitative data on the resolving power and depth discrimination of a confocal LSM are specified, it is necessary to distinguish clearly whether the objects they refer to are point-like or extended, and whether they are reflective or fluorescent. These differences involve distinctly varying 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).



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:

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

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:

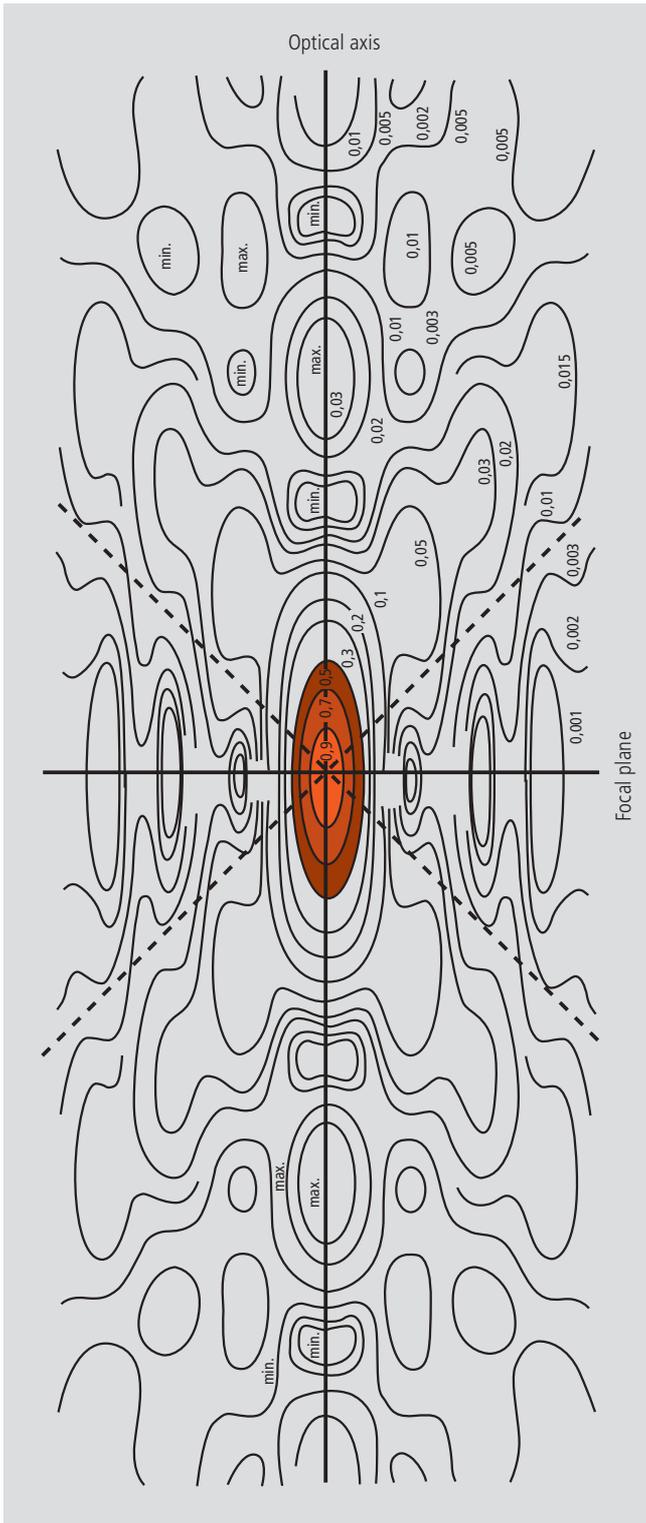
$$\approx \frac{1.77 \cdot n \cdot \lambda_{exc}}{NA^2} \quad (2a)$$

Lateral:

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

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_{III}). 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_{III}). The intensity at the focus is normalized as 1. (Born & Wolf, Principles of Optics, 6th edition 1988, Pergamon Press)

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:

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

- 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.

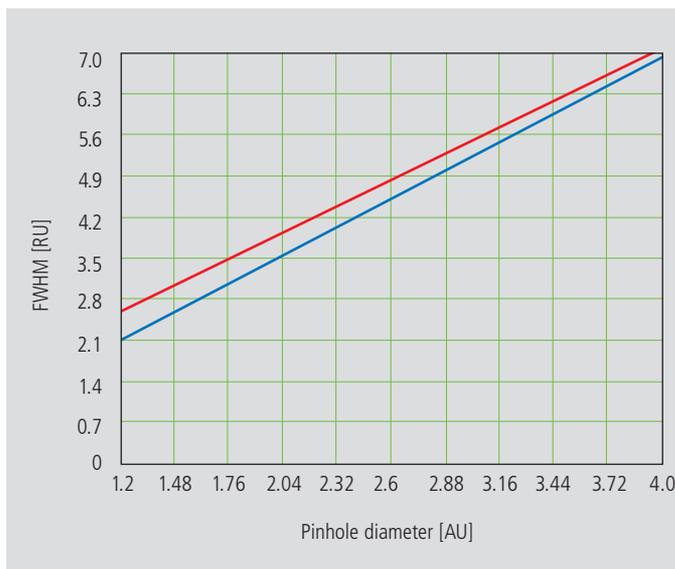
Likewise, in the case of geometric-optical confocality, there is a linear relationship between depth discrimination and pinhole diameter. As the pinhole diameter is constricted, depth discrimination improves (i.e. the optical slice thickness decreases). A graphical representation of equation (4) is illustrated 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: $NA = 0.6$; $n = 1$; $\lambda = 520$ 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

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

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 wavelength¹:

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

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

Axial:

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

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

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

Lateral:

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

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 $PH < 1AU$, 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.

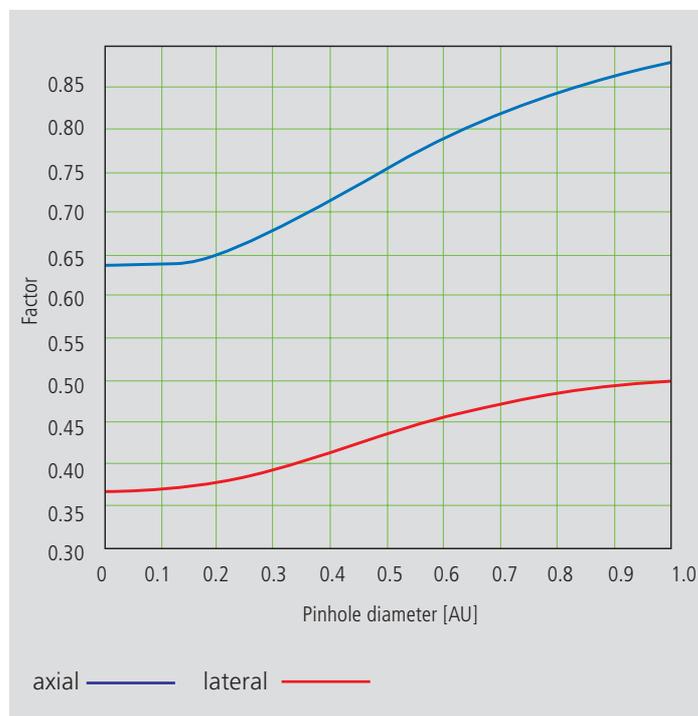


Fig. 10 Theoretical factors for equations (7) and (8), with pinhole diameters between 0 and 1 AU.

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 ($NA, \bar{\lambda}, n$).

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

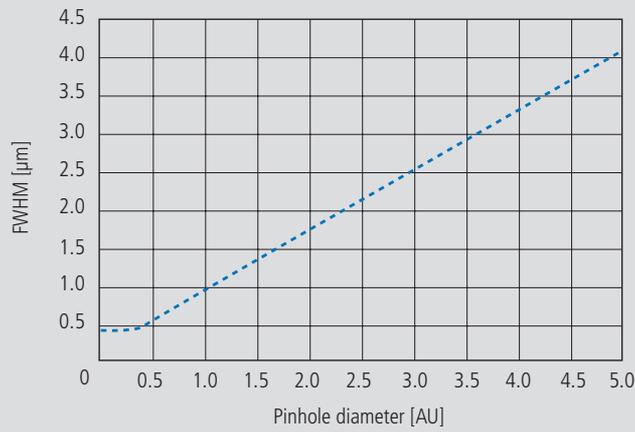
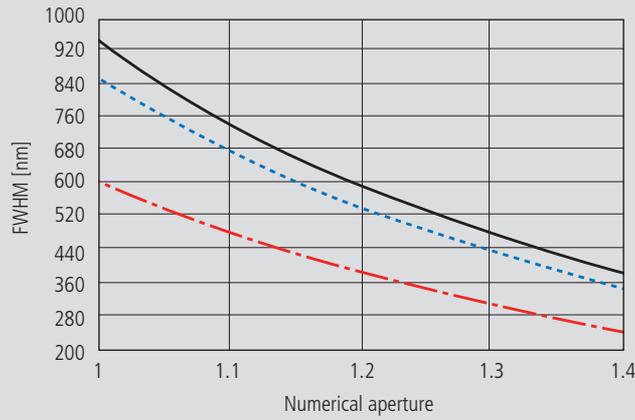


Fig. 11

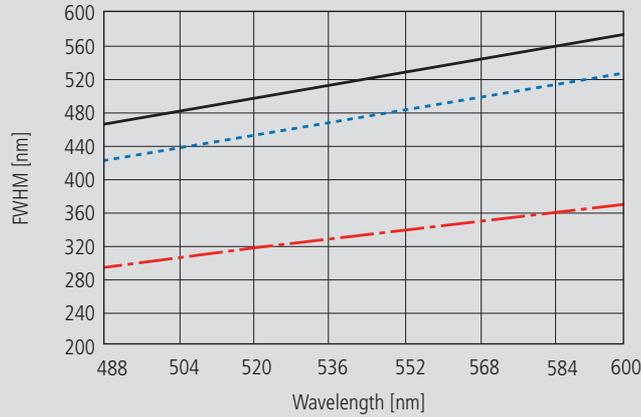
a) Variation of pinhole diameter

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



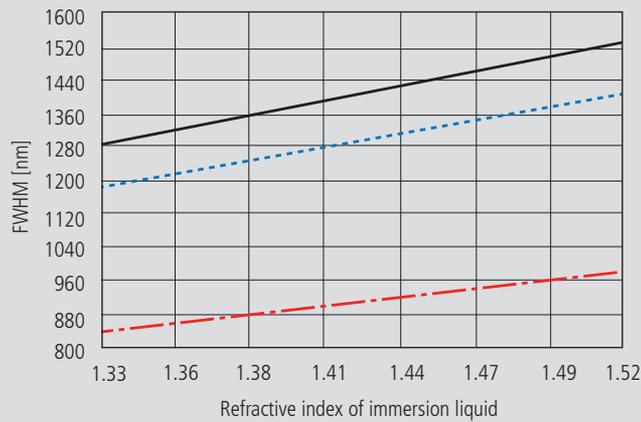
b) Variation of numerical aperture

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



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

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



d) Variation of refractive index

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

Overview

Conventional microscopy	Confocal microscopy 1 AU < PH < ∞	Confocal microscopy PH < 0.25 AU
<p>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.</p>	<p>1. Optical slice thickness¹⁾</p> $\sqrt{\left(\frac{0.88 \cdot \lambda_{em}}{n \cdot \sqrt{n^2 - NA^2}}\right)^2 + \left(\frac{\sqrt{2} \cdot n \cdot PH}{NA}\right)^2}$ <p>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.</p>	<p>1. Optical slice thickness</p> $\frac{0.64 \cdot \bar{\lambda}}{(n \cdot \sqrt{n^2 - NA^2})}$ <p>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.</p>
<p>2. Axial resolution (wave-optical depth of field)</p> $\frac{n \cdot \lambda_{em}}{NA^2}$ <p>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.</p>	<p>2. Axial resolution</p> $\frac{0.88 \cdot \lambda_{exc}}{(n \cdot \sqrt{n^2 - NA^2})}$ <p>FWHM of PSF_{III} (intensity distribution at the focus of the microscope objective) in Z direction. No influence by the pinhole.</p>	<p>2. Axial resolution</p> $\frac{0.64 \cdot \bar{\lambda}}{(n \cdot \sqrt{n^2 - NA^2})}$ <p>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.</p>
<p>3. For comparison: FWHM of PSF in the intermediate image (Z direction) – referred to the object plane.</p> $\frac{1.77 \cdot n \cdot \lambda_{em}}{NA^2}$	<p>3. Approximation to 2. for NA < 0.5</p> $\frac{1.77 \cdot n \cdot \lambda_{exc}}{NA^2}$	<p>3. Approximation to 2. for NA < 0.5</p> $\frac{1.28 \cdot n \cdot \bar{\lambda}}{NA^2}$
<p>4. Lateral resolution</p> $\frac{0.51 \cdot \lambda_{em}}{NA}$ <p>FWHM of the diffraction pattern in the intermediate image – referred to the object plane) in X/Y direction.</p>	<p>4. Lateral resolution</p> $\frac{0.51 \cdot \lambda_{exc}}{NA}$ <p>FWHM of PSF_{III} (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.</p>	<p>4. Lateral resolution</p> $\frac{0.37 \cdot \bar{\lambda}}{NA}$ <p>FWHM of total PSF in X/Y direction plus contrast-enhancing effect of the pinhole because of stray light suppression.</p>

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 ($x = t \cdot v_{\text{scan}}$). 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).

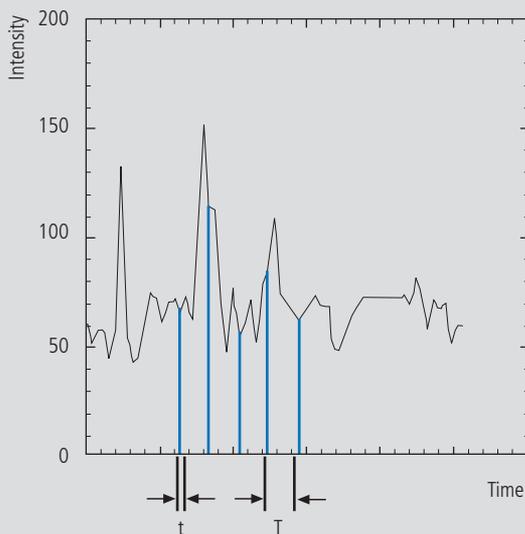
Types of A/D conversion

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 ($t \ll T$)



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).

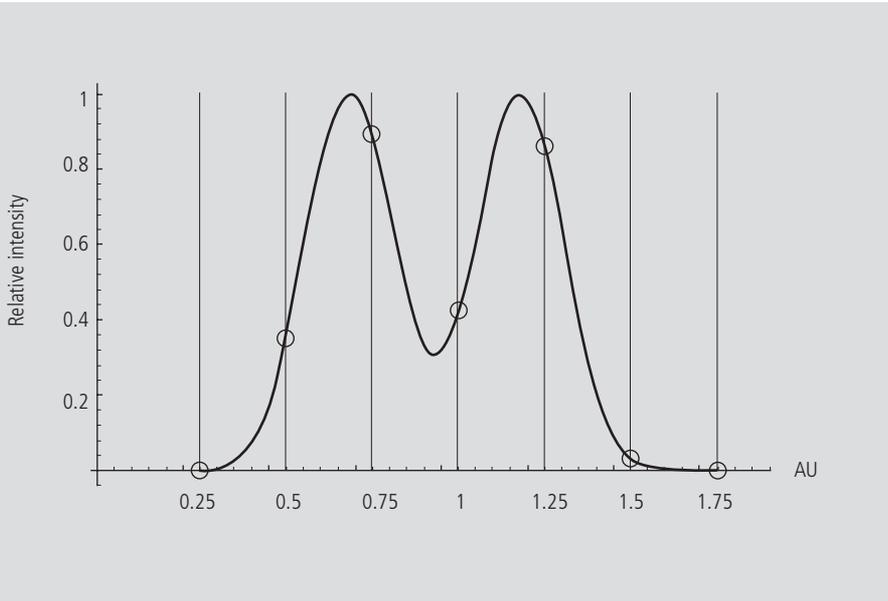
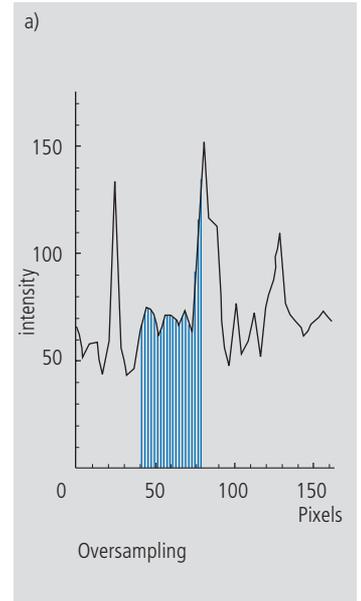


Fig. 14 Oversampling, correct sampling and undersampling of a continuous signal.



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:

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

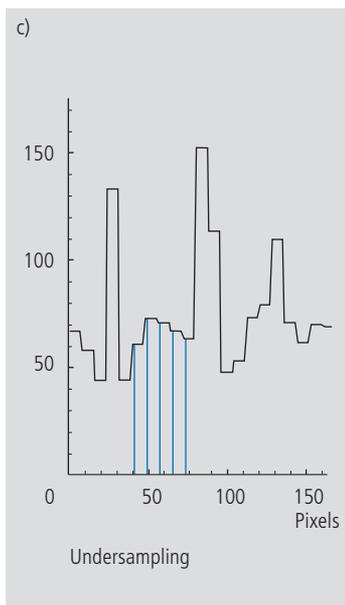
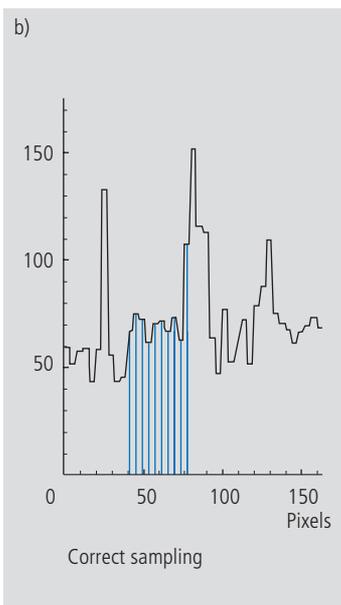
Number of pixels = number of pixels per line
Zoom factor (Z) = scanning zoom set in the software
 (Example: Zoom factor 2 reduces the edge length of the scanned field by a factor of 2)

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 fulfill the Nyquist theorem can therefore be calculated as follows:

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

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 over-sampling. 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).

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.

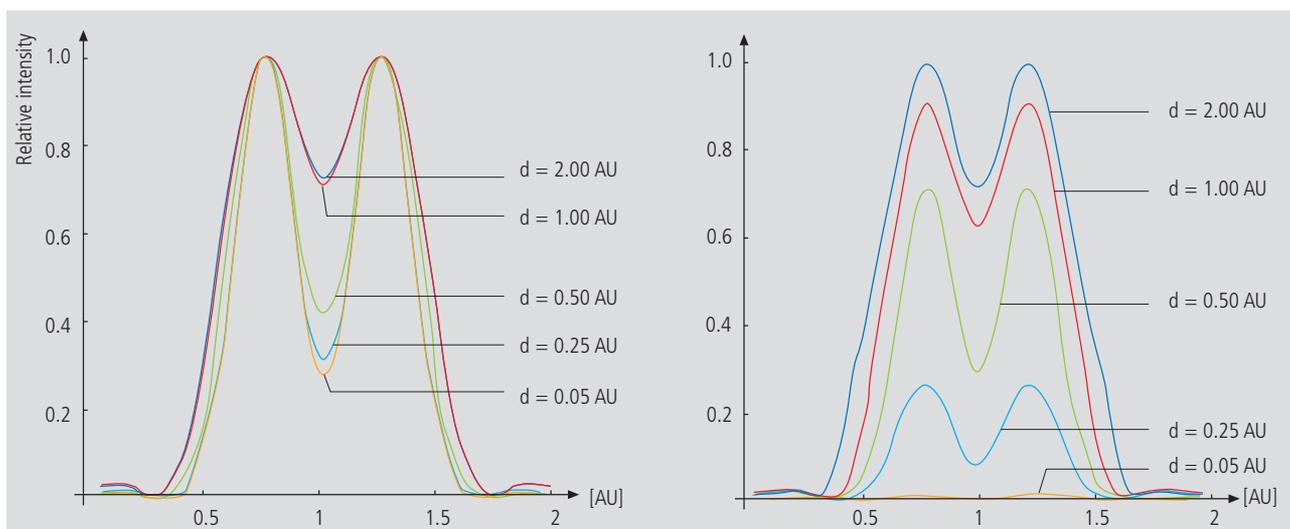
With a pinhole diameter < 1 AU, resolution improves (better point separation thanks to a deeper dip), which is penalized by a drastic loss in energy.

Moreover, it should be considered that it depends on the signal level which 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.

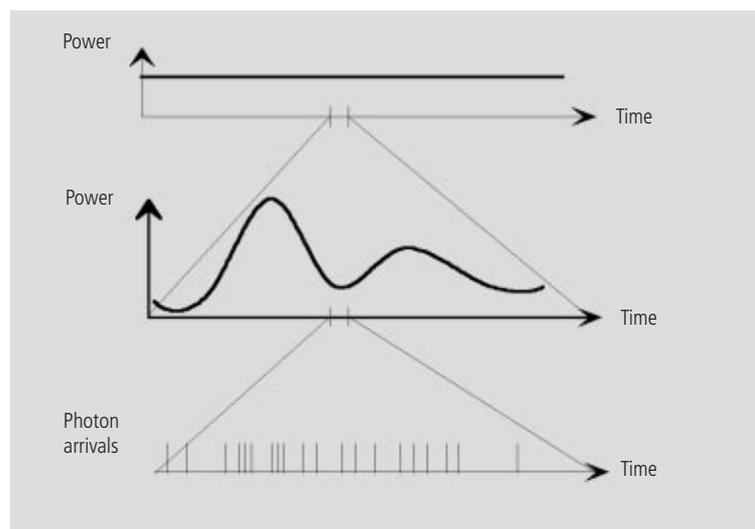
Therefore, it makes sense to talk of resolution probability rather than of resolution. Consider a model which combines the purely optical understanding of image formation in the confocal microscope (PSF) with the influences of shot noise of the detected light and the scanning and digitization of the 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 ($\leq 50\%$ 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.

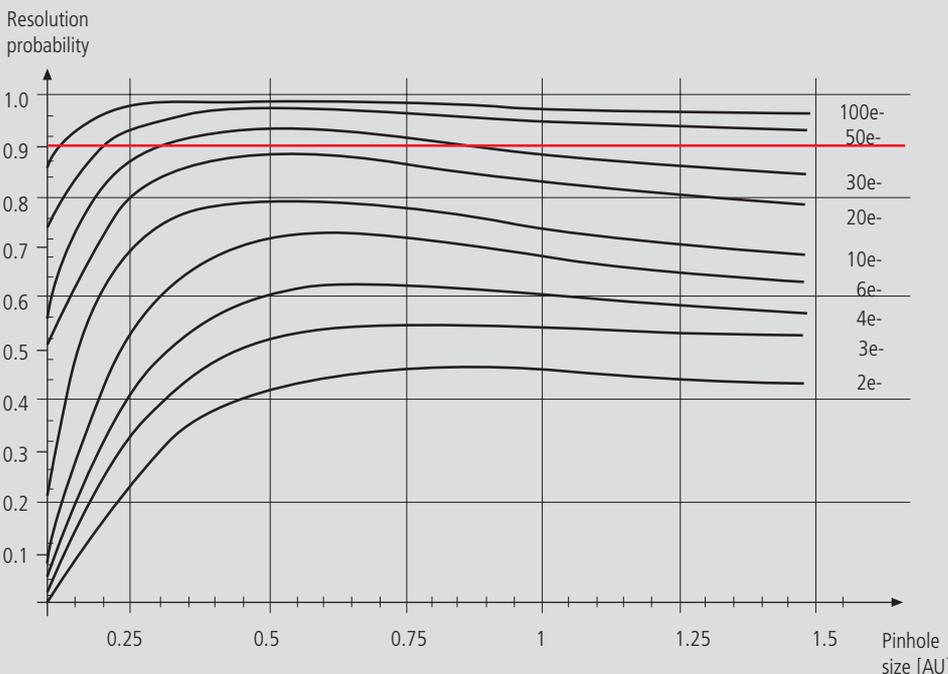


Fig. 17 The graph shows the computed resolution probability of two self-luminous points (fluorescence objects) spaced at 1/2 AU, as a function of pinhole size and for various photoelectron counts per point object (e-). The image raster conforms to the Nyquist theorem (critical raster spacing = 0.25 AU); 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 μ W 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 probability 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 number of photons detected increases. As fluorescence 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 acquisitions (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 specimen, as the exposure time per pixel remains constant. Photon statistics are improved by the addition of photons from several scanning runs ($SNR = \sqrt{n \cdot N}$; $N = \text{const.}$, $n = \text{number of scans averaged}$). By comparison, a longer pixel time directly improves the photon statistics by a greater number N of photons detected per pixel ($SNR = \sqrt{N}$, $N = \text{variable}$), but there is a greater probability of photobleaching or saturation effects of the fluorophores.

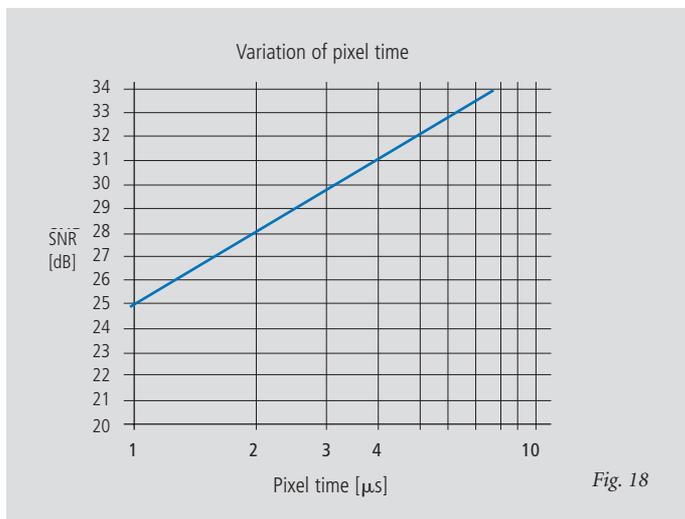


Fig. 18

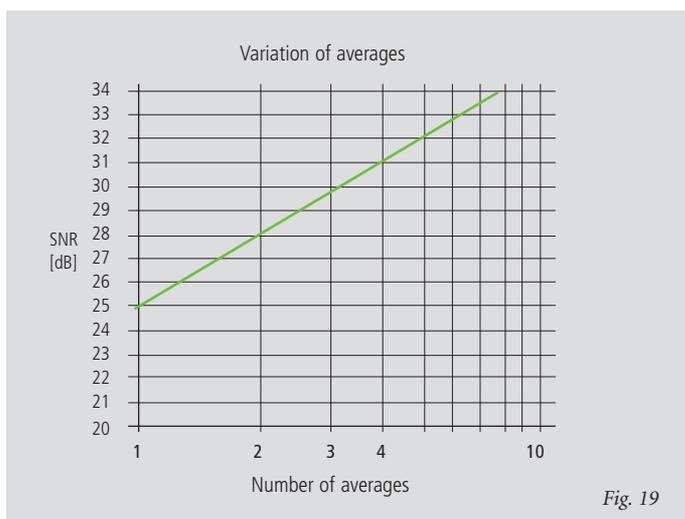
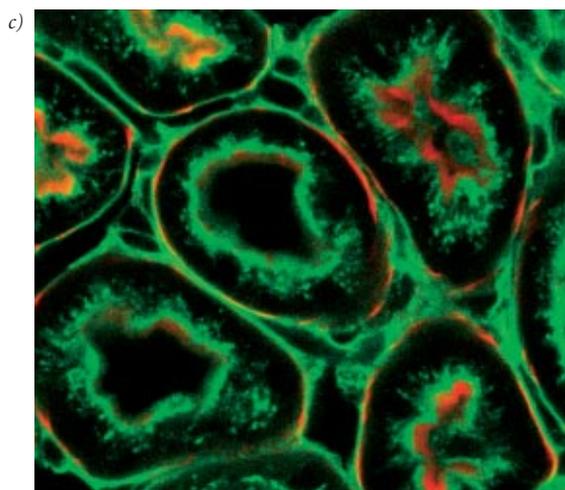
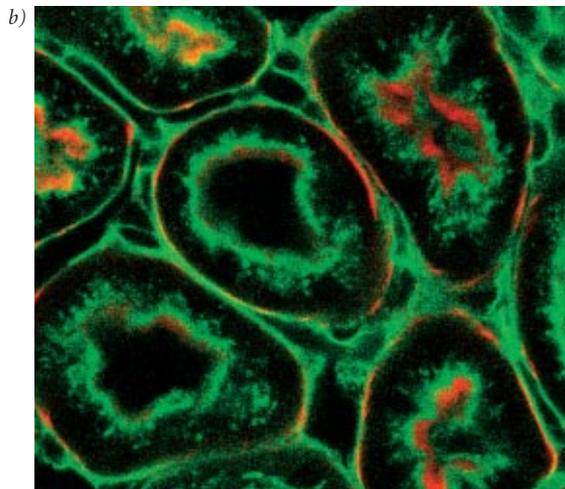
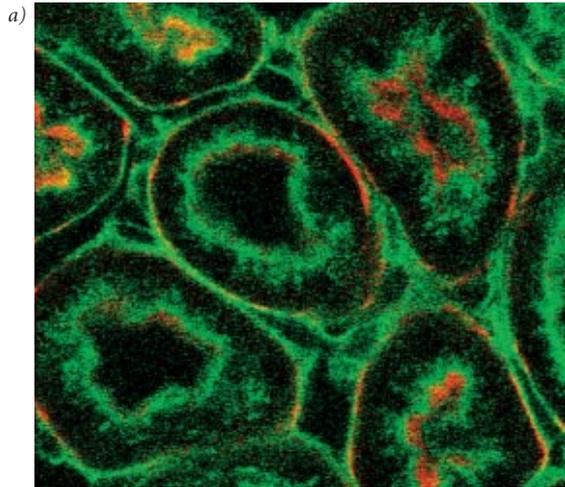


Fig. 19

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).



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 beamsplitter¹ but a dichroic beamsplitter optimized for the particular fluorescence.

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 μ s in a), 6.4 μ s (no averaging) in b), and 6.4 μ s plus 4 times line-wise averaging in c).

¹An 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

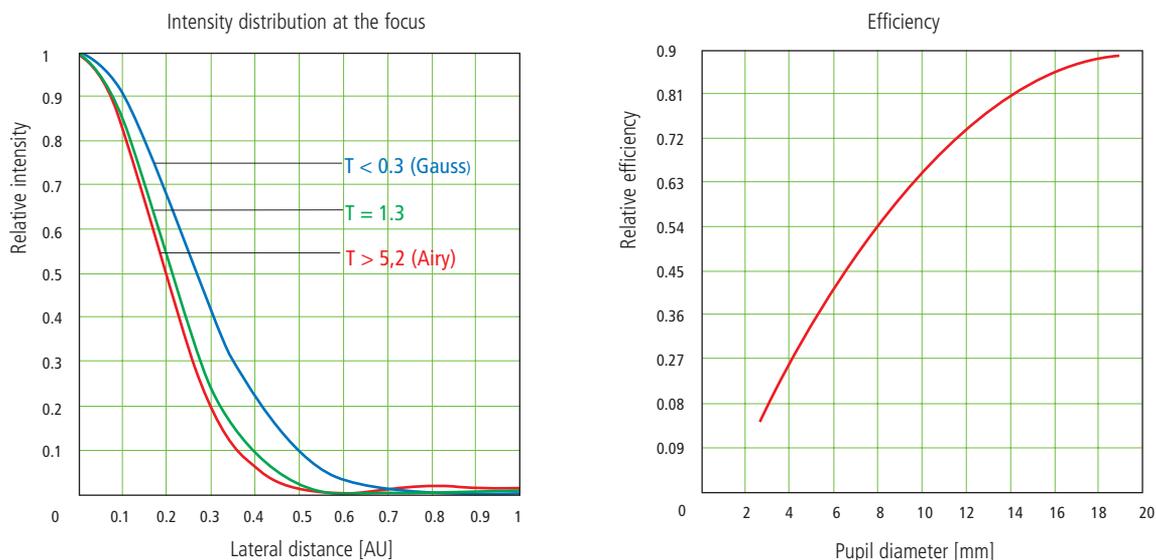
All descriptions in this monograph suggest a confocal LSM with a ray geometry providing homogeneous illumination at all lens cross sections. The focus generated in the object has an Airy distribution, being a Fourier transform of the intensity distribution in the objective's pupil plane. However, the 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 $T \leq 0.5$; 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



The truncation factor T is defined as the ratio of laser beam and pupil diameter of the objective lens used:

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

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

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

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 $\bar{\lambda} = 496 \text{ nm}$ $\rightarrow 1AU = 0.465 \mu\text{m}$

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 $NA = 1.3$, $\bar{\lambda} = 496 \text{ nm}$ and $n = 1.52$ $\rightarrow 1RU = 0.446 \mu\text{m}$

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 μ W 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/cm². This corresponds to an excitation photon flux of $6.34 \cdot 10^{23}$ photons/cm² 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/cm² 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/cm² sec.

At rates greater than $1.26 \cdot 10^{24}$ photons/cm² 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 μ W 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/ μ sec

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 $t = \frac{n}{F_{\max}}$, and referred to the maximum emission rate, this corresponds to a lifetime of the fluorescein molecule of about $115 \mu\text{s}$.

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 ($NA = 1.2$, $100 \mu\text{W}$ excitation power, $\lambda = 488 \text{ nm}$), a photon flux of $F \sim 23 \text{ photons}/\mu\text{sec}$ results. In combination with a sampling time of $4 \mu\text{sec}/\text{pixel}$ 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,

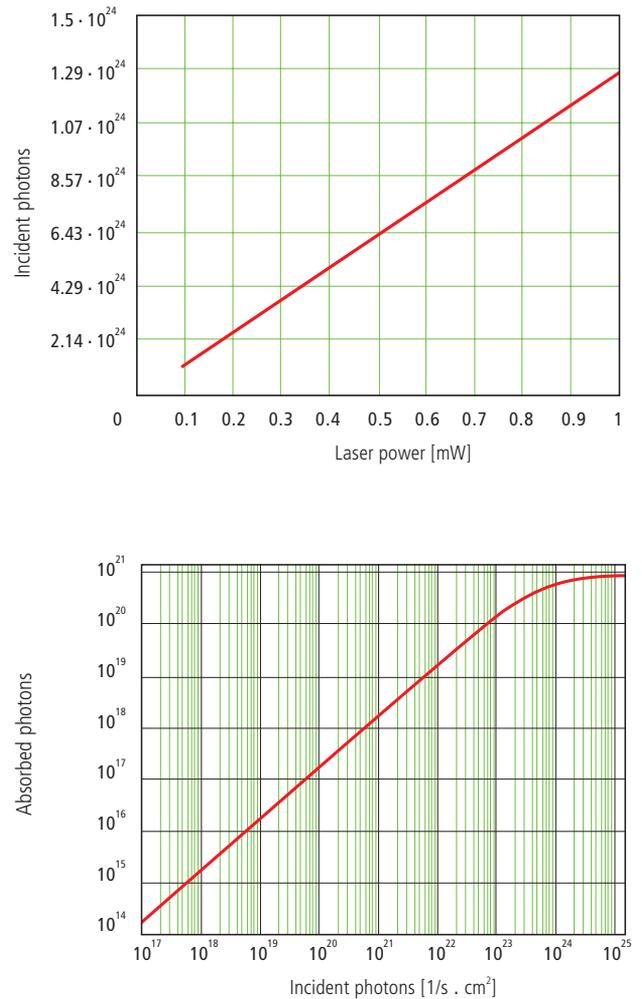


Fig. 22 Excitation photon flux at different laser powers (top) and excited-state saturation behavior (absorbed photons) of fluorescein molecules (bottom).

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 $\lambda = 488 \text{ nm}$ and $NA = 1.2$ the sampling volume can be calculated to be $V = 12.7 \cdot 10^{-18} \text{ l}$. Assuming a dye concentration of $0.01 \mu\text{Mol/l}$, 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 $h \cdot \nu$ 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

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

where $QE(\lambda)$ = 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 $q = 0.05$

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

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Subject to change.

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

Laser

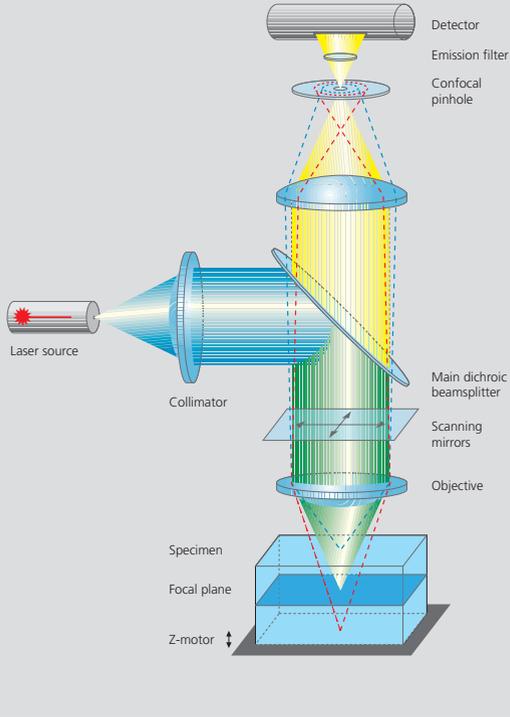
- **Light source** – projected into specimen
- **Laser power:** adjustable via attenuation device (AOTF, AOM, MOTF) and tube current setting (Ar)
- **Lifetime Ar:** prolonged by using lower tube current; but laser noise will be increased (8 A = minimum noise)
- **Stand-by mode:** prolongs laser lifetime; 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 objective focus



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 amplifier:** 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")
- **HET:** separates excitation and emission light
- **NET:** 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: λ ≥ 505 nm → detection)

Objective Lens

- **Optical image formation** – determines properties of image quality such as resolution (x, y, z)
- **Numerical Aperture (NA):** determines image 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)

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!

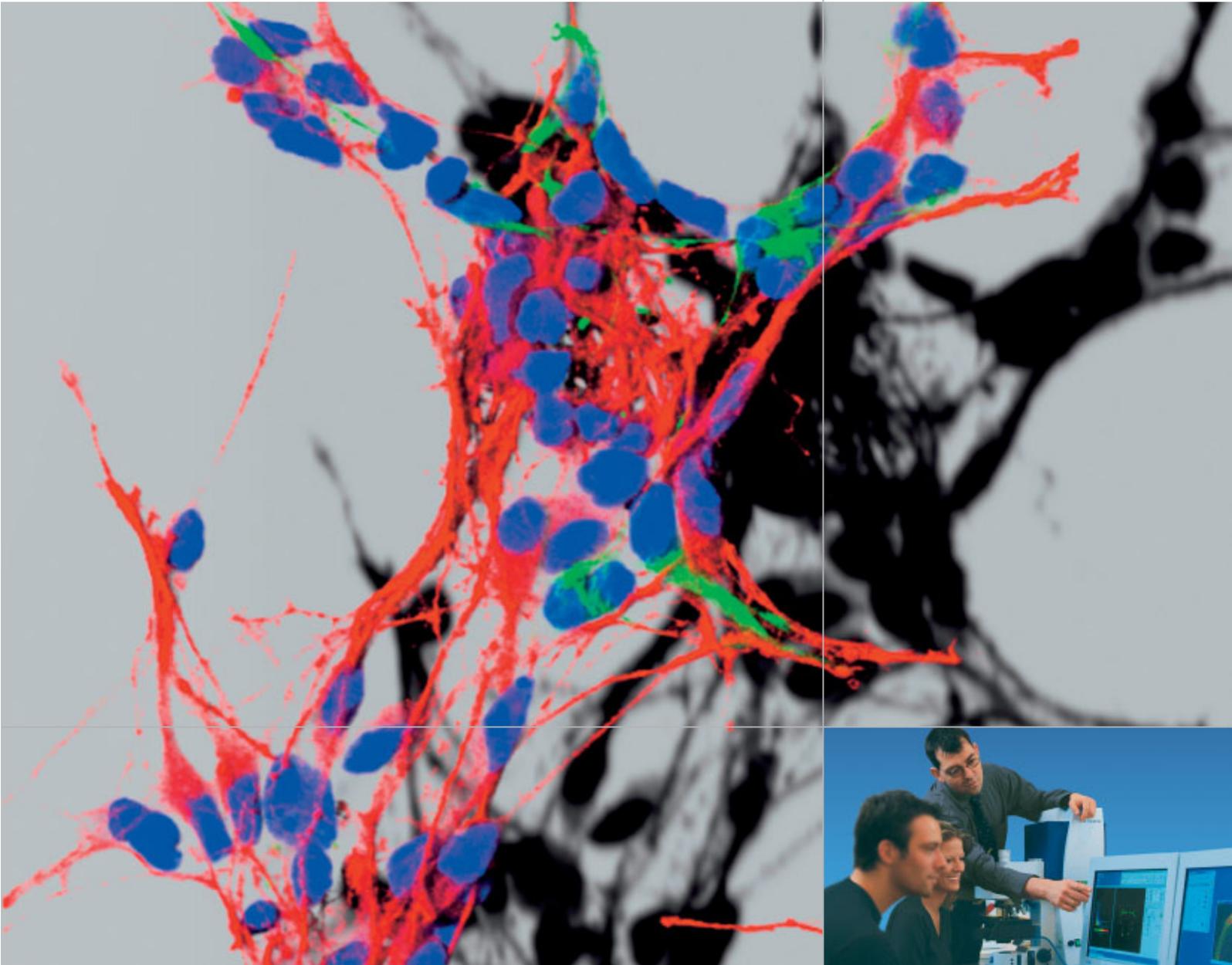
- Use Multitracking: very fast switching of excitation wavelengths; prevents crosstalk of signals between channels; predefined configurations available.
- Use ROI (Region Of Interest) function: significantly reduces excited area of specimen and increases acquisition rate at constant SNR; several ROIs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

The Confocal Laser Scanning Microscope



We make it visible.

Confocal Laser Scanning Microscopy



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



We make it visible.

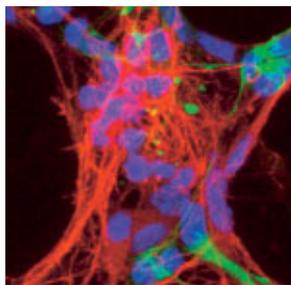
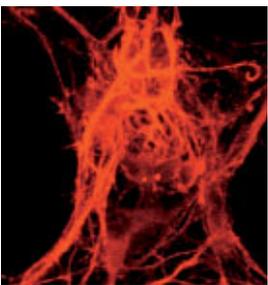
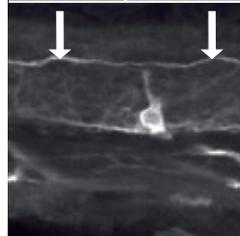
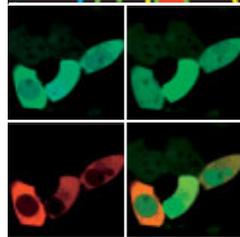
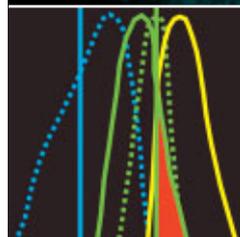
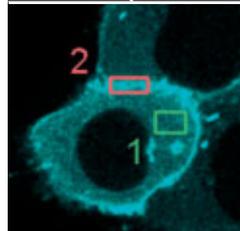
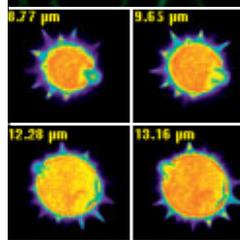
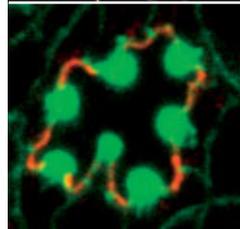
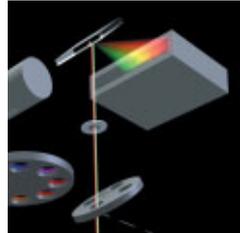
Confocal Laser Scanning Microscopy

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.

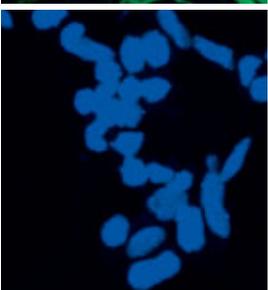
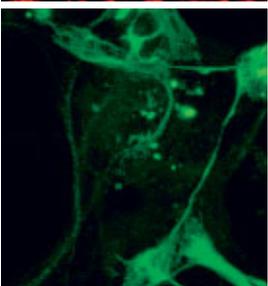
In the search for answers to these questions, imaging systems based on the classical light microscope have come to play an unprecedented role, thanks to many technical innovations and a high degree of automation. Many experiments have only become possible because of the new functions provided by modern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

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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

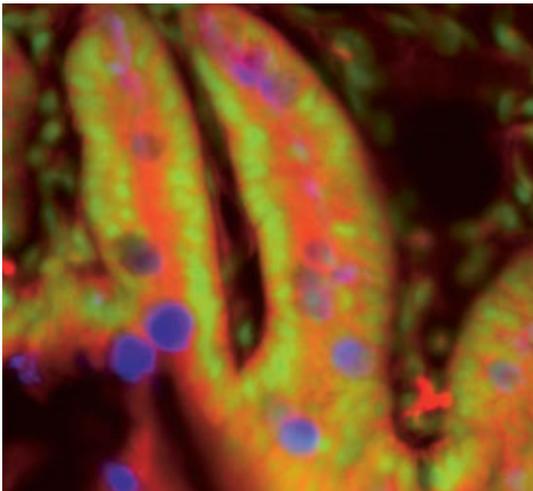
Confocal laser scanning microscopes (LSMs) are distinguished by their high spatial and temporal resolving power. They clearly outperform classical light microscopes especially by their axial resolution – a quality that enables users to acquire optical sections (slices) of a specimen. An object can thus 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 fluores-

cence 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.

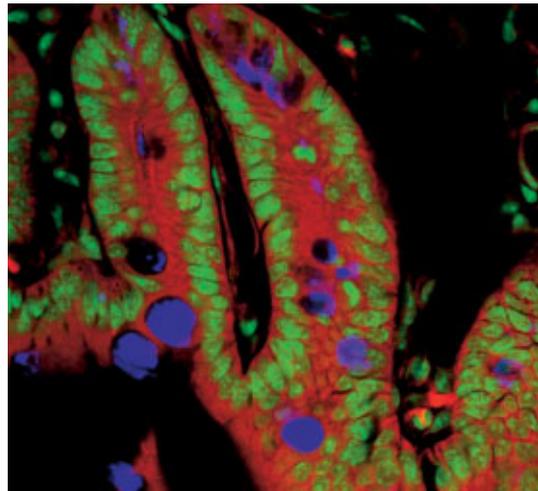
Because of its versatility, laser scanning microscopy has become an established mainstream method in biomedical research – a tool permitting scientists to follow innovative experimental paths. This article will show which basic functions and applications of laser scanning microscopy can be taught in 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 are blurred in the non-confocal image are visible clearly and in greater contrast.

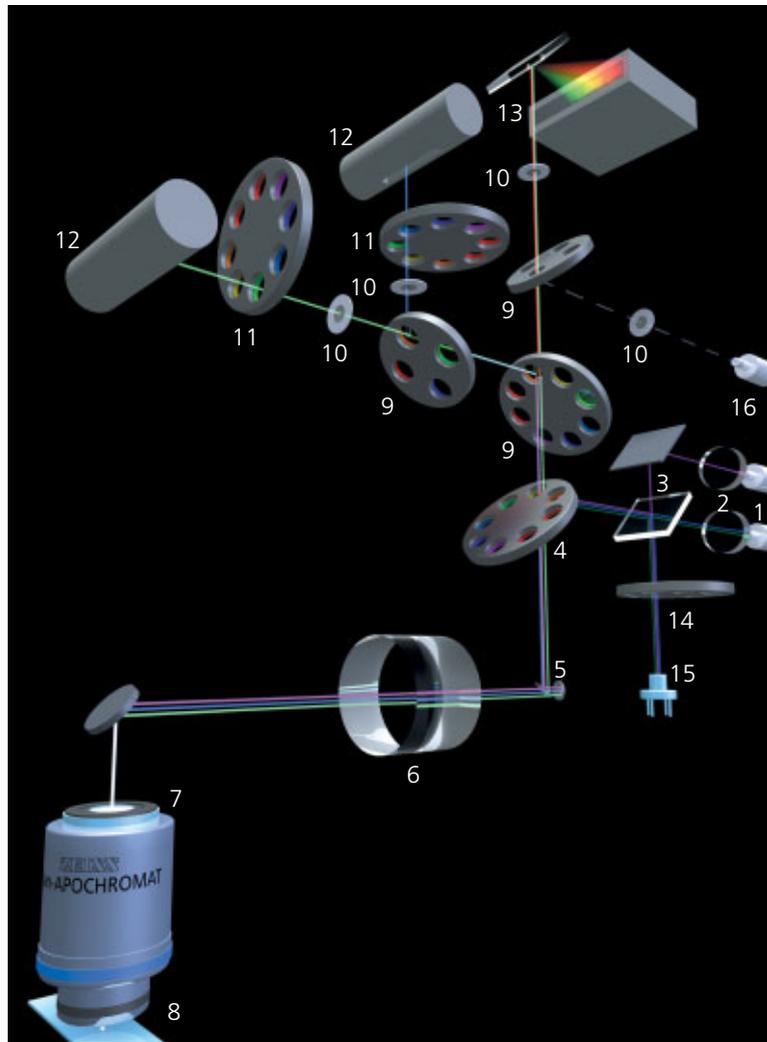
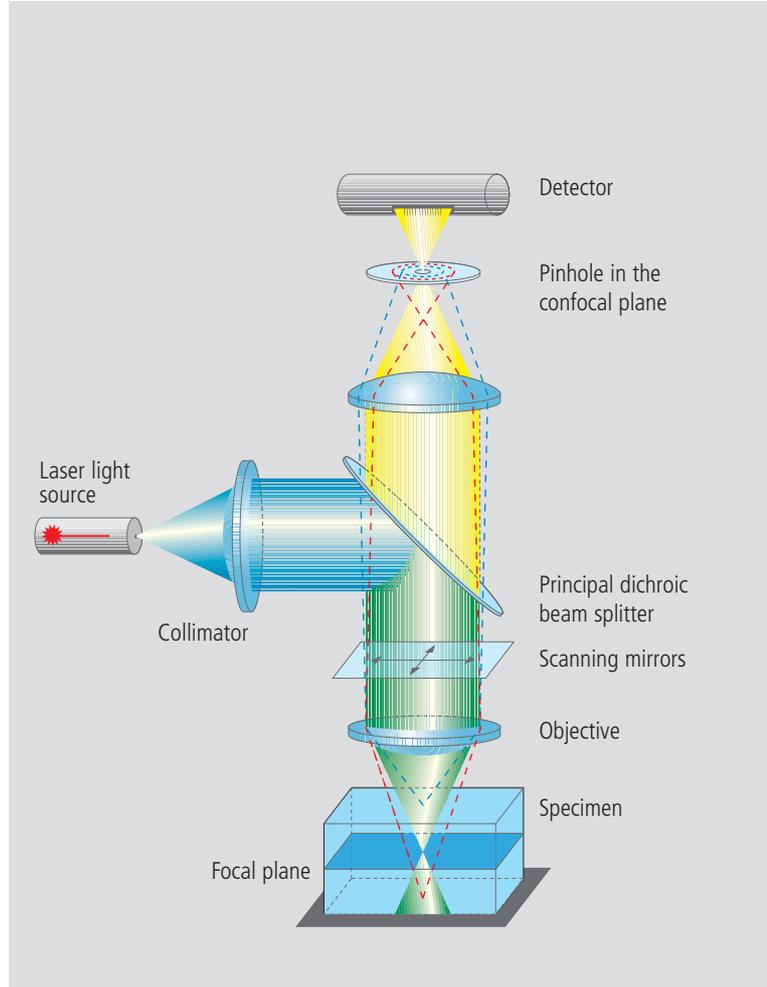


The Confocal Principle

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.

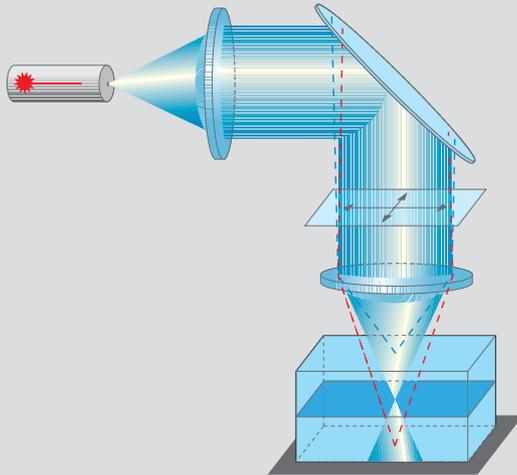
An LSM can be easily understood as a modified light microscope supplemented by a laser module that serves as a light source, and a scanning head (attached to the microscope stand) that is used to detect the signal. Signal processing is effected by an electronic system contained in a box. The whole system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



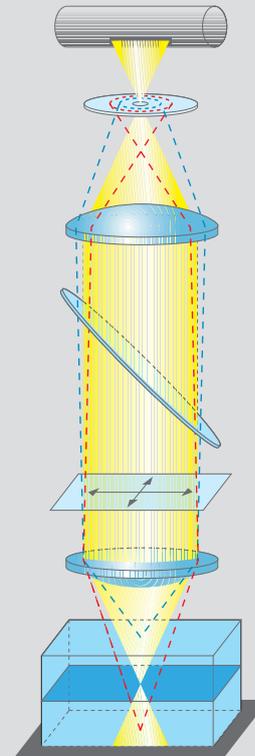
Excitation light path

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

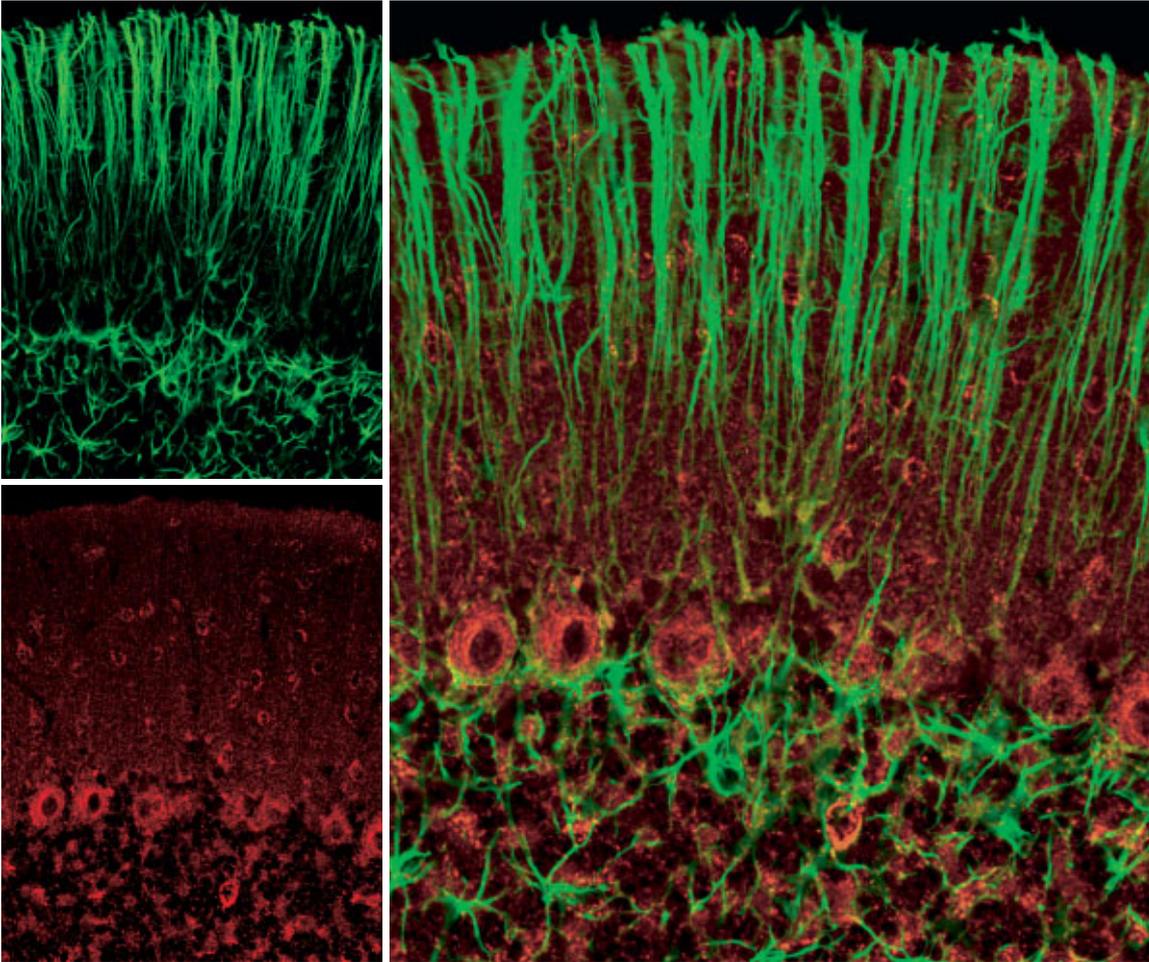
Two-Dimensional Images

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.

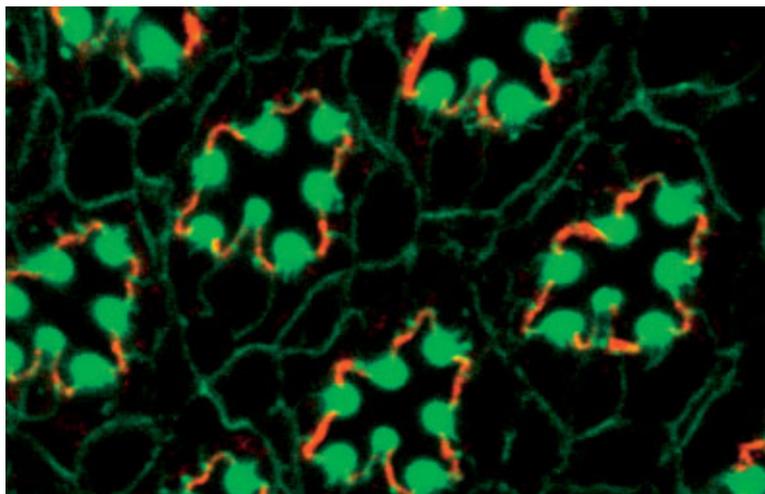
The thickness of the optical section (slice) and the focal position are selected so that the structures of interest are contained in the slice. The lateral resolution of a 2D image is defined by the pixel size in X and Y. The pixel size, in turn, varies with the objective used, the number of pixels per 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 *Visual* 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.

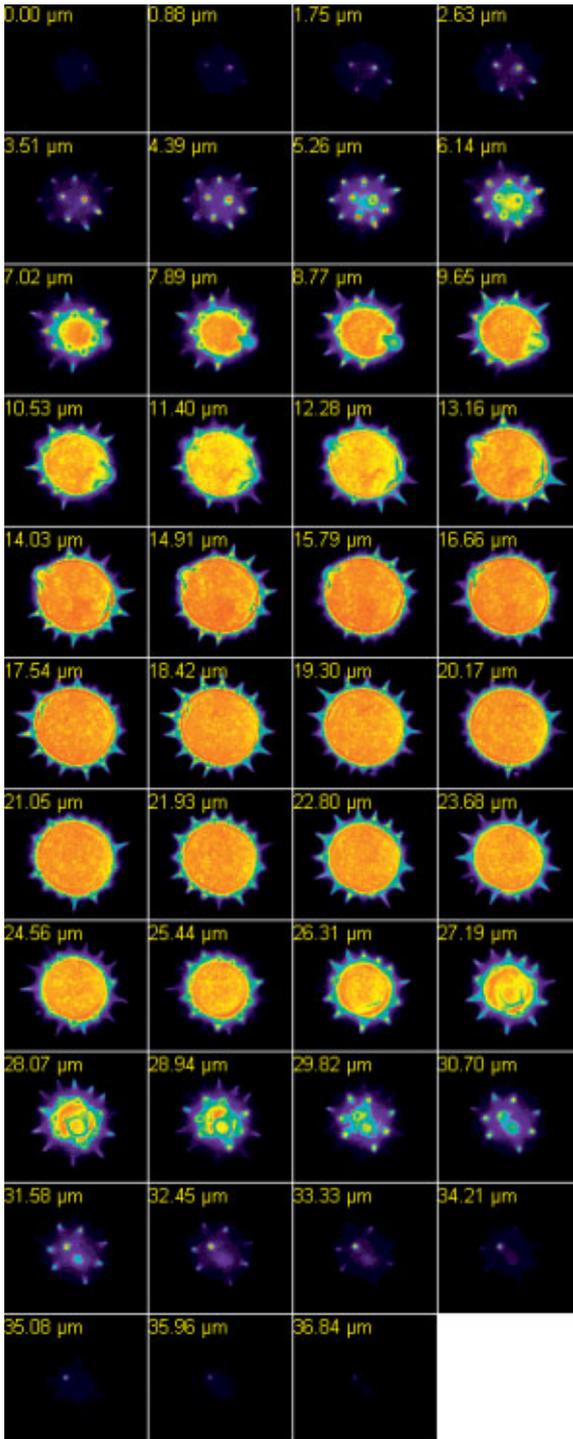
If the sample to be examined is labeled with more than one fluorochrome, it is necessary to adjust the optical slice thicknesses of the various image channels. The slice thickness is a function of the numerical aperture of the objective, the wavelength used, and the pinhole diameter. It differs for channels 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.

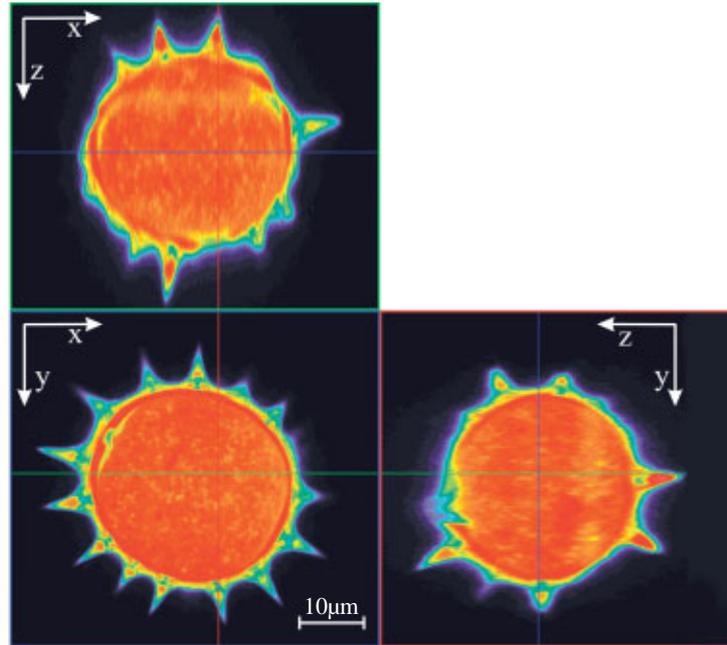
The procedure for a three-dimensional image

- 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



XYZ image stack of a pollen grain. A series of XY images acquired in different focus positions represents the Z dimension of the specimen.



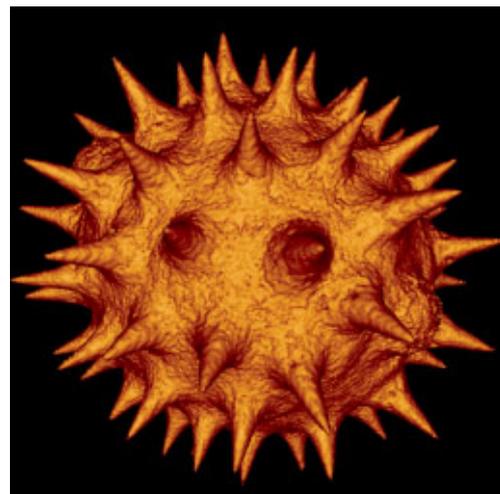
The orthogonal projection of the three-dimensional image data stack permits the raw data stack to be sectioned anywhere in any of the three mutually perpendicular planes.

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.



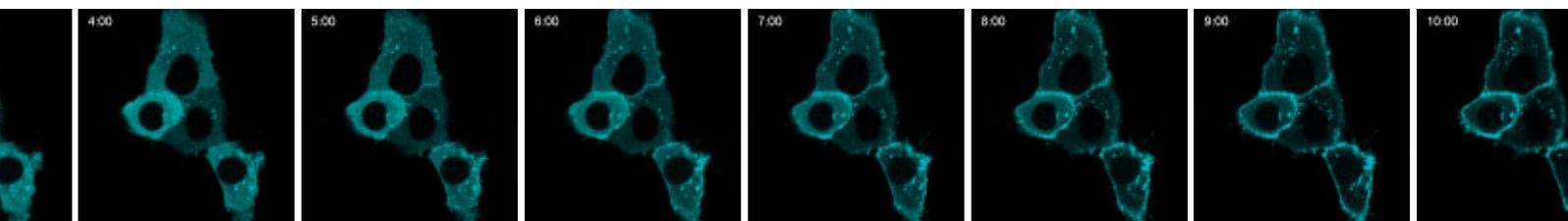
Time Series

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

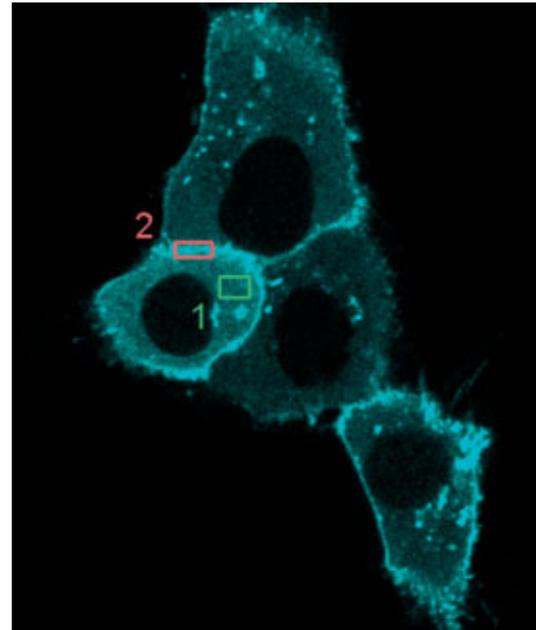
- 1** Define the image dimensions to be recorded versus time (*XY image, Z stack, λ stack*)
- 2** Optimize the recording conditions at minimum laser output to avoid or minimize bleaching
- 3** Define the number of images to be taken and the time interval between two successive images (*Time Interval 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.

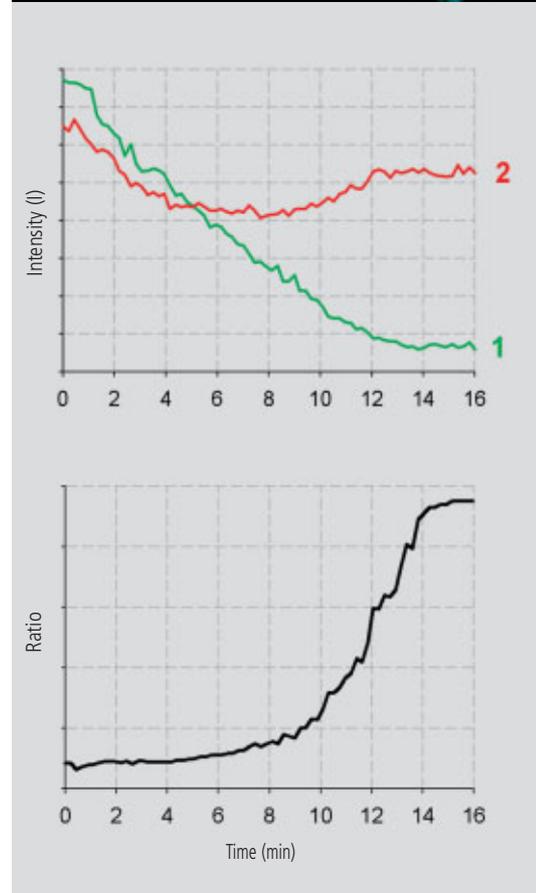
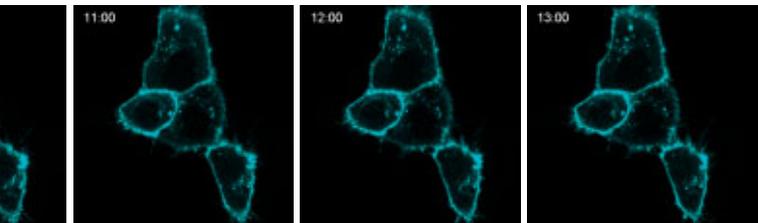
ROI 1: Cytosol

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.

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.

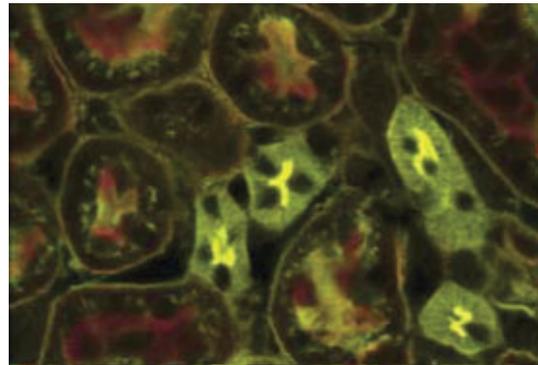
In a pure emission crosstalk between two fluorochromes A and B, the two emission spectra will overlap, but the laser lines will excite the dyes independently of each other; i.e. there is no overlap of the excitations.

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.

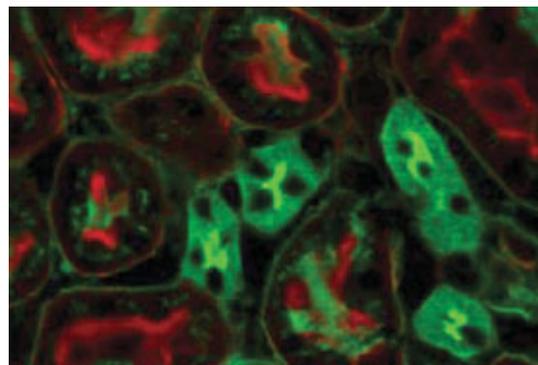
Emission crosstalk

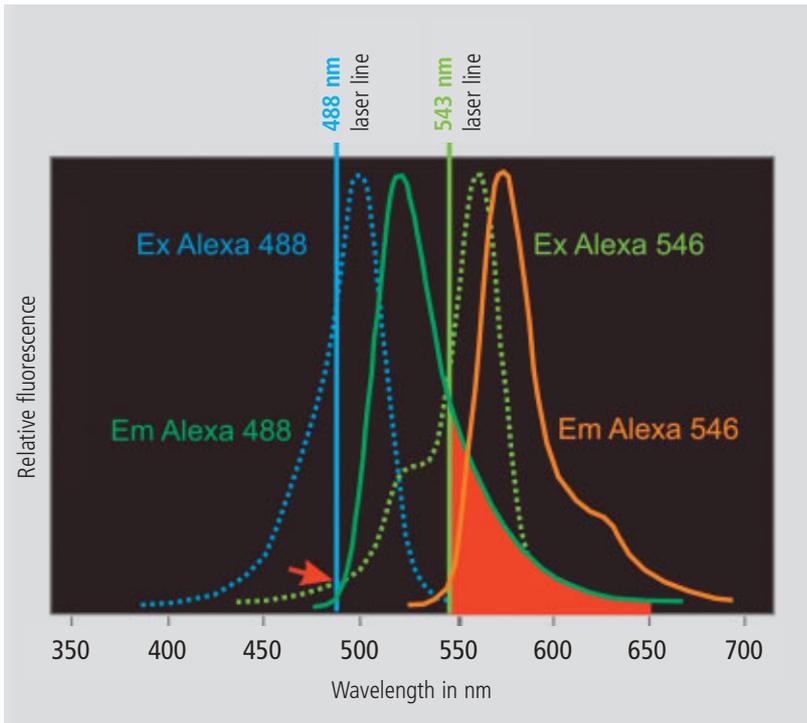
Section through a mouse kidney, double-labeled with Alexa 488 (wheat germ agglutinin) and Alexa 568 (phalloidin). Simultaneous excitation with 488 and 543 nm. The emission of Alexa 488 is detected in both the green (BP 505-530 nm) and red (BP 560-615 nm) channels. Because of this bleed-through, the areas 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

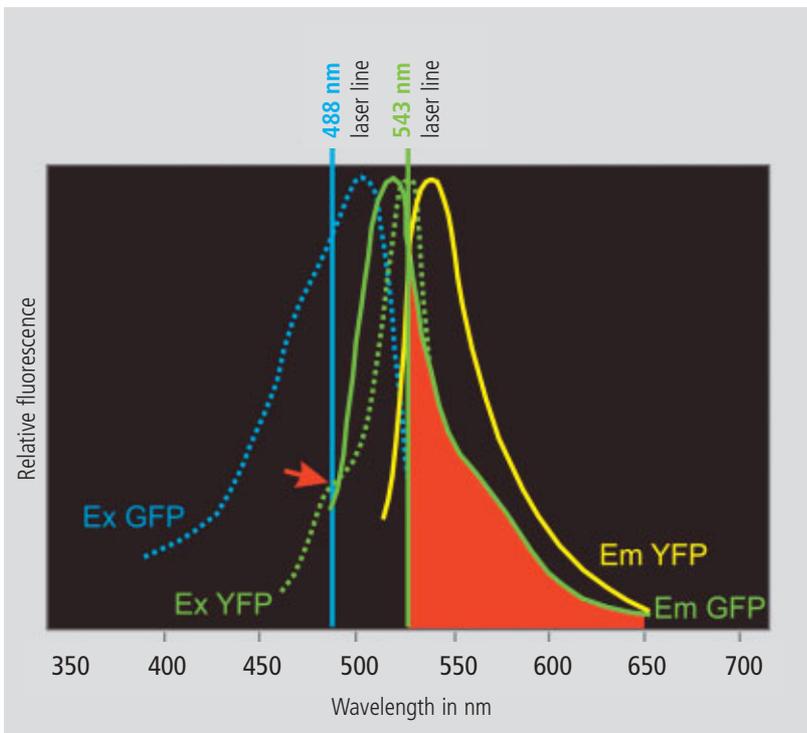
The excitation efficiency of the two fluorochromes is determined by the point of intersection between the laser line used and the excitation spectrum (dotted line).

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.ncicrf.gov/ccr/flowcore/welcome.htm>; modified

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).

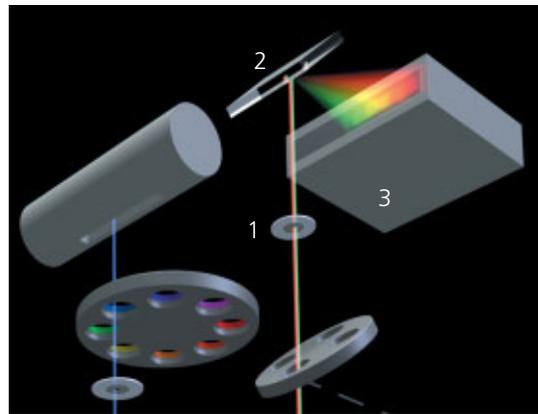
The LSM 510 META is a system for the fast acquisition of images of high spectral resolution. The hardware enabling this functionality consists of a spectrally dispersive element, a photomultiplier (PMT) with 32 parallel detection channels (META Detector), and special electronic circuitry for detector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

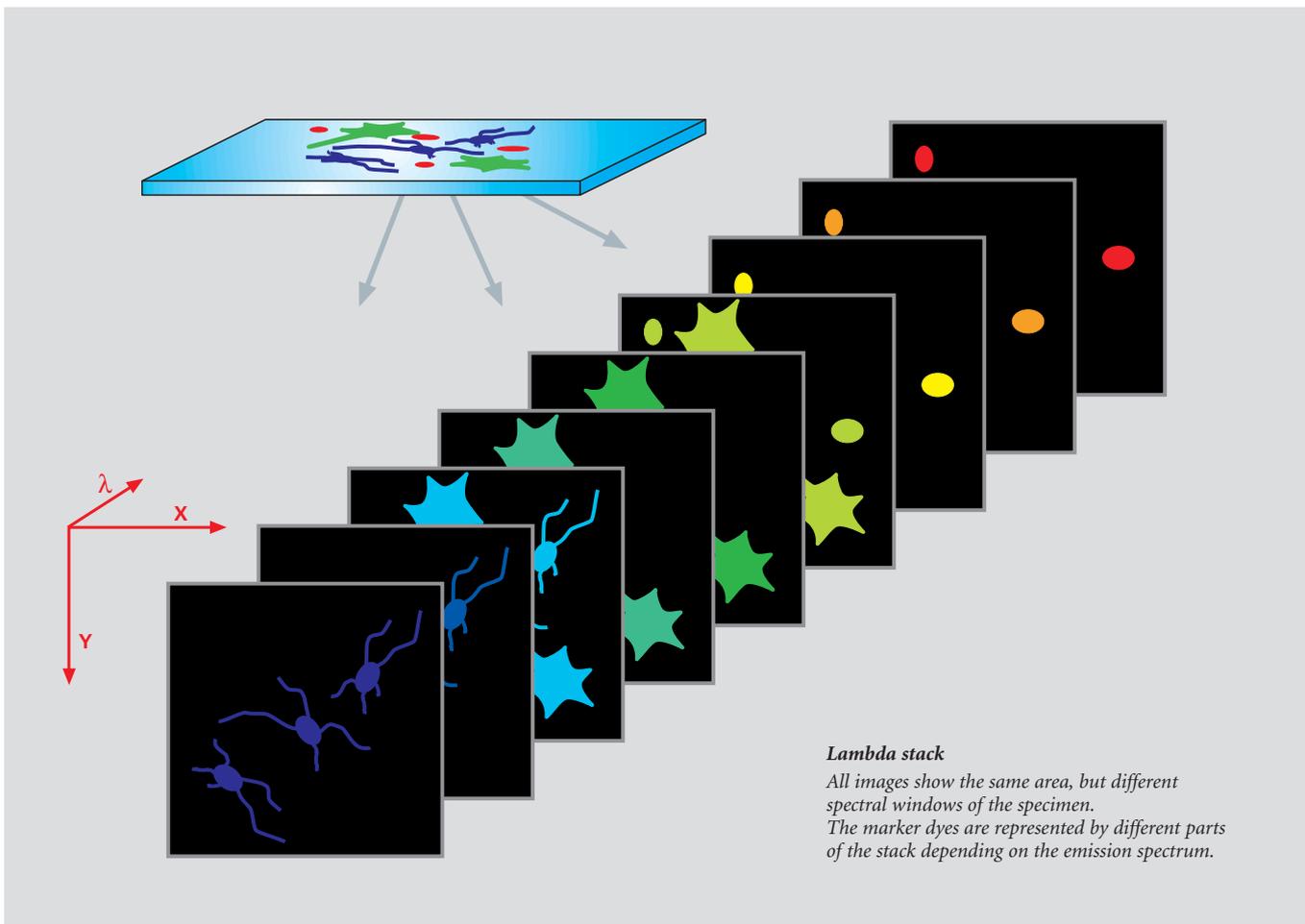
Part of the beam path in the LSM 510 META scanning head

- 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



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.

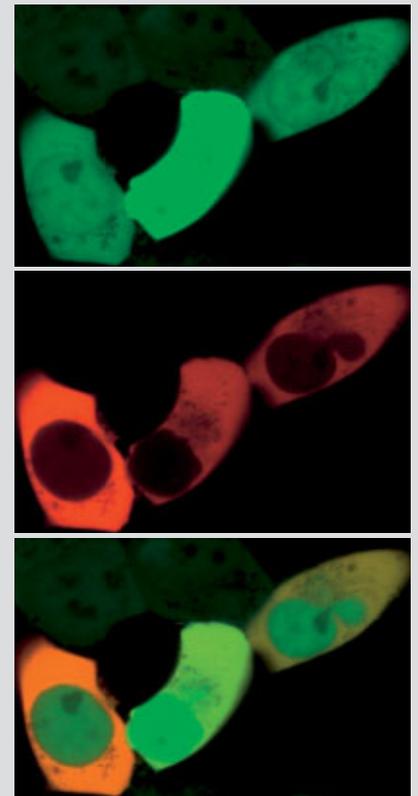
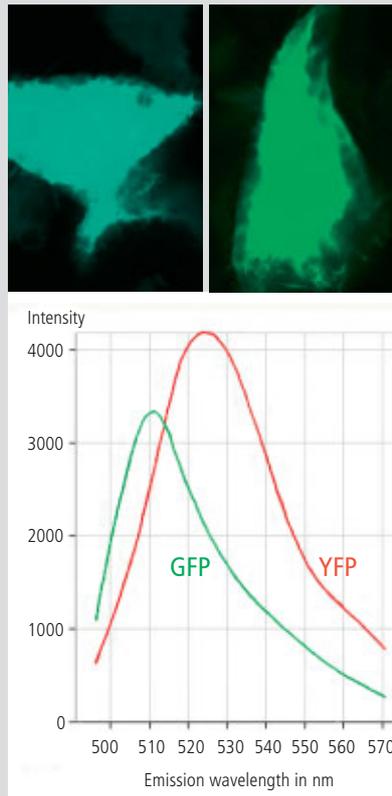
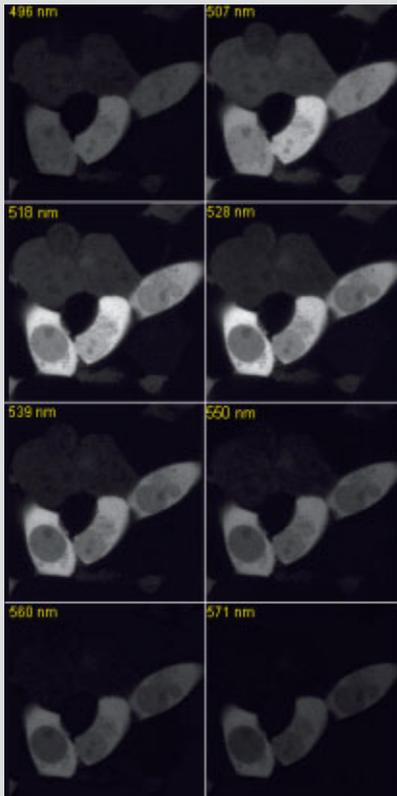
Linear Unmixing

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 $S(\lambda)_{\text{dye A, B and C}}$ overlap, the cumulative spectrum $\Sigma S(\lambda)$ measured can be expressed as

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

By means of known reference spectra $S(\lambda)_{\text{dye A, B and C}}$, 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.



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

Spectral Imaging

Channel Unmixing

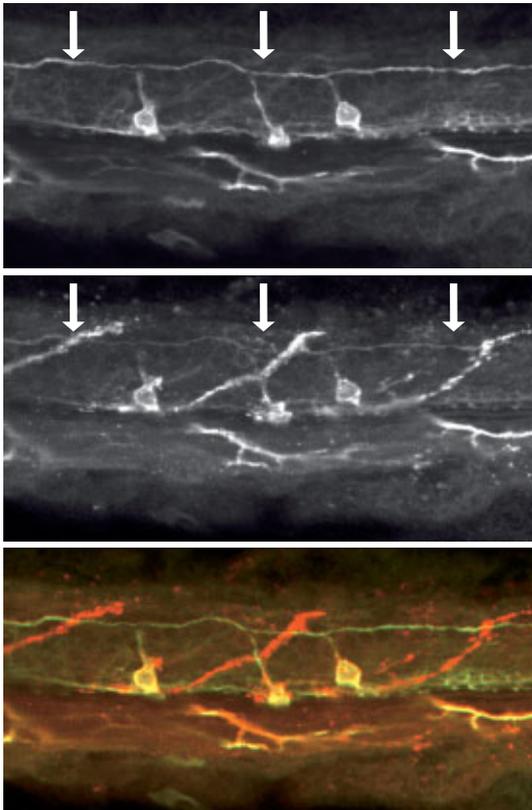
If the emission spectra of fluorescent markers overlap only slightly, the signals can be separated with the *Channel Unmixing* function.

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.

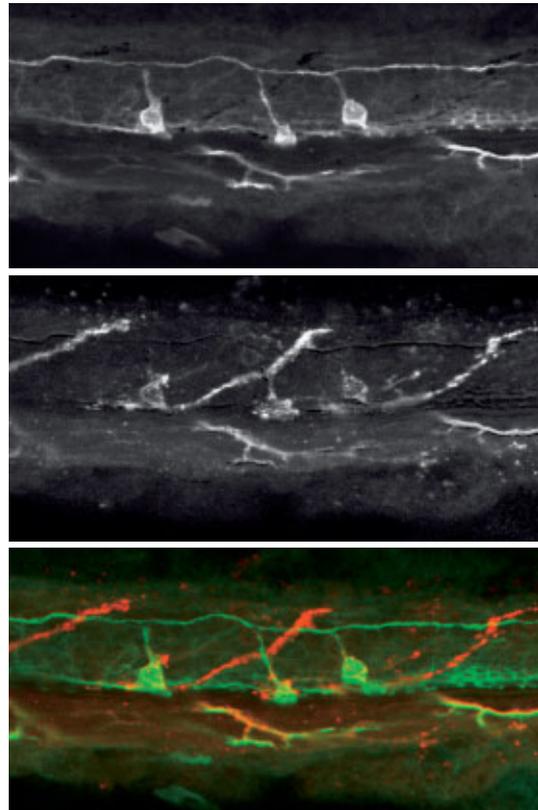
Double labeling of the nervous system of a zebra fish embryo

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

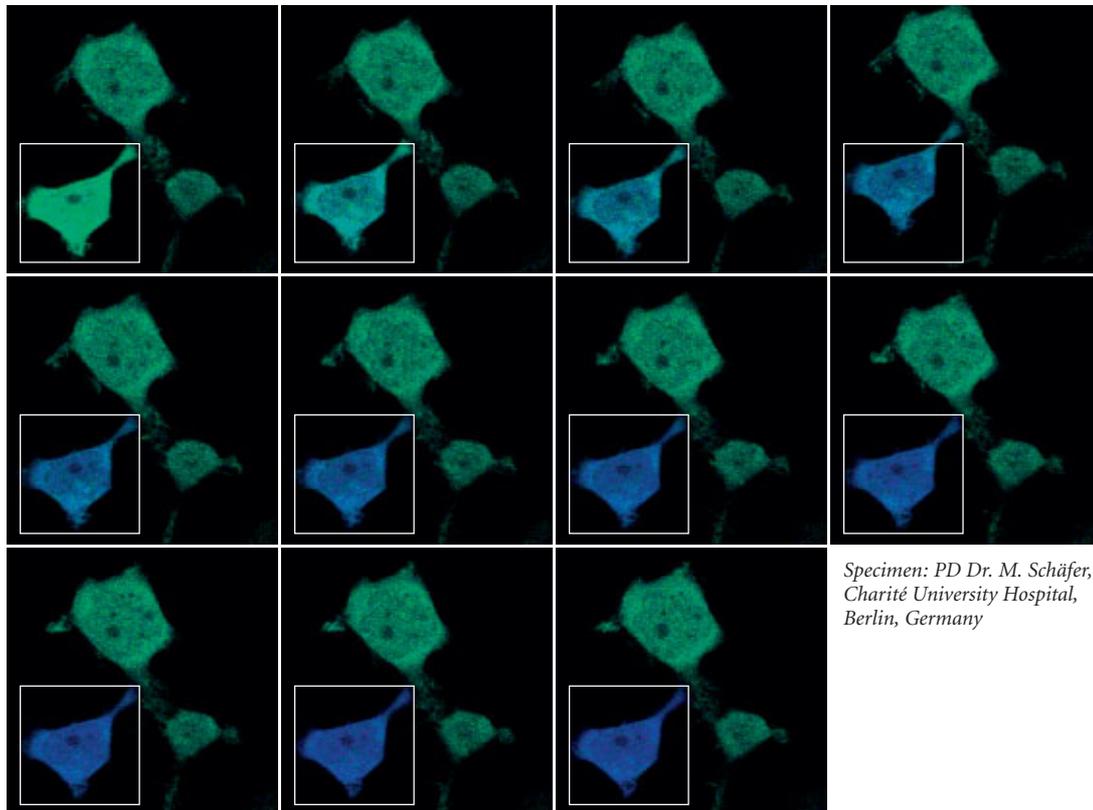
Expression of a FRET-positive protein construct (CFP linker citrin) in HEK 293 cells.

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.

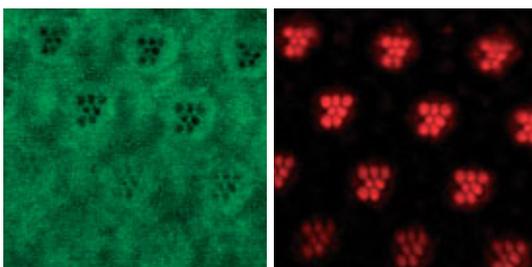
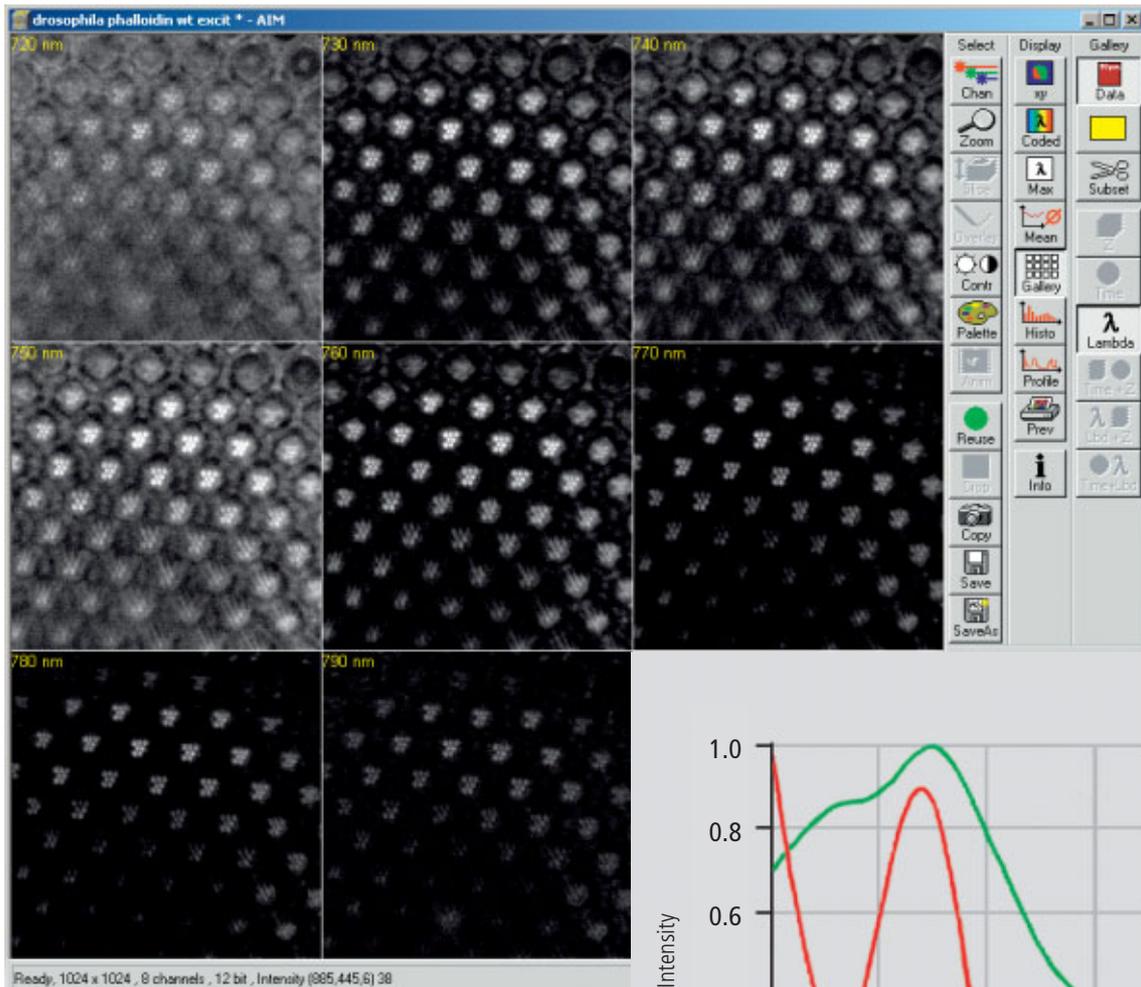
In *Excitation Fingerprinting*, this property is used for the acquisition of excitation lambda stacks. For that purpose, the multiphoton laser is controlled by the LSM software to shift its excitation wavelength by a defined interval before every new image. The image stacks thus recorded can be used 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.



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

Courses

Laser scanning microscopy and related techniques

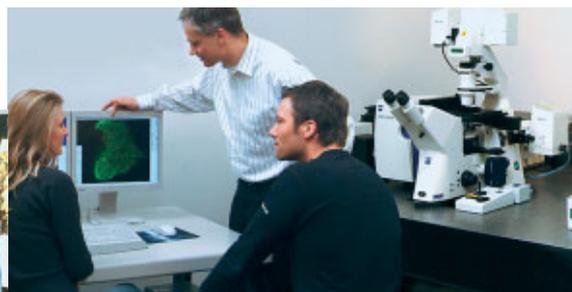
Laser scanning microscopy has become a mainstream technique in biomedical research. Thorough familiarity with its theoretical principles and application know-how is a prerequisite for successful experimentation.

Carl Zeiss offers seminars that teach the fundamental theory and explain biomedical research methods, followed by intensive practical hands-on training in small groups.

The participants in these courses have access to various combinations of microscopes and latest generation LSM systems made by Carl Zeiss.

There is no better way to efficiently acquire new know-how and skills in using up-to-date LSM equipment.

- **Laser scanning microscopy in biomedical applications**
- **Multiple fluorescence labeling and quantitative colocalization**
- **Dynamic investigation of living cells – FRAP, FLIP, FRET, photoactivation and photoconversion**
- **Confocal laser scanning microscopy in materials research & quality inspection – principles and applications**
- **Fluorescence correlation spectroscopy in biomedical research**



For details, see
www.zeiss.de/courses

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.

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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

For further information, please contact:

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www.zeiss.de/lsm

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1 Purpose

This section describes the operation of the Laser Scanning Microscope with the ZEN 2009 software.

When starting up and operating the microscope system, mind the operating instruction manuals for the Axio Imager.Z2, Axio Imager.M2, Axio Observer.Z1 and Axio Examiner microscopes:

- M70-2-0020 Axio Imager.Z2 / M2, Operating Manual
- B 40-0111 Axio Observer.Z1, Operating Manual
- M60-2-0003 Axio Examiner, Operating Manual
- M60-2-0007 Axio Scope.A1, Operating Manual

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 / 64-bit Microsoft® WINDOWS VISTA operating system.

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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.

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.

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*.krm
- 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

1.4 Software Operation

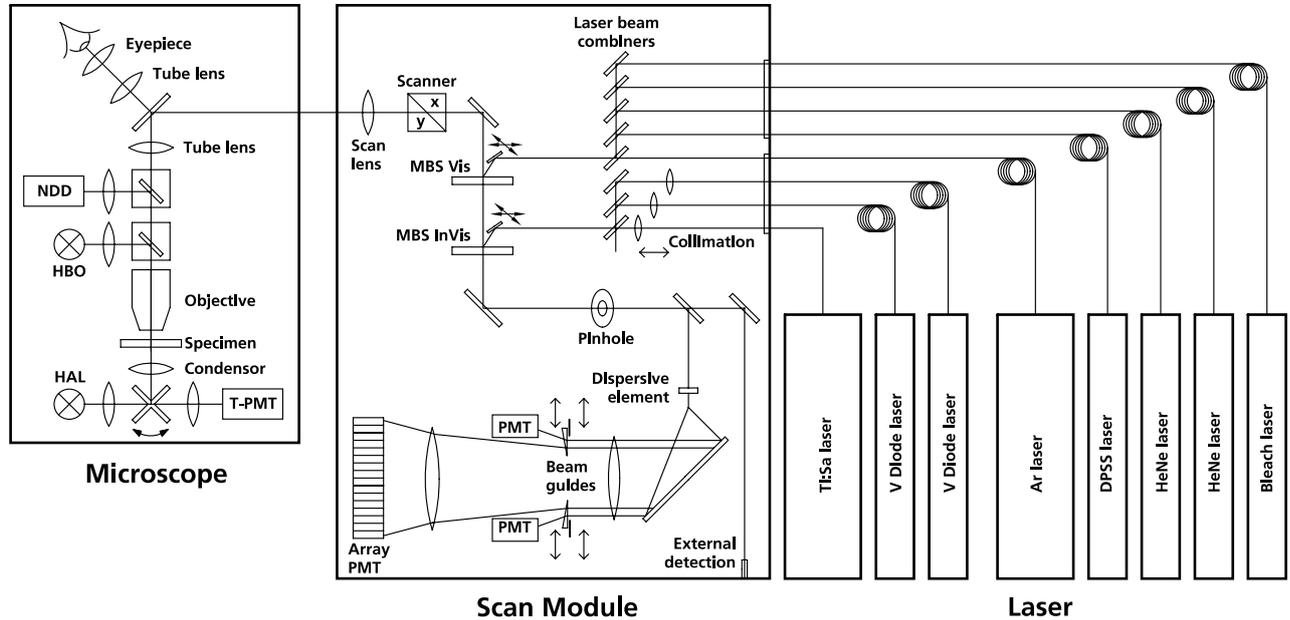
The ZEN software can be operated using the mouse, the PC keyboard, or both.

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

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

2 Hardware Aspects

2.1 Optical Diagram of the LSM 710 (Schematic)



- HAL Halogen Lamp
- HBO Mercury Vapor Short-Arc Lamp
- MBS Main Beam Splitter
- NDD Non-Descanned Detector
- PMT Photomultiplier
- T-PMT Transmission-Photomultiplier

Fig. 2-1 Optical path, schematic (34-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 beam splitter (MBS), which separates the emissions from the excitation light. The fluorescence signals are separated from each other by the beam guides and directed to individual photomultipliers.

2.2 Performance and Features of the LSM 710

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 RP or SP) or upright (Axio Imager.Z2, Axio Imager.M2 or Axio Examiner) 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 and 458 nm and the tunable laser "In Tune" covering the whole range from 488 to 640 nm. The V Laser Module provides wavelengths of 405 and 440 nm (440 nm laser not in combination with In Tune). A Ti:Sa Laser provides pulsed laser light from 680 to 1080 nm for Multiphoton imaging (NLO). Coupling of the laser light (except the Multiphoton laser) is through polarization-preserving single-mode optical fibers. One variable beam collimator each for the V 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 2,3 or 34 internal image acquisition channels, usable for fluorescence, and an additional transmitted-light channel are ideal for the investigation of multiple fluorescence specimens. The diameter of the pinhole and the XY positions can be optimized, and the desired emission can be selected. This adjustment also includes positioning along Z. For the simultaneous registration of multiple fluorescence signals, identical optical sections can be obtained in each confocal channel.

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 contrast image.

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 11 × 11 mm².

The scanning field size can be freely selected between 4 × 1 and 6144 × 6144 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.

2.3 Microscope Equipment of the LSM 710 System

The LSM 710 system is equipped either with the inverted Axio Observer.Z1 BP or SP microscope or with the upright Axio Imager.Z2, Axio Imager.M2 or Axio Examiner 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 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.
- 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 software-controlled switching mirror is mounted on the illuminator support. Alternatively, the light is directed to the LSM 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 710 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.

2.4 Computer Hardware and Software

The LSM 710 is controlled via a standard high-end Xeon PC. Linking to the electronic control system is made via Gigabit Ethernet interface. The PC comes with 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 16-bit A/D converters, each of which can generate 65536 brightness levels.

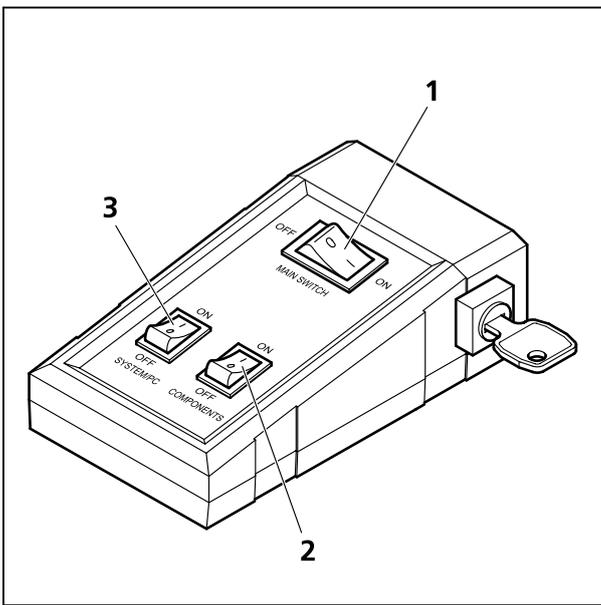
The software provides an enormously wide range of image processing functions, including all standard 2D/3D (stereo, projection) functions identical to sophisticated 3D reconstruction capabilities (surface and alpha rendering), digital processing of voxels and 3D measurement functions (surface areas, volumes).

3 Startup and Shutdown of the System

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 witch (Fig. 3-1).

Refer to the operating manual and CHAPTER 7 ANNEX of the printed manual for handling of any Titanium-Sapphire-Laser used for Multiphoton (NLO) Microscopy.



- 1 Main switch ON/OFF
- 2 COMPONENTS switch ON/OFF
- 3 SYSTEM PC switch ON/OFF

Fig. 3-1 Power remote switch

- To start the system switch the main switch (Fig. 3-1/1) to **ON**.
- When set to **ON** the power remote switch labeled **System/PC** (Fig. 3-1/3) 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 (Fig. 3-1/2) 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 VISTA operating system desktop appears on the screen, showing a number of icons.



Maintenance Tool icon

With a calibration objective and correct system configuration, the maintenance tool allows convenient self adjustment of the LSM 710 system. The optical beampath, relative pinhole position and scanner adjustment can be set and checked automatically on the LSM 710.



Stand Select icon

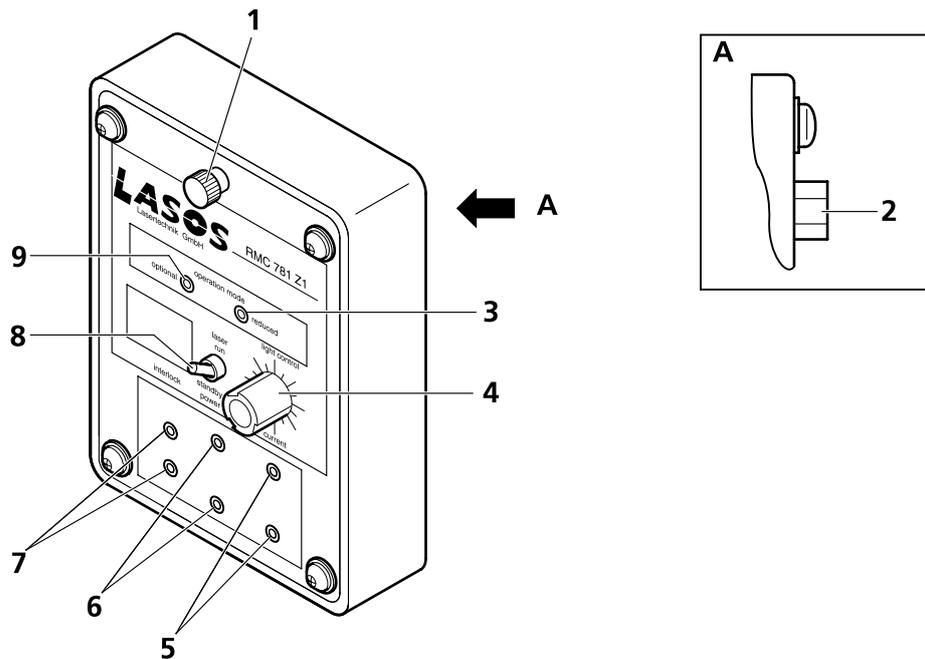
The **Stand Select** tool permits a new or updated database to be assigned to the LSM software program. This function should preferably be performed by authorized service personnel. See printed manual CHAPTER 6 TOOLS, ADDITIONAL SOFTWARE.



ZEN icon

Start ZEN software for operating the LSM laser scanning microscopes.

3.1.1 Handling of the Argon Laser



- | | |
|--|--|
| <ul style="list-style-type: none"> 1 Holding-down bolt 2 Connection to the main power supply unit 3 Red LED (output power too high - strong reduction of lifetime) 4 Light control potentiometer (laser power) 5 Current measuring connection | <ul style="list-style-type: none"> 6 Power measuring connection 7 Connection for external interlock loop 8 Switch LASER RUN/STANDBY 9 Green LED (Laser power stabilized; optimum power range regarding lifetime) |
|--|--|

Fig. 3-2 AR laser control

- First turn the key switch at the power supply of the argon laser from Standby to On. The laser is then held in idle mode for about 5 minutes independent of the run-idle-switch.
- Flip the run-idle-switch from IDLE to RUN (run-idle-switch button (Fig. 3-2/8) up).
- After switching to RUN mode the laser power increases up to full power within 50 s (provided the 5 minutes idle time at start up are already over). The time it takes to get to the full power mode is also dependent on the setting of the potentiometer, lower power levels require less time for ramp up.
- The green LED lights up if the set power is stabilized and the laser is ready to use (Fig. 3-2/9).
- The tube current can be set with the light control potentiometer (Fig. 3-2/4). The adjustment range is from standby (left stop) to 7 A (green LED (Fig. 3-2/9)); tube currents greater 7 A will show a red LED (Fig. 3-2/3).
- Warm up time after cold start to keep the specification for long-term stability is around 1 h.

 The red LED indicates the power range which is critical for the lifetime of the laser. An operation in this power range is possible for a brief period, but not permanently.

 Caution: Even in idle mode laser radiation is emitted.

- The 514 nm laser line becomes available at a tube current of 5 A. It is recommended to use this line at 6 A. Tube currents higher than 6 A will decrease the lifetime (red LED)!
- The tube current can be monitored via the PIN 4 (Fig. 3-2/5) with a voltmeter. The output can be measured in mV/A; means 6 A will be shown as 0.6 V.
- If the laser is not active over a longer time (e.g. 1 h), it should be set to standby. This protects the laser and increases the lifetime. It is better, to have the laser for a longer time in standby mode, than to switch it on and off several times per day.

 The specified lifetime applies for a nominal power of 25 mW (or 40 mW) for all lines, with an operating regime of < 12 h/day. The required tube current might increase over time.

3.1.2 Starting ZEN

The ZEN software is started by double clicking the ZEN icon. The login panel (Fig. 3-3/a) appears on top of the ZEN Main Application window. 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. 3-3/b).

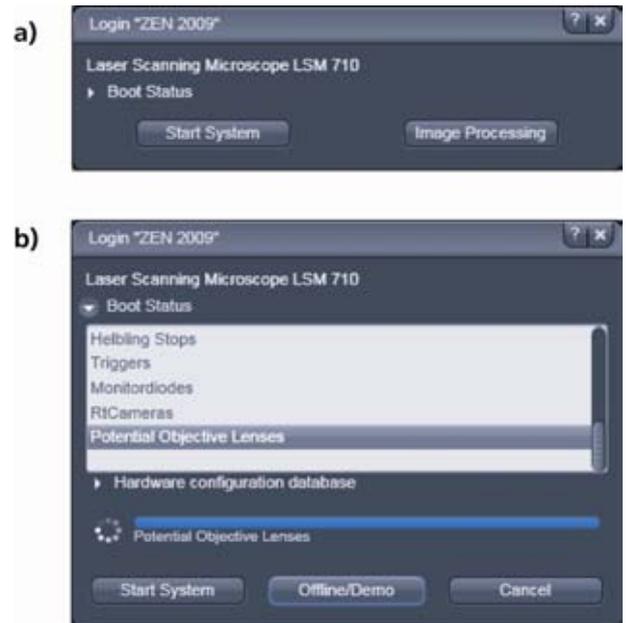


Fig. 3-3 Starting the ZEN software

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 **Maintain Tab: LSM Options**). The lasers will then automatically be switched off when you exit the LSM program.

3.2.1 Exiting ZEN Software

- Make sure you have saved all your image data.
- Switch off all the lasers which might still be running.
- Close the ZEN software window. This terminates the ZEN software.
- Shut down WINDOWS VISTA
- About 20 seconds after WINDOWS VISTA is shut down your computer turns off.

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 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 710 microscope system, including the computer, off power.

4 Introduction to the Software Application Layout

4.1 Overview on the Screen Layout

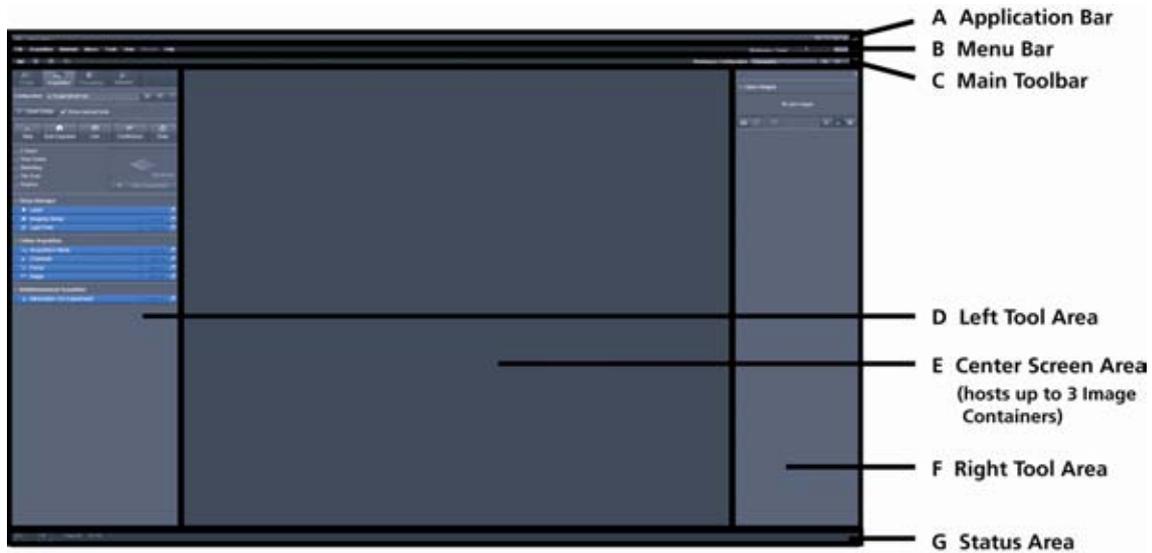


Fig. 4-1 ZEN Main Application window after Startup with empty image container

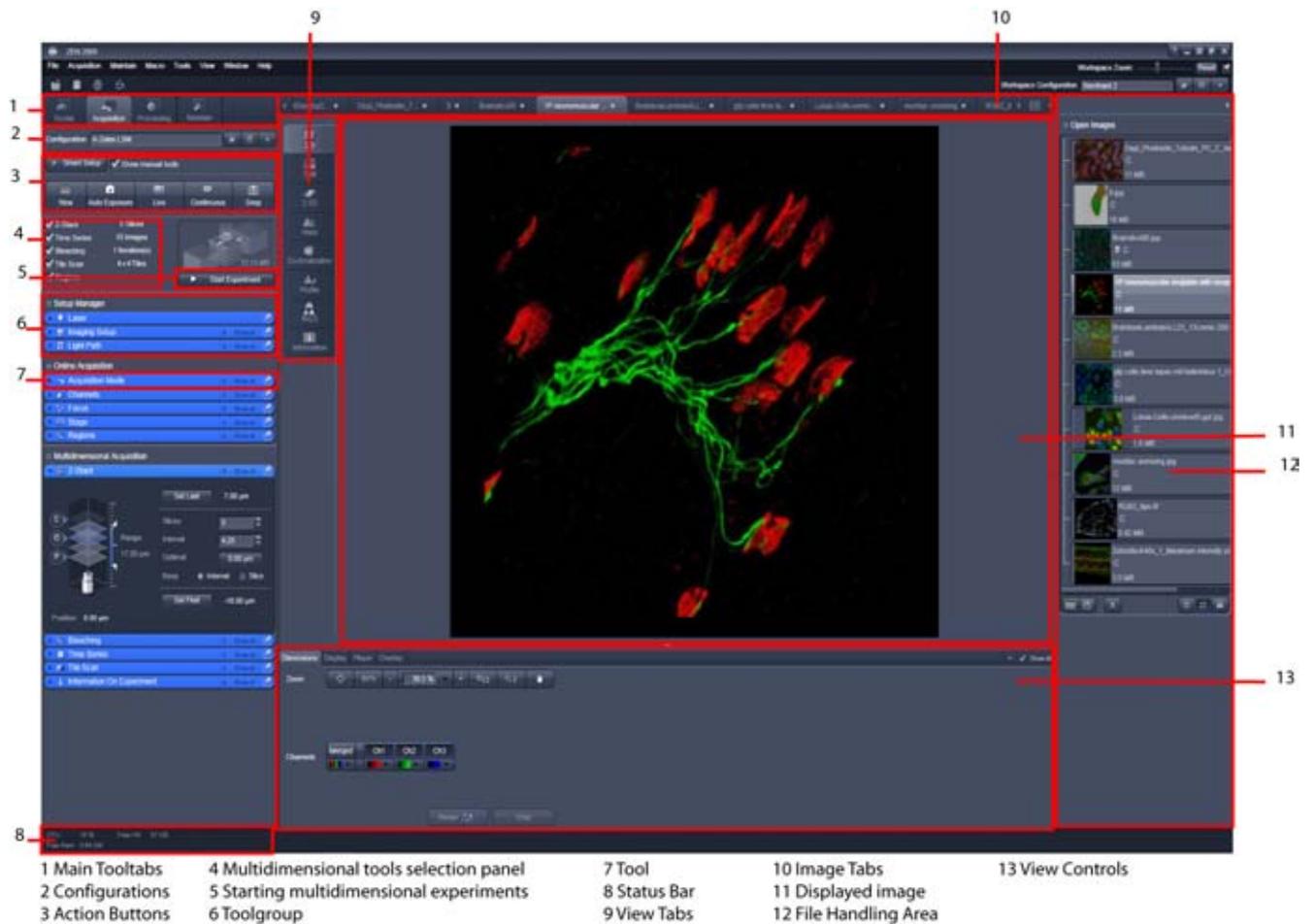


Fig. 4-2 ZEN Main Application window after Startup with several images loaded

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. 4-1/D) the user finds the tools for image acquisition, image processing and system maintenance, easily accessible via 4 **Main** tabs (Fig. 4-2/1). All functions needed to control the microscope can be found on the **Ocular** tab, to acquire images use the **Acquisition** tools (Fig. 4-2/3 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. 4-1 and Fig. 4-2).

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:

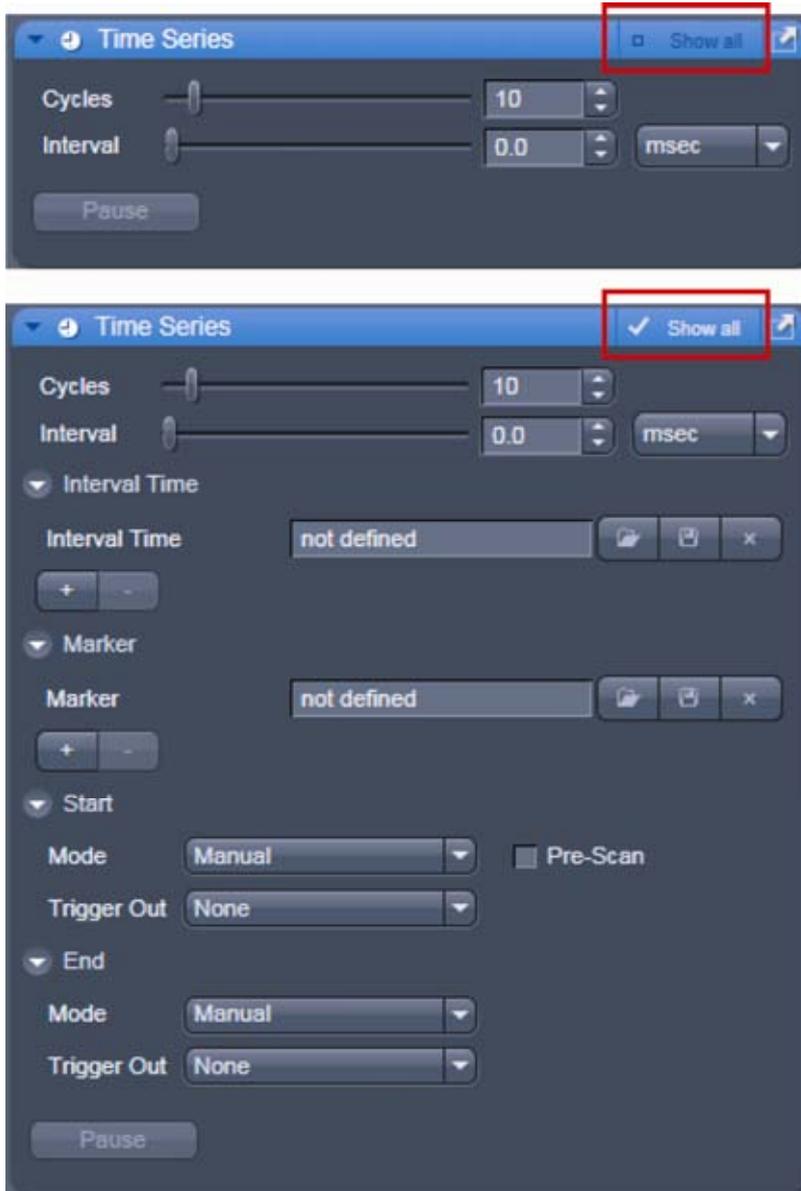


Fig. 4-3 Show all mode

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. 4-3).

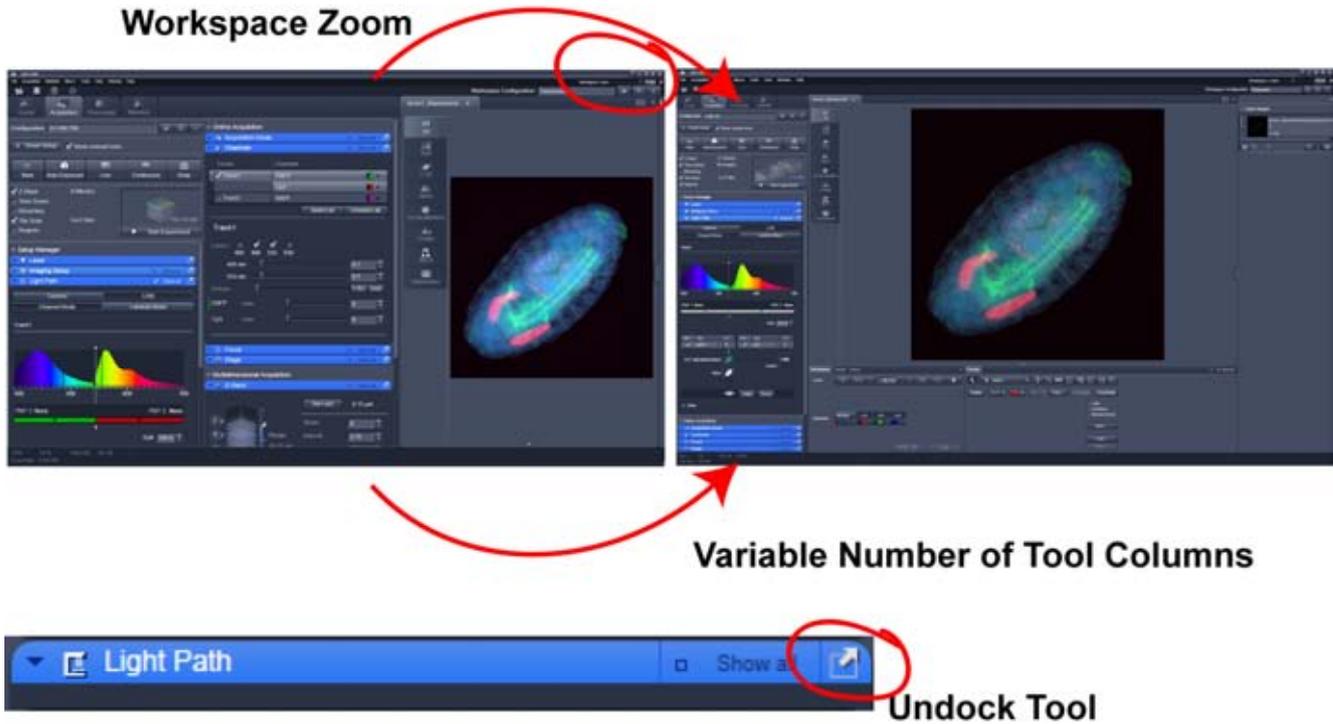
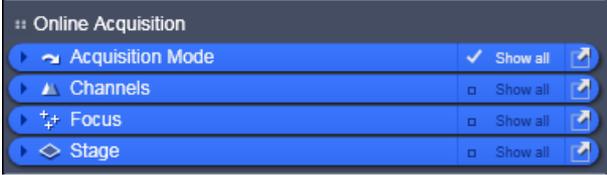
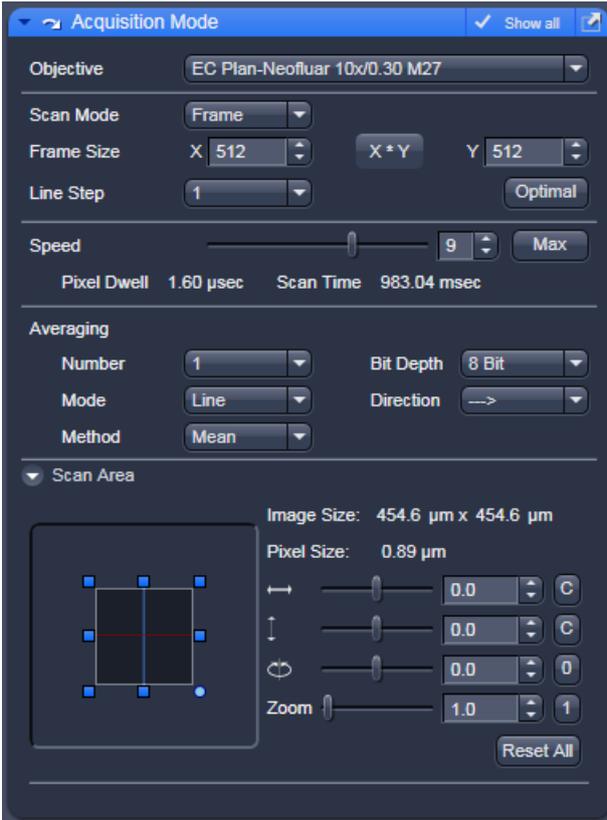
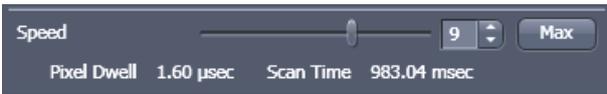
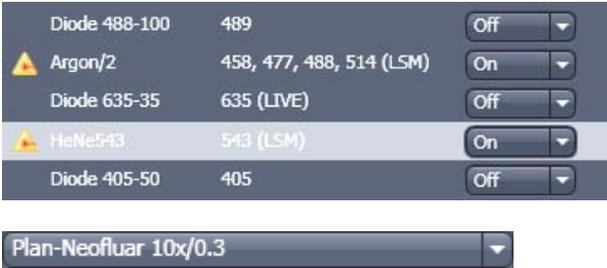


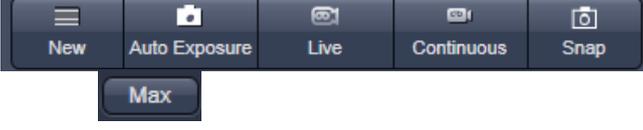
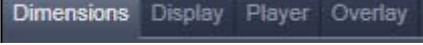
Fig. 4-4 ZEN window Layout configuration

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. 4-4).
- 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. 4-4).
- 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.

4.3 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.

	<p>Slider with input ("spin") box and arrows</p> <ul style="list-style-type: none"> – 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.
	<p>Button</p> <ul style="list-style-type: none"> – Selection / performance of a function via mouse click.
	<p>Tab</p> <ul style="list-style-type: none"> – Selection of functions of a tab via mouse click (e.g.: Track1 or Track2 tab).
	<p>Load – Save - Delete</p> <ul style="list-style-type: none"> – Buttons to load, save or delete a configuration, overlay set, track setting etc.

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

4.5 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**.

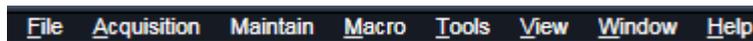


Fig. 4-5 Menu bar, left side

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.



Fig. 4-6 Menu bar, right side

Clicking on  moves all undocked tools back to the **Left Tool Area** (see also section **Tool Groups and Tools**).

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
Open Opens an existing document in the active image container	Auto Exposure Starts the Find procedure. This balances Gain settings.	Camera Opens the Camera Color Adjustment tool	Visual Macro Editor Opens the Visual Macro Editor	VSTA Macros	Small Thumbnail View Displays the image tabs with small thumbnails	Close all Closes all windows, a separate save dialog window will be displayed if images have not been saved
Save Saves the active document	Find Focus Starts the Autofocus. A Line / Z Scan is performed and the focus position is determined upon contrast values.	Hardware Administrator			Large Thumbnail View Displays the image tabs with large thumbnails	
Save As Saves the selected document using various image formats	Live Starts the fast scanning procedure, scans with lower resolution and higher speed.	Test Grid			1 Container Sets ZEN to work only with one image container	Help Help Opens the ZEN help
Export Exports the currently active document using various file formats	Continuous Scans an image continuously with the currently active scanning parameters.				2 Containers Sets ZEN to work with two image containers	About Opens a panel with information about the ZEN release
New File browser Opens a document containing a ZEN File Browser.	Snap Scans one single image.				3 Containers Sets ZEN to work with three image containers	
Login Opens the Login ZEN dialog to change the software operation mode.	Start Experiment Starts a multidimensional experiment (Z, T, ...).				Automatically layout container The size of the containers is automatically adjusted depending on the screen space available	
Spectra Database Opens the spectra database	Stop Stops the scanning immediately				Shared view Controls Shares the view controls below the images between containers	
Exit Closes the ZEN Software					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	

4.6 Main Toolbar

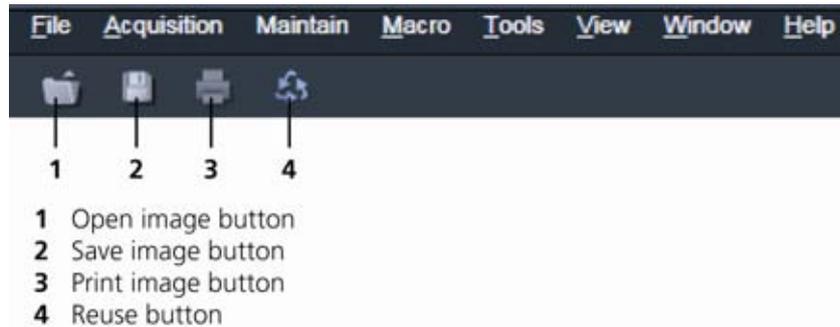


Fig. 4-7 Main toolbar, left side

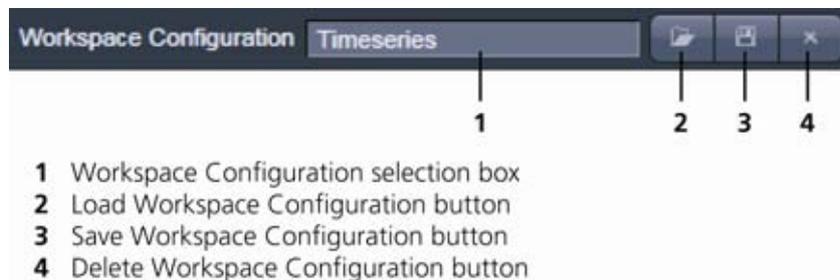
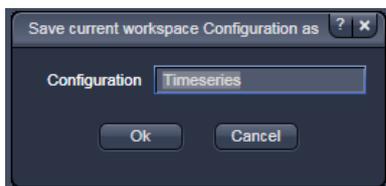


Fig. 4-8 Main toolbar, right side

Workspace Configuration

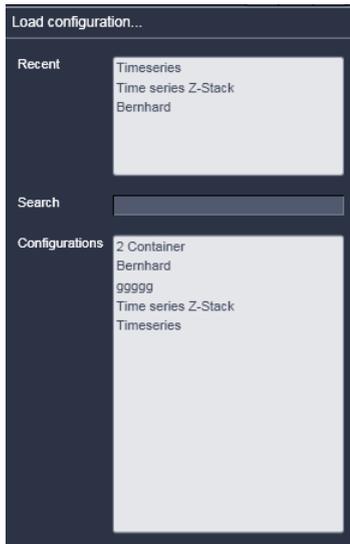
This function allows loading, saving or deleting a workspace configuration. These workspace configurations are saved settings that allow to restore a pre-defined layout of the whole ZEN application window, including status, size and position of tools and windows, workspace zoom, number of tool columns.

- To save a workspace configuration, click on the  button, the following dialog will appear:



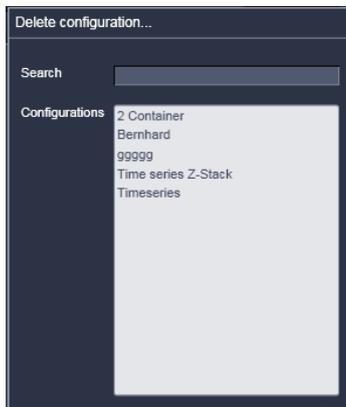
The name of the workspace configuration can be entered. Clicking **OK** saves the configuration.

- To load a workspace configuration, click on the  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  button. The configuration to be deleted can be selected from a list.



4.7 Status Bar



Fig. 4-9 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 than one process is running then the single process information will be shown in the gallery.



Fig. 4-10 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.



4.8 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

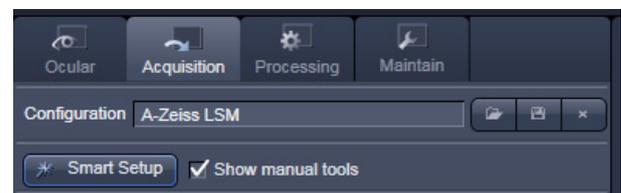
Groups special tools, e.g. **Setup Manager** or **Online Acquisition**.

Setup Manager

The tools of this tool group (Laser, Imaging Setup and light path) are only visible if the check box **Show manual tools** is checked. Those tools are necessary if the system should be operated in full manual mode. The use of the automated functions of ZEN like smart setup include the functions of those tools.

 The tools **Laser**, **Imaging Setup** and **Light Path** are not displayed by default in the software. They can be shown if the check box **Show manual tools** is checked.

Fig. 4-11 Left tool area



Online Acquisition

Tools continuously needed for all acquisition

Multidimensional Acquisition:

Tools for all multidimensional imaging

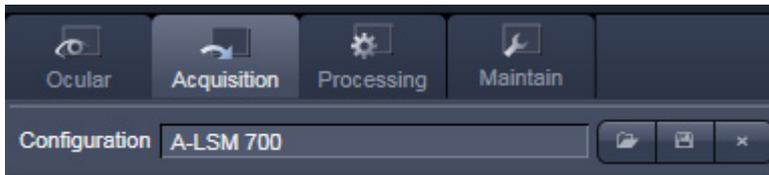
The tools of this tool group will only appear if selected in the selection panel below the start buttons. Once selected by the check box, the tool will appear in the multidimensional acquisition tool group. The tool is then active and will be used after hitting the **Start Experiment** button. The experiment type is represented above the button by a graphic.



Tools / tools window

Used for setting the microscope and software functions.

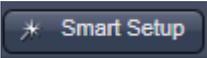
Imaging Configuration



This function allows selecting an imaging configuration. The configuration can be chosen in a list box. Clicking on a configuration loads the selection.



Smart Setup

- Clicking the **Smart Setup** button  will open the **Smart setup** dialog (see section **Smart Setup**).

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.

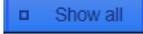
To position the tool window freely on the desktop, use the drag and drop function.

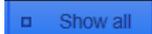
Open a tool window

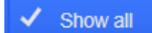
Clicking on the tool header opens or closes the tool pull down window. The state is indicated by a changing icon (, ) left hand in the tool window header.

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  button shows or hides additional functions of the tool.

 Only the basic functions are shown; this functionality is adequate for most applications.

 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.

4.8.2 Context Menu of the Left Tool Area

There are 2 different context menus in the Left Tool Area with different functionalities available.

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. 4-12 Context menu of multidimensional acquisition 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.

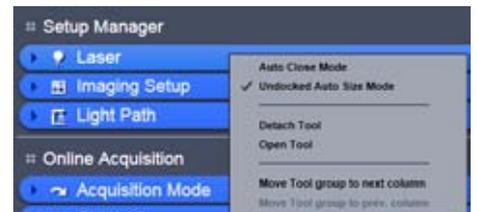


Fig. 4-13 Context menu of the tool groups area

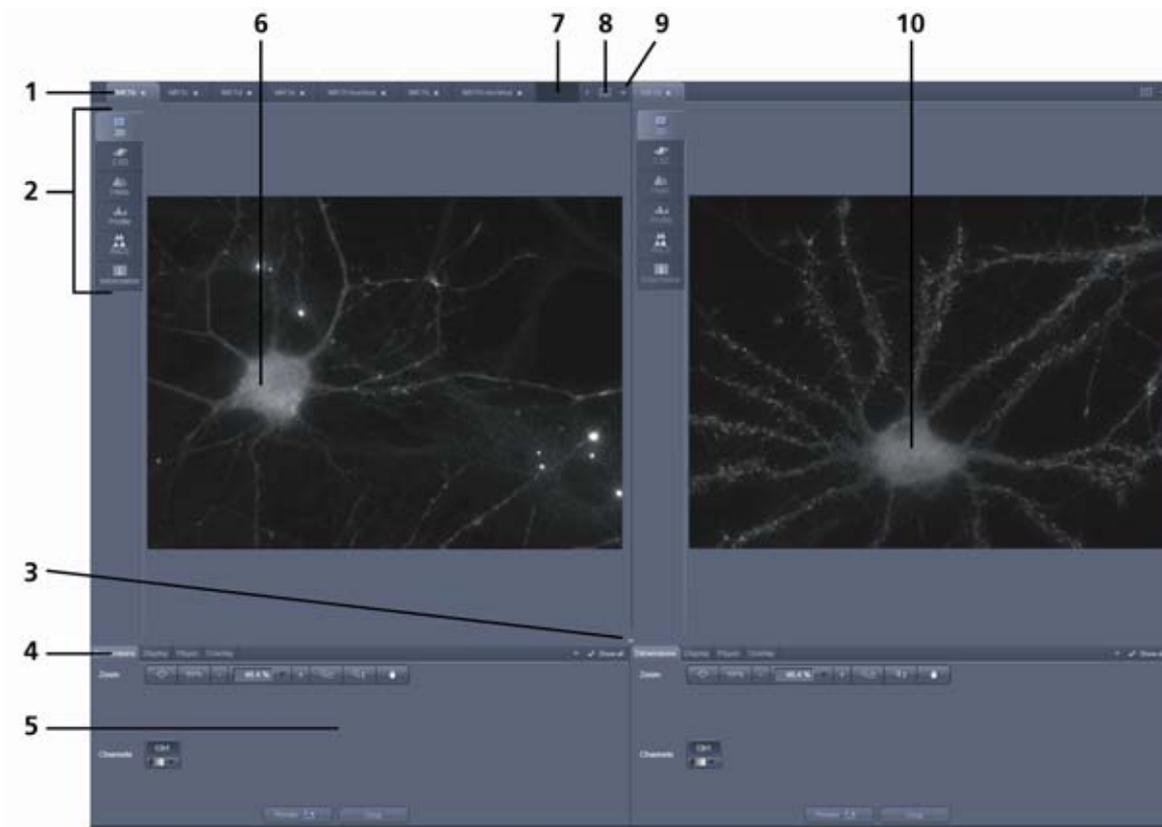
4.9 Center Screen Area

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.

4.9.1 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. 4-14 Center screen area

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 **Context Menu of the Center Screen Area**).

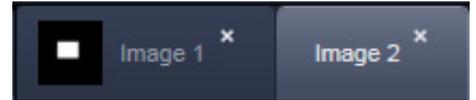


Fig. 4-15 Image tabs

Image view tabs

Used to select one of the various image views (see also sections **2D View** to **Information View**).

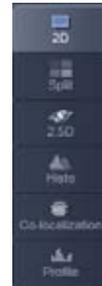


Fig. 4-16 Image view tabs

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 **2D View**).

Image control area

Shows the tools of the selected image display or analysis functions.



Fig. 4-17 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.

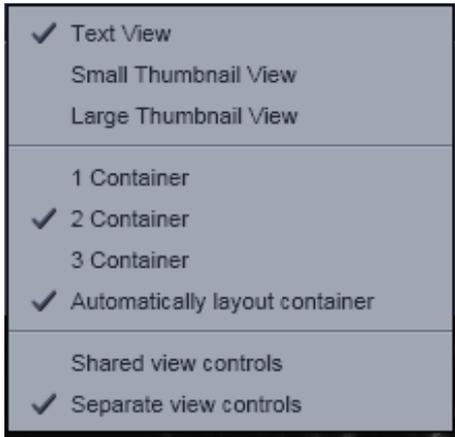


Fig. 4-18 Context menu

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

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

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.

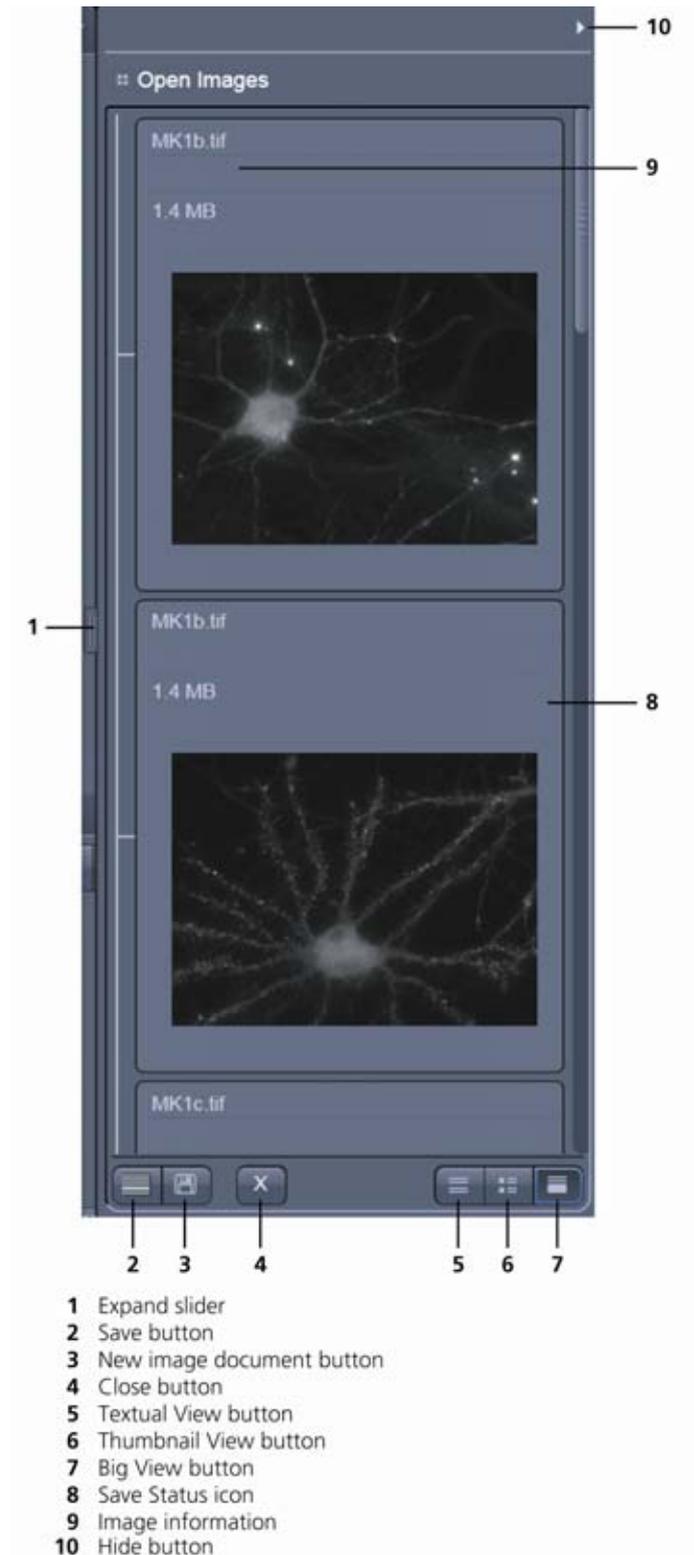
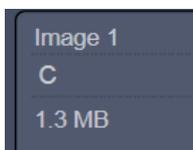


Fig. 4-19 Right tool area

**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.

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.

5.1 Ocular Tab

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.

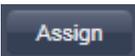
Pressing the **Online** button sets the Ocular tool active – the specimen can be observed and all changes in the ocular tool take effect immediately. When pressing the **Offline** button the system stays in the imaging mode. Changes in the ocular tool do only take effect once the Ocular tab is set in the active mode (**Online**) again. When leaving the Ocular tab the software remembers the state of the **Online / Offline** buttons.

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.

These configurations can be assigned to max. 8 buttons below the configuration panel by clicking

the 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.

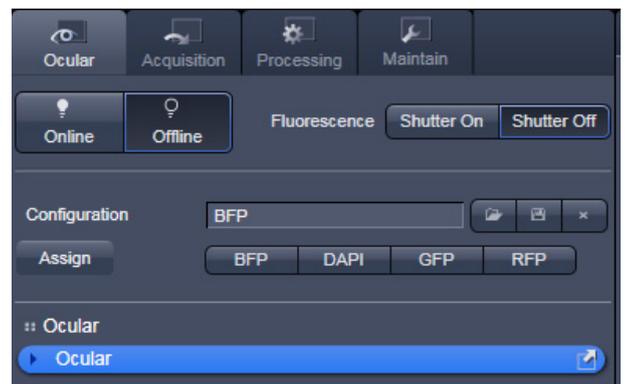


Fig. 5-1 Ocular tab



Fig. 5-2 Configuration panel



Fig. 5-3 Assign panel

5.1.1.1 Controls for Axio Imager.Z2

- 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.

(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. 5-5/1) to switch the beam path of the tube in LSM position.
- Control the brightness of the halogen lamp with the potentiometer (Fig. 5-5/4) 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.

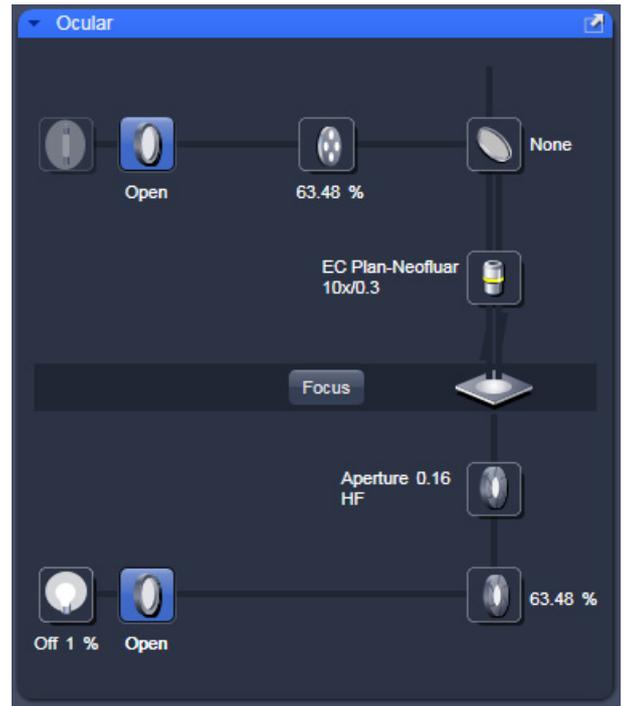


Fig. 5-4 Axio Imager.Z2 controls in the Ocular panel

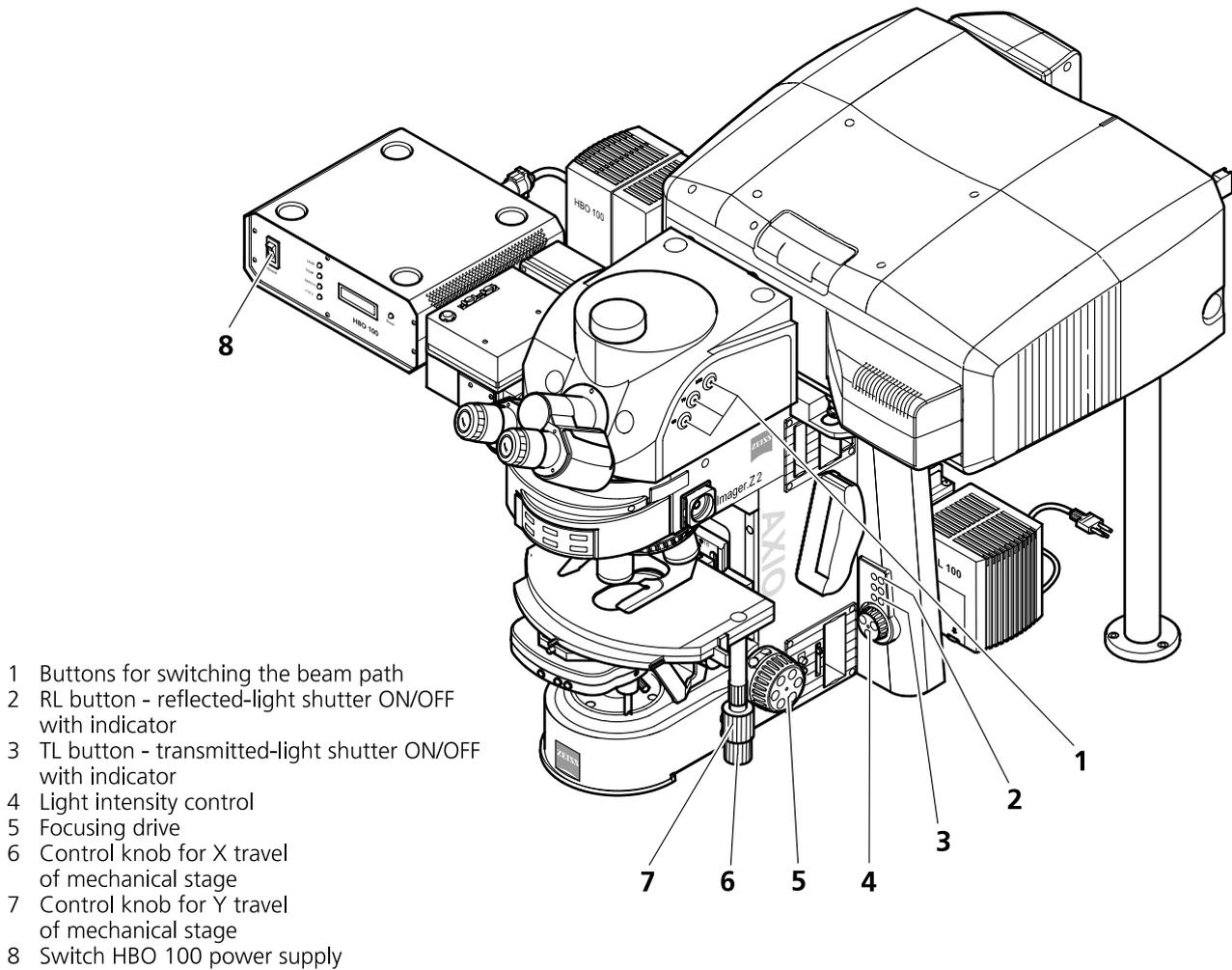


Fig. 5-5 **LSM 710 with Axio Imager.Z2**

- 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. 5-5/5) 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. 5-5/6 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. 5-5/8)
- 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.Z2 is performed in fixed steps.

5.1.1.2 Controls for Axio Observer.Z1

- 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).

(1) Conventional setting of the microscope Axio Observer.Z1

The Recording of microscope settings works as described for the microscope Axio Imager.

For the conventional setting of the Axio Observer.Z1, 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. 5-7/4) 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. 5-7/3 and 2).

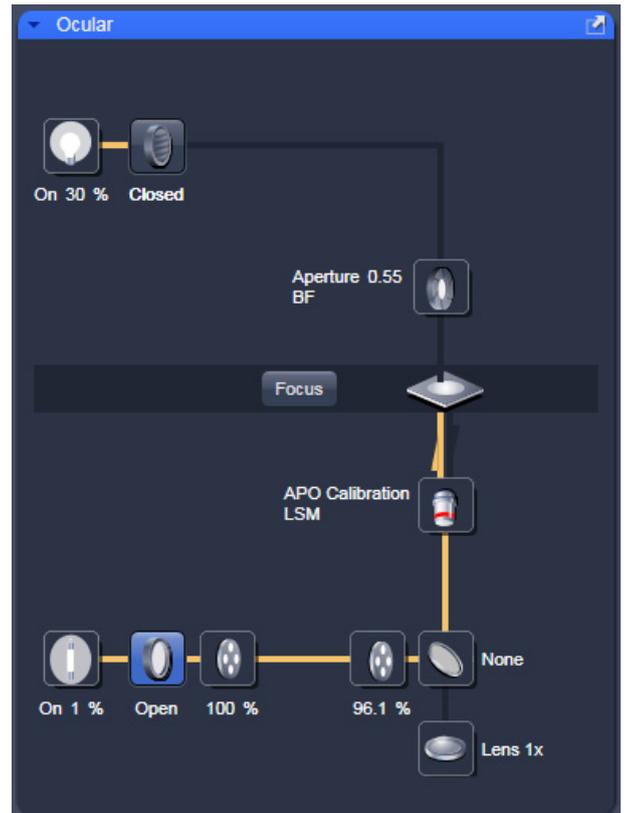


Fig. 5-6 Axio Observer.Z1 controls in the Ocular panel

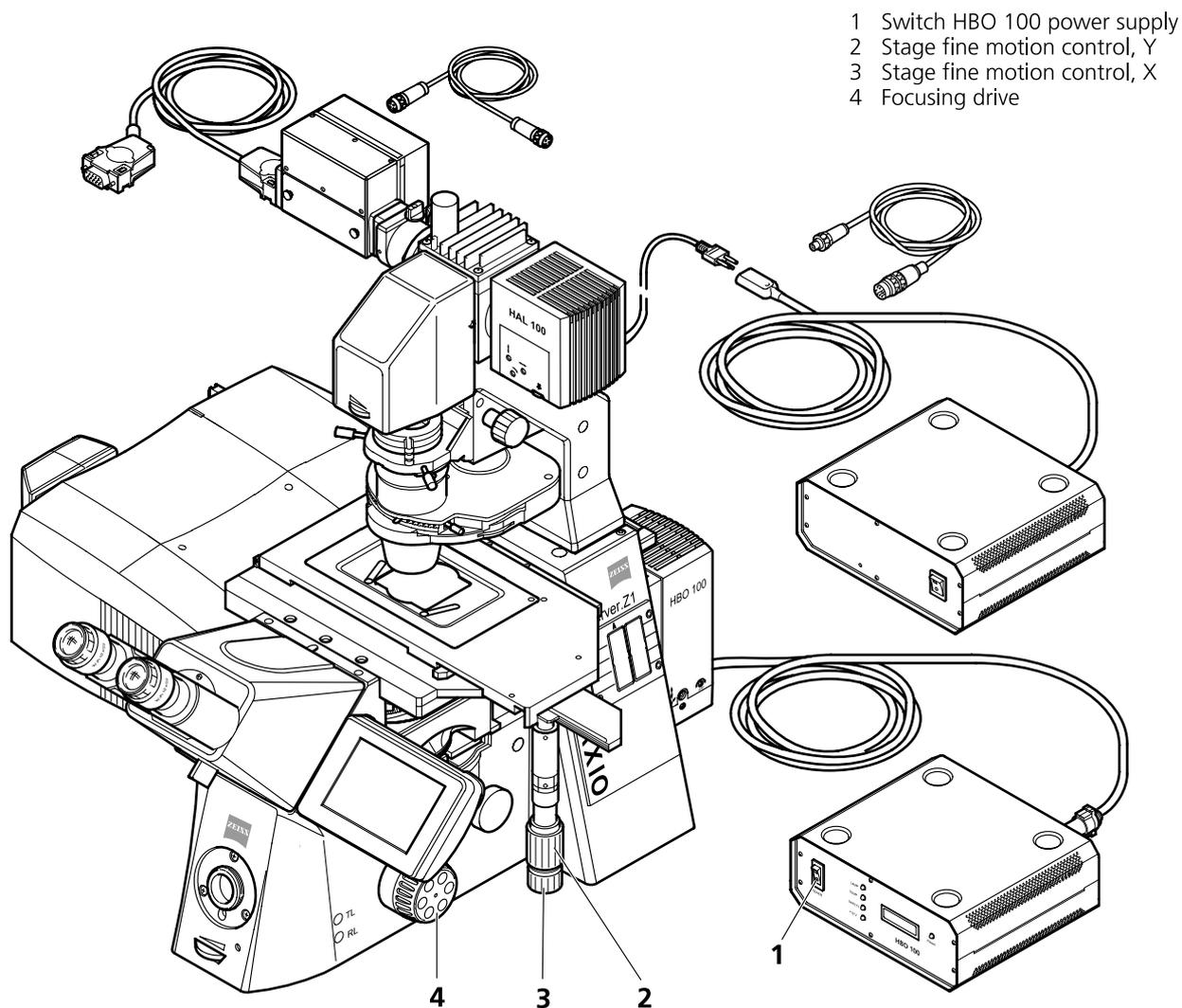


Fig. 5-7 LSM 710 with Axio Observer.Z1

(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. 5-7/1).
- 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.

5.1.1.3 Controls for Axio Examiner

For setting the Axio Examiner, proceed in the same way as with Axio Imager.Z2 and Axio Observer.Z1. Since the objective turret of the Axio Examiner is not motorized the setting of the desired objective has to be done manually.

The objective in use must be indicated in the **Acquisition Mode** tool.

5.1.1.4 Controls for the Axio Scope with Motorized Z-drive

The Axio Scope (only available for LSM 700) has only a motorized z-drive, all other components are not encoded and have to be set in the software manually for correct functionality. Especially, the objectives are changed manually. The used objective must be set in the **Acquisition Mode** tool.

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 CO₂ can be set by using the input boxes.

The measured values are displayed next to the input boxes.

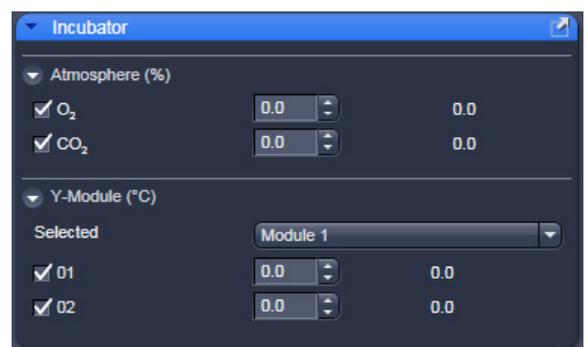


Fig. 5-8 Ocular tool, incubator control

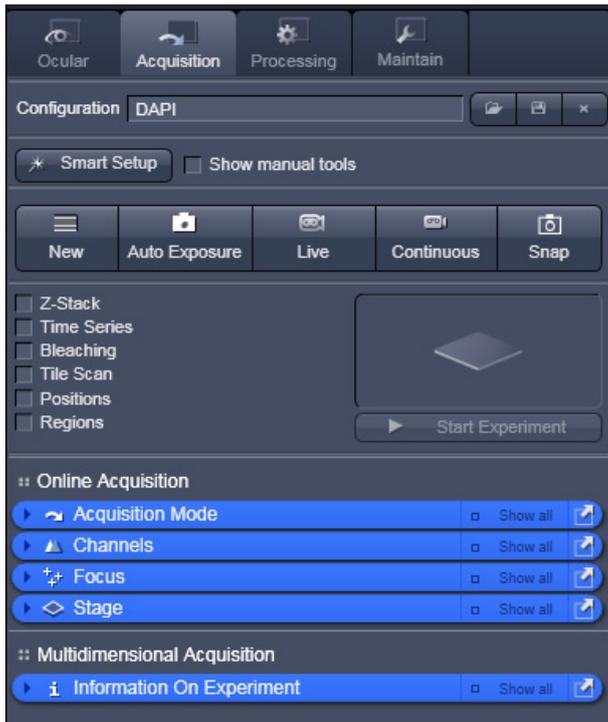


Fig. 5-9 Acquisition tool tab



Fig. 5-10 Main Tool Acquisition tab: Action buttons

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 **Starting ZEN**) this tab is not available. The same is true for the license free LE version of **ZEN**.

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.

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.)



5.2.2 Smart Setup

The tool **Smart Setup**, is an intuitive, user-friendly interface which can be used for almost all standard applications. It configures all the system hardware for a chosen set of dyes.



Fig. 5-11 Smart setup button

Configure your experiment (Fig. 5-13/1):

- Simply choose the dye(s) used from the list dialog, and assign signal display color(s), see also Fig. 5-12.

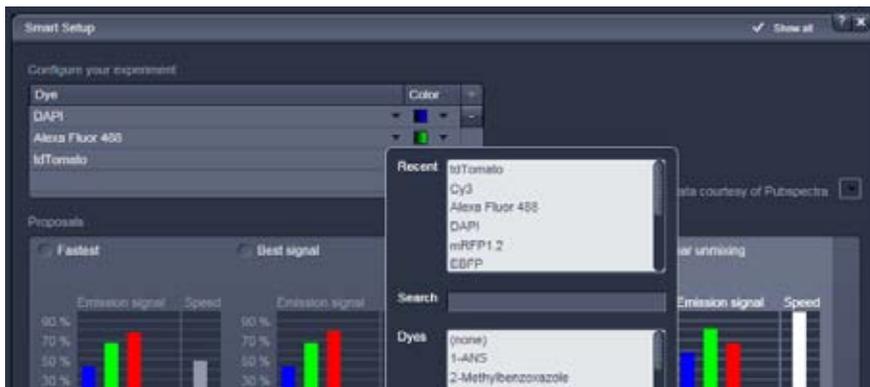
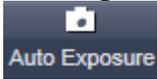


Fig. 5-12 Configure your experiment

Dye (Fig. 5-13/2):

- Once finished with the input, **Smart Setup** suggests alternative considerations: One for **fastest** imaging, one for the **best signal, best compromise** between both speed and best signal and the optimal setup for later **linear unmixing** of the dyes.
- Clicking **Apply**, automatically sets the ideal hardware parameters for the dyes chosen. If the option **Linear Unmixing** is chosen, the system is set in the lambda mode automatically. Clicking the **Auto**



Exposure button will optimize the settings of the Gain (Master) and offset for the given laser power and pinhole size. Further image optimization from this point can be done easily.

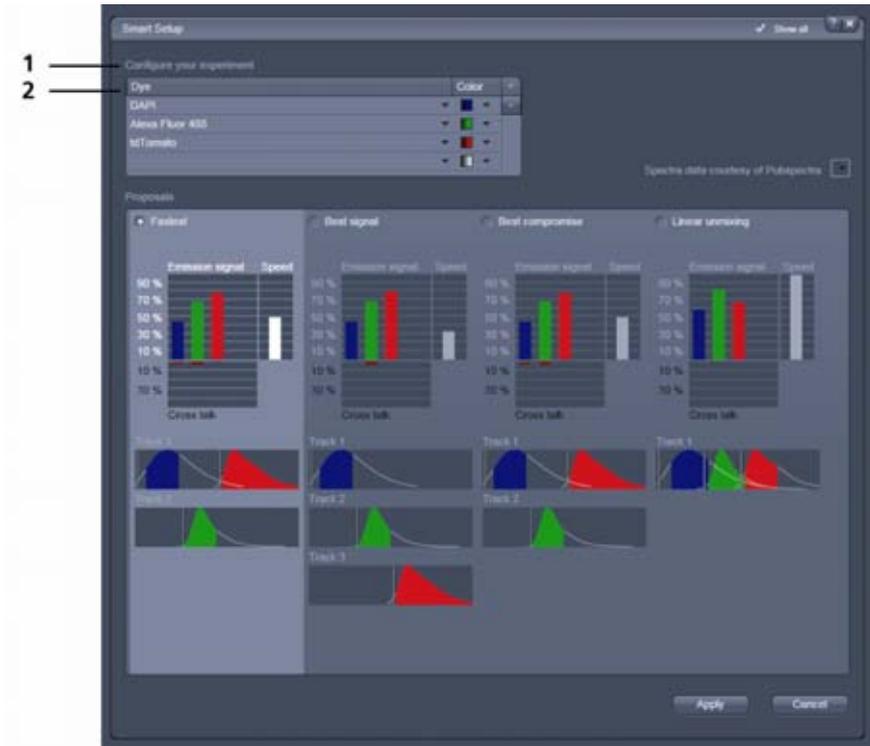


Fig. 5-13 Smart Setup menu

For questions or suggestions send an e-mail to smartsetup@zeiss.de.

5.2.3 Tool Group Setup Manager: Laser Tool

 The tools **Laser**, **Imaging Setup** and **Light Path** are not displayed by default in the software. They can be shown if the check box **Show manual tools** is checked.

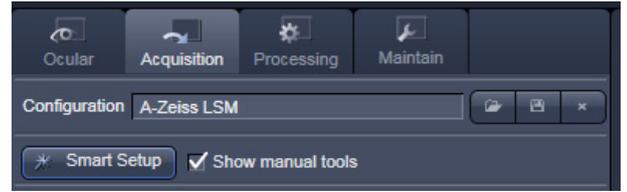


Fig. 5-14 Show manual tools function

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.

The additional laser information panel (available in **Show all** mode) shows the relevant and currently set **Maximum Power**, **Wavelength**, **Status** and **Output [%]** values of the currently highlighted laser (if these values are provided by the laser). Click the arrow next to Laser Properties to open this part of the window. Depending on the type of laser the provided information may vary.

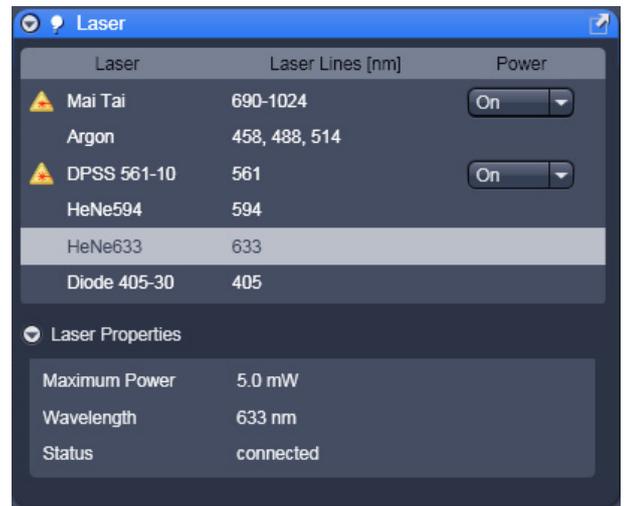


Fig. 5-15 Tool group Setup Manager: Laser tool

 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 power remote switch.



Fig. 5-16 Laser menu with Diode 405-30 activated



Fig. 5-17 Mode pull down menu

Diode lasers

The LSM 710 can be equipped with Diode 440 and / or Diode 405 lasers that can be used in continuous wavelength (cw) mode or in pulsed (ps) mode (Fig. 5-16).

Selection of the mode is available in the Mode pull down menu in the **Laser properties** menu (Fig. 5-17).

You can select between **Continuous Wave** or **Pulsed - 20 MHz, Pulsed - 50 MHz** and **Pulsed - 80 MHz** mode.

The diode lasers are directly modulated. They can be further attenuated by ticking the **ND04** check box, which will place a neutral density filter in front of the lasers

Please note that pulsed modes were specifically designed for Fluorescence Lifetime Microscopy (FLIM) and should be preferentially used for this application. Since laser powers in the pulsed mode are substantially less than in the cw mode, the pulsed mode is not suitable for bleaching experiments. Also signal-to-noise ratios in intensity imaging are less in the pulsed mode compared to cw operation, hence the cw mode should be chosen for high quality imaging.

5.2.4 Tool Group Setup Manager: Imaging Setup Tool



Fig. 5-18 Imaging Setup Tool

 The Tool group **Setup Manager** is only visible when **Show manual** tools is ticked.

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 **Tool Group Setup Manager: Light Path Tool**) and stored as an imaging configuration.

Three different modes are available for imaging. Select the modes depending on the application.

Channel Mode:

This mode uses one or more detectors as single channels with defined imaging parameters. All hardware parameters set to detect one or 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 **sequentially**. 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.

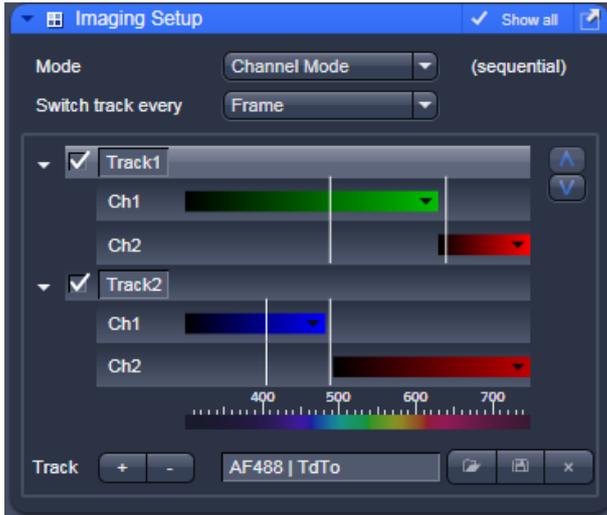


Fig. 5-19 Imaging Setup tool: Channel Mode

Within the **Imaging Setup** tool (Fig. 5-19) 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. With the **Show all** mode active, 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.

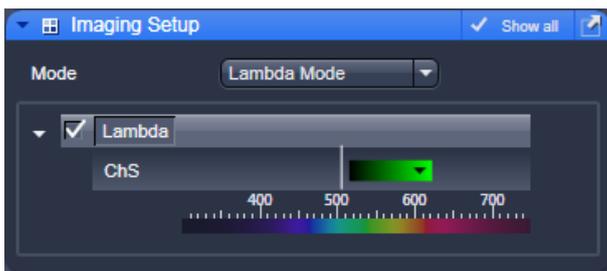


Fig. 5-20 Imaging Setup tool: Lambda Mode

Lambda Mode:

The **Lambda Mode** (Fig. 5-20) 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 QUASAR 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 10 nm. According to the detection range that is set for the QUASAR 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 **Unmixing View**) using the acquired Lambda stack and allow to clearly separate even heavily overlapping emission signals.

Online Fingerprinting:

The imaging mode **Online Fingerprinting** (Fig. 5-21) 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 QUASAR detector are defined in the loaded configuration or using the **Light Path** tool.



Fig. 5-21 Imaging Setup tool:
Online Fingerprinting

5.2.5 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.

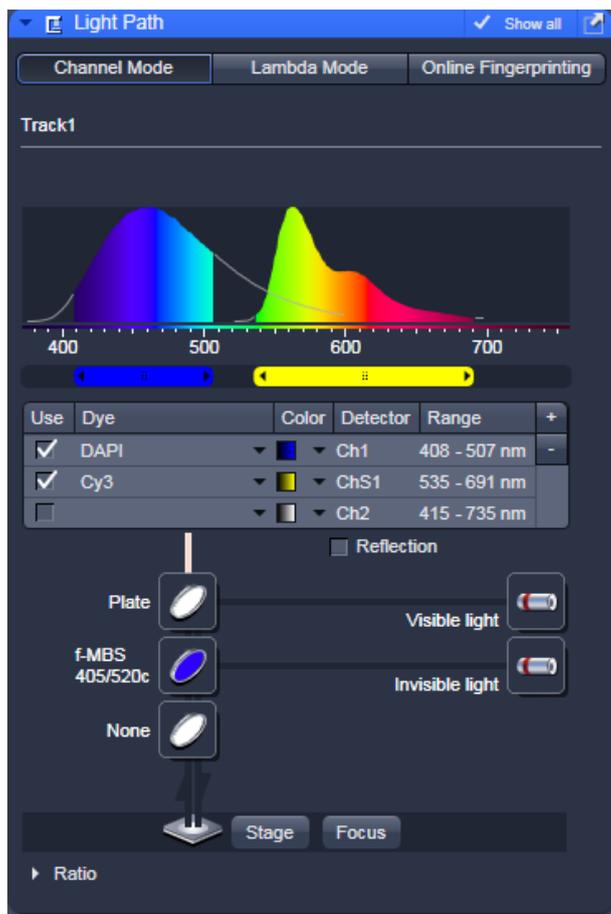


Fig. 5-22 Light Path tool

The **Channel Mode** tab (Fig. 5-22) displays the current hardware settings within the LSM 710 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. 5-22 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 (MBS)

- MBS 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.
- To select a beam splitter, click on the icon and chose the desired one from the drop down menu.

(2) Detection sliders

To select an emission filter, click on the sliders and set the detection bands according to the dry emission spectra on the top.

 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.

5.2.5.1 Activation/Deactivation of Channels

- 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. 5-23).

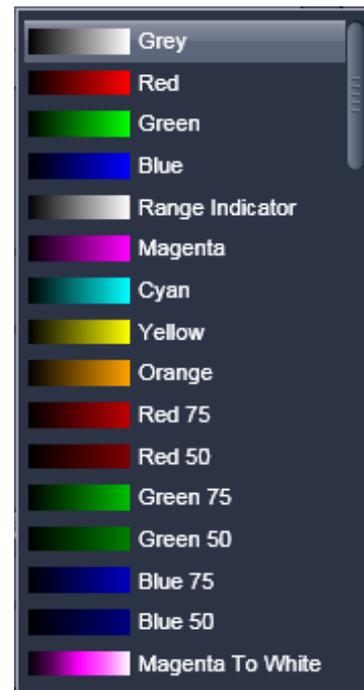


Fig. 5-23 Drop down menu for selection of the channel color

5.2.5.2 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 710 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. A click on the icon opens a drop down menu from which the desired filter can be chosen.

5.2.5.3 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 **Tool Group Online Acquisition: Focus Tool** and section **Tool Group Online Acquisition: Stage Tool**.

5.2.5.4 Setting of Laser Line and Laser Attenuation

The **Laser** icon provides access to the control of the laser lines and their attenuation (Fig. 5-24). 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. 5-24 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.

5.2.5.5 Multiple Tracks Configuration

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 **Tool Group Setup Manager: Imaging Setup Tool**).

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 Gain (Master), Digital Offset, Digital Gain and Data Depth (see section **Tool Group Online Acquisition: Channels Tool**), the scan parameters like **Frame Size, Zoom, Rotation & Offset**, and **Scan Direction** parameters (see section **Tool Group Online Acquisition: Acquisition Mode Tool**) are stored.

5.2.5.6 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. 5-25 Ratio channels in the Light Path tool

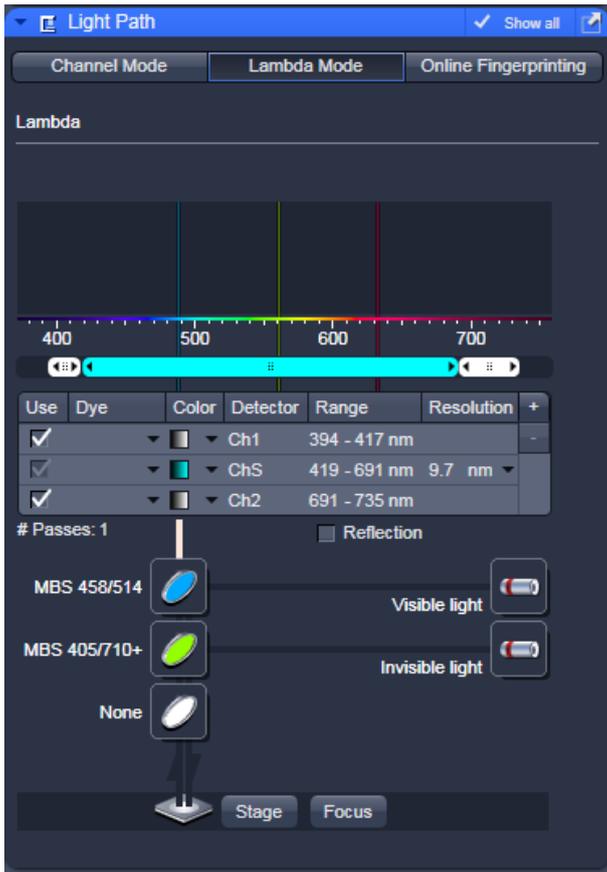
A click on the arrow next to **Ratio** in the lower part of the **Light Path** tool window (Fig. 5-25)

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 **Tool Group Online Acquisition: Channels Tool**).

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.



5.2.5.7 Imaging in Lambda Mode

(to be selected in the **Imaging Setup** tool or in the **Light Path** tool).

The **Lambda Mode** is recording the overall emission from the sample onto a wavelength-dispersive element and is imaged on a 34-channel detector. All 34 photomultipliers of the detector cover a spectral width of approximately 360 nm, a single PMT covers a spectral range of 9.7 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 complete fluorescence signal 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 QUASAR detector settings are performed in the **Light Path** tool (Fig. 5-26).

The QUASAR detector is displayed for spectral imaging if lambda mode has been chosen as imaging configuration in the **Imaging Setup** tool.

Fig. 5-26 Light Path tool window; Lambda Mode activated

The **Light Path** tool displays the laser lines, the main (dichroic) beam splitter, and the spectral range of the QUASAR detector.

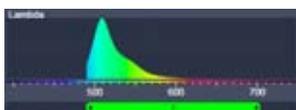
You can change the settings using the following function elements:



Activation / deactivation of the excitation wavelengths (checkbox) and setting of excitation intensities (slider).



Selection of the main dichroic beam splitter (MBS) through selection from the relevant list box.



Definition of number of PMTs out of the 34 PMT Detectors to fit the required spectral range.



Access to the focus control (see section **Tool Group Online Acquisition: Focus Tool**)



Step width in spectral range (9.7 recommended).



Activation / deactivation of reflection.

QUASAR detector settings

Use the two sliders to define the spectral detection range of the QUASAR detector.

Resolution: Step size can be 3.2, 4.9, 9.7, 19.4, 23.3 or 38.9 nm.

5.2.5.8 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:

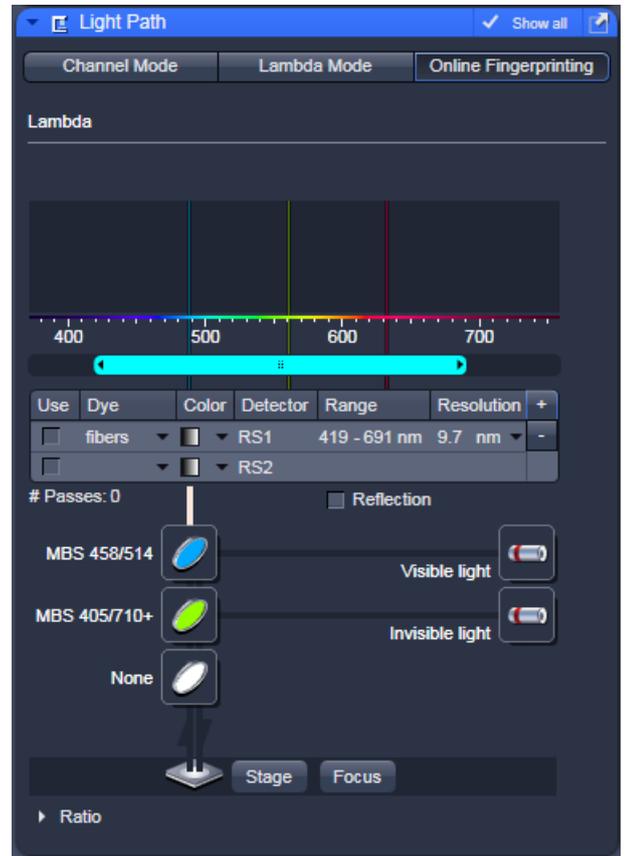


Fig. 5-27 Light Path tool window; Online Fingerprinting Mode

RS1 ... 8

Use	Dye	Color	Detector	Range	Resolution	+
<input checked="" type="checkbox"/>		■	RS1	460 - 674 nm	9.7 nm	-
<input checked="" type="checkbox"/>		■	RS2			

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. 5-28 Light Path tool: Camera tab

5.2.5.9 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 condenser 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.

5.2.6 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 710, LSM 7 *LIVE* or camera.

Depending on the selected scan mode the additional parameters for image acquisition will change.

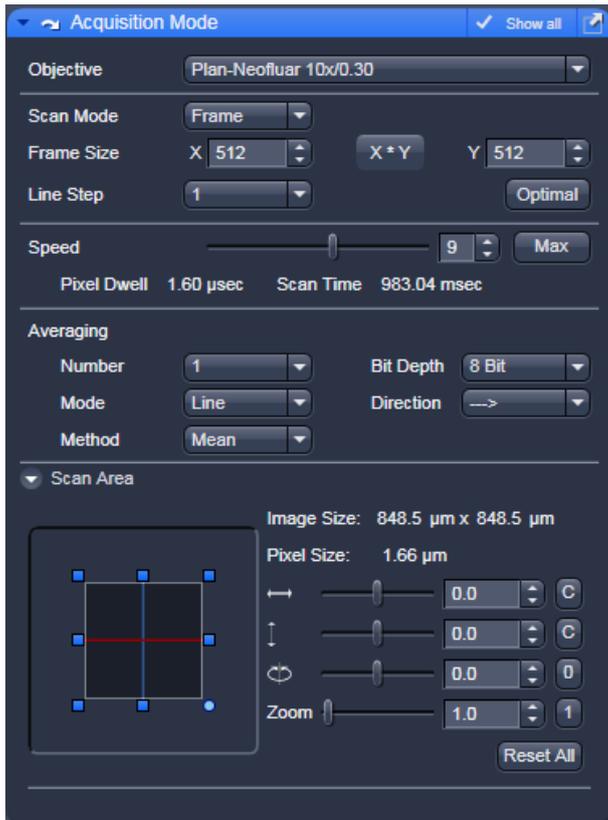


Fig. 5-29 Acquisition Mode - Frame with LSM tab selected in the Light Path tool

5.2.6.1 Frame Mode for LSM 710

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:

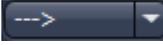
Objective, Frame Size, Line Step Factor, Speed, Pixel Depth, Scan Direction, Averaging and Zoom including rotation and offset

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 6144 pixels (integers). The value for **X** must always be an integral multiple of 4. The maximum value for **X** is also 6144 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 15 preset steps via slider or input box. Recommended: 8 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 ... 9	10	11	12	13	14	15
Zoom:	0.6-1	1.1-1.6	1.7-2.7	2.8-4.1	4.2-6.7	6.8-8.4	> 8.5

- Select **8 Bit**, **12 Bit** or **16 Bit** Data Depth, i.e. 256, 4096 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.

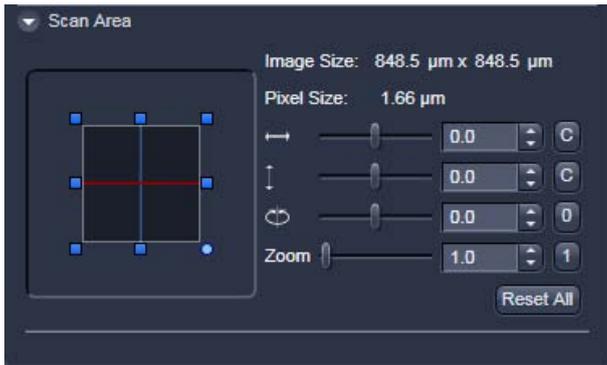


Fig. 5-30 Scan Area panel

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 0.6 to the maximum of 40, and is displayed in the relevant input box. The value 0.6 corresponds to factor 1, and value 40 to factor 66, related to the field of view. From zoom factor 5 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°.

5.2.6.2 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.

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.

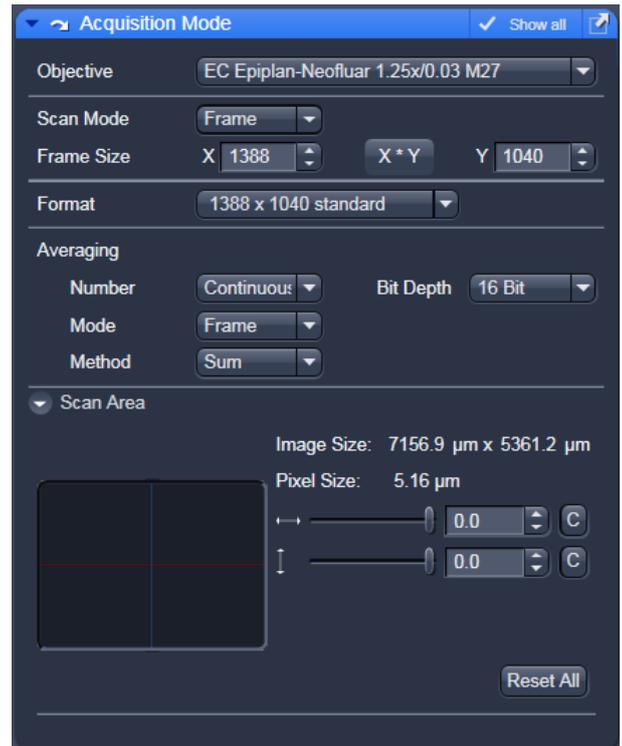


Fig. 5-31 Acquisition Mode - Frame with Camera tab selected in the Light Path tool.

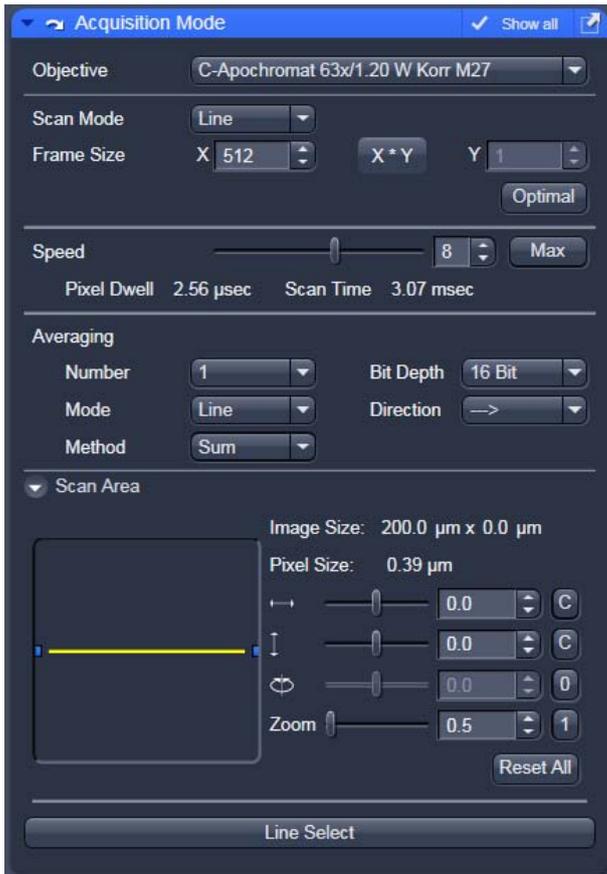


Fig. 5-32 Acquisition Mode – Line with LSM tab selected in the Light Path tool

5.2.6.3 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 **Frame Mode for LSM 710**. **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.

5.2.6.4 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. 5-33).

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 **Frame Mode for LSM 710**.

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**.

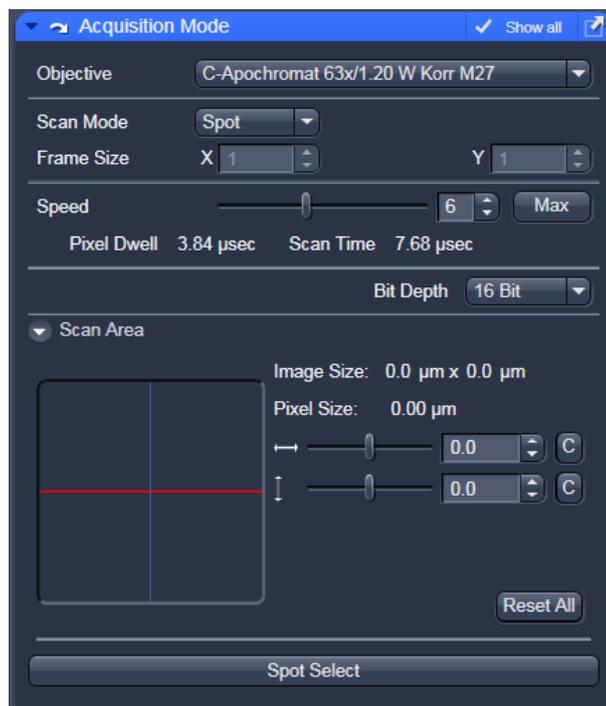


Fig. 5-33 Acquisition Mode tool - Spot Mode



Fig. 5-34 Channels tool using channel mode (in this case multiple tracks) for imaging

5.2.7 Tool Group Online Acquisition: Channels Tool

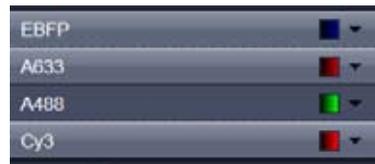
The **Channels** tool provides the control of the parameters of the individual detection channels.

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** EBFP list entry displays the controls for the selected channel only.

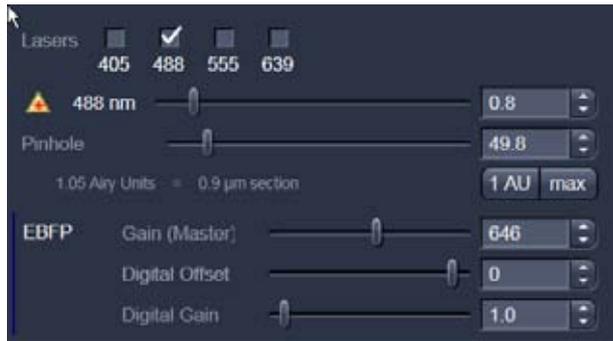
Using shift-clicks – the controls can be displayed for any given combination of channels or tracks.



If entered in Smart Setup, 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 Channel 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 1200)
 - 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.

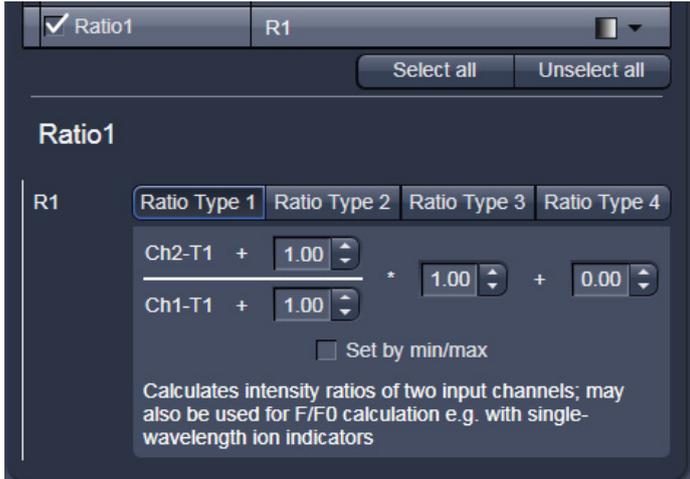


Fig. 5-35 Channel Settings panel of a Ratio Channel

5.2.8 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.

5.2.9 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 .

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 **Manually** button.

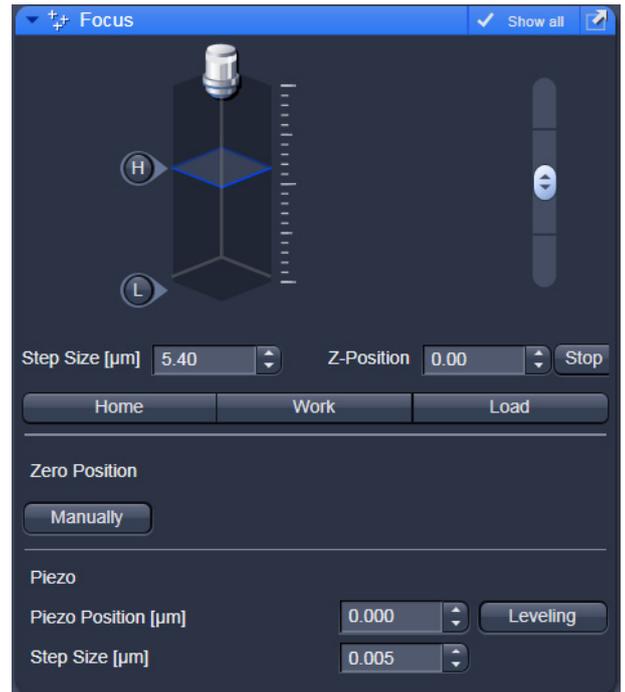


Fig. 5-36 Focus tool window

In addition a focus software joy stick is available to focus with different speeds.

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 printed manual CHAPTER 7 ANNEX of the printed manual for further information on the Piezo objective focus and Z-Piezo stage insert.



Fig. 5-37 Stage tool window

5.2.10 Tool Group Online Acquisition: Stage Tool

The following software description applies to systems which are equipped with a motorized scanning stage.



If an LSM 710 scan head is attached to the side port of an inverted microscope (Axio Observer) take care not to catch your fingers when moving the motorized XY scanning stage to the maximum position 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 between 1 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 from the drop down menu by clicking **Move To**. Three different values for the **Speed Settings** are available. They can be assigned interdependently to **Speed 1-3**.

5.2.11 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. 5-38).

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. 5-38 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.

5.2.12 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.

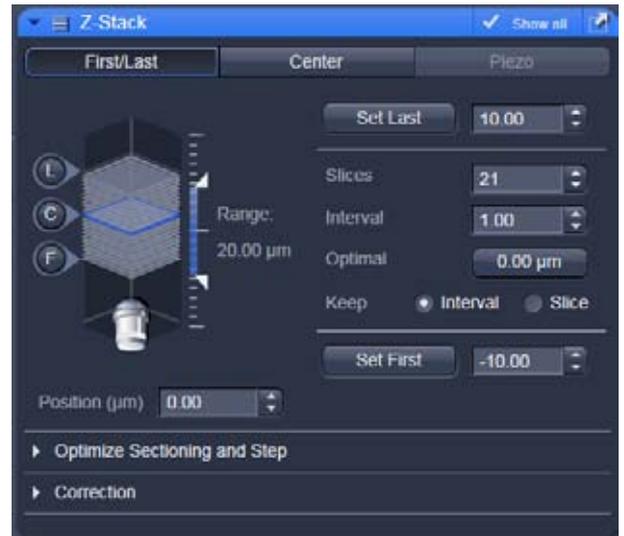


Fig. 5-39 Z-Stack tool First/Last mode with Z-Stack Navigation displayed

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. 5-39) or to use **Center** (Fig. 5-40). In case a **Piezo** focusing device is attached to the system, the Piezo device can be chosen for the acquisition of the stack.

5.2.12.1 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 **Tool Group Online Acquisition: Focus Tool**), 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 Range 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.Z2 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.

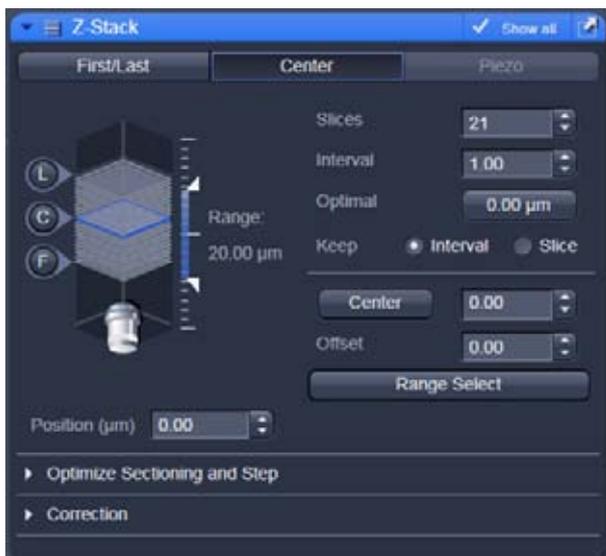


Fig. 5-40 Z-Stack tool Center mode with Z-Stack Navigation displayed

5.2.12.2 How to Proceed Setting a Z-Stack Using Center Mode

The **Center** mode (Fig. 5-40) 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  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 , , and  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 Center 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.  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.

5.2.12.3 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. 5-41). 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 from each other and affects the Range of the stack. The Focus Position is not changed.

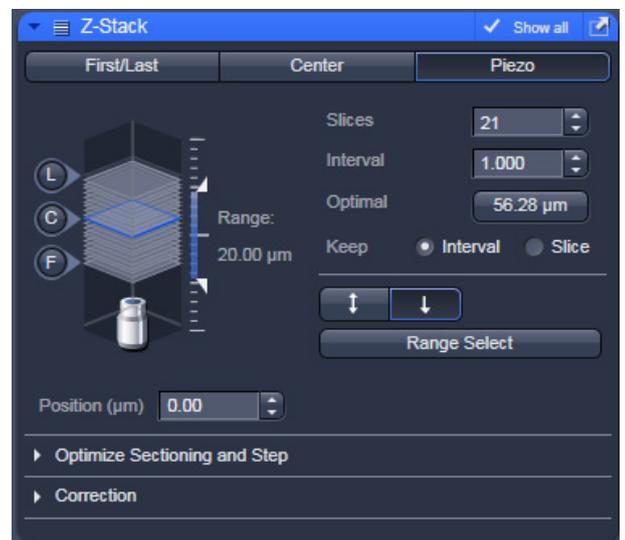


Fig. 5-41 Z-Stack tool Piezo mode with Z-Stack Navigation displayed

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. 5-42 Optimize Sectioning and Step: Optimal Interval is set starting with one Airy unit for all channels



Fig. 5-43 Optimize Sectioning and Step: Match Pinhole to Step resulting in equal optical sections for all channels

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.

5.2.12.4 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 $1.5 \mu\text{m}$, the optimal interval is $0.75 \mu\text{m}$ (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.

5.2.12.5 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.



$$\text{Ratio} = \frac{n}{n'}$$



Fig. 5-44 Auto Z Brightness Correction

Auto Z Brightness Correction (Fig. 5-44) 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.

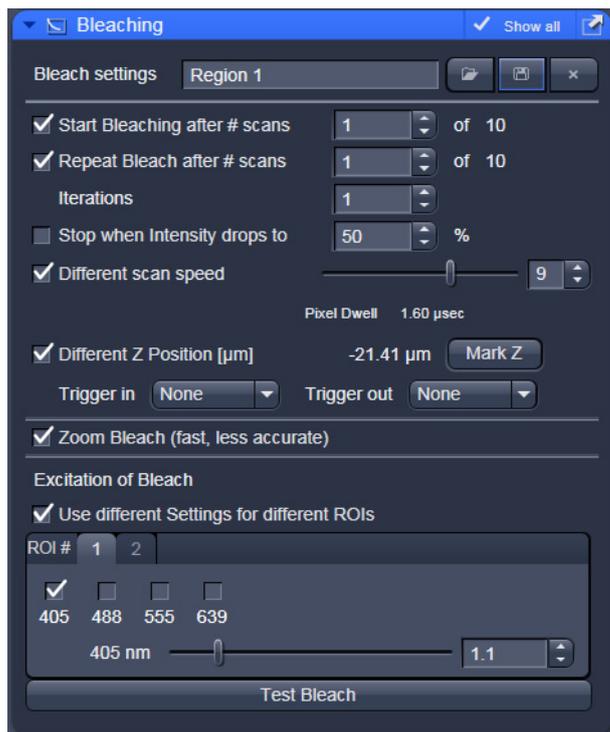


Fig. 5-45 Bleach Control window

5.2.13 Tool Group Multidimensional Acquisition: Bleaching

The use of this function permits setting the bleach parameters using the LSM 710 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 **Tool Group Online Acquisition: Regions Tool**) 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 printed manual 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 **Tool Group Online Acquisition: Regions Tool**). Only a region activated under Bleach is considered for bleaching. In the **Excitation of Bleach** area (Fig. 5-46) 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.

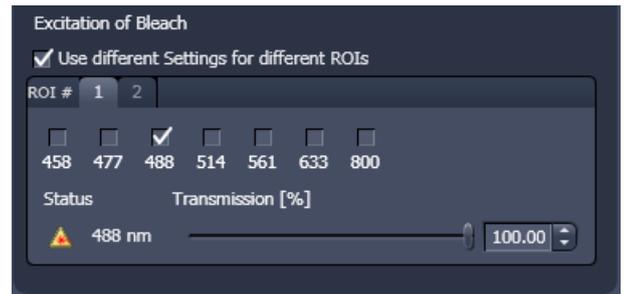


Fig. 5-46 Excitation of Bleach area for setting laser line and intensity for bleaching



Fig. 5-47 Time Series tool

5.2.14 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.

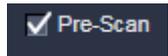
The **Marker** functionality provides the possibility to indicate the start or end of an external process in the data of the image series by connecting this external process to the selected **Trigger In** for that Marker. The **Description** of the Marker is then visible in the image data using the Mean ROI functionality or the image gallery display mode (see section **Gallery View of the ZEN File Browser**). 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 . 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.

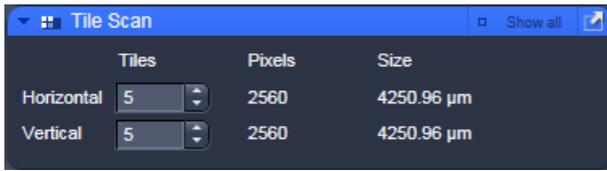


Fig. 5-48 Tile Scan tool

5.2.15 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. 5-49 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 single 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.

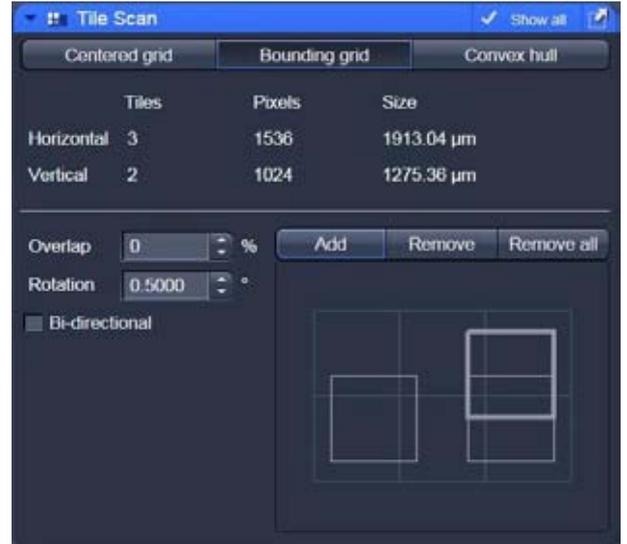


Fig. 5-50 Tile Scan: Bounding grid

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.

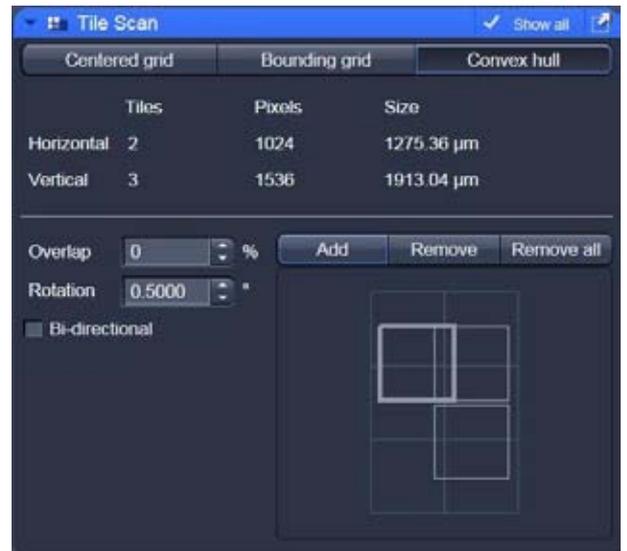


Fig. 5-51 Tile Scan: Convex hull

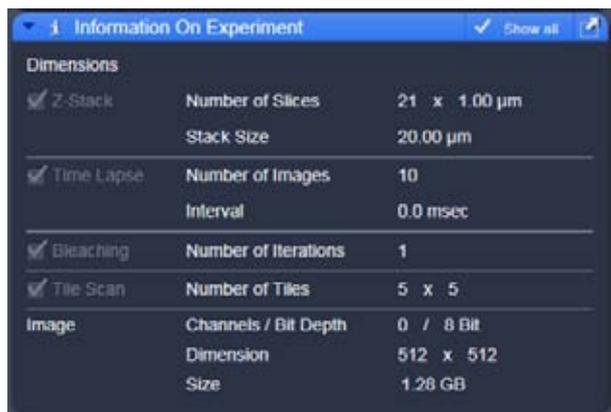


Fig. 5-52 Information on Experiment

5.2.16 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.

5.2.17 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.

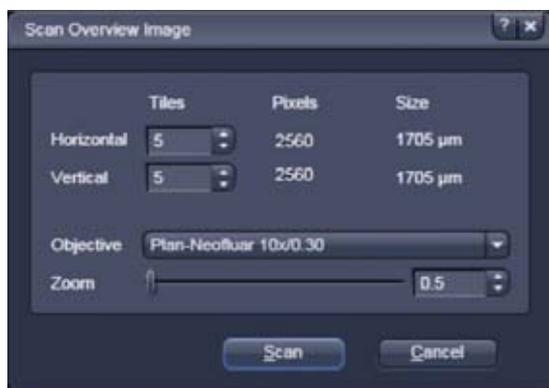


Fig. 5-53 Positions Tool, Position List tab; sub-tab Scan Overview Image

Using the first tab, **Position List** (Fig. 5-53), 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 **Tool Group Online Acquisition: Stage Tool**).

 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. 5-53) to define the number of **Tiles**, the **Objective** (chosed 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.

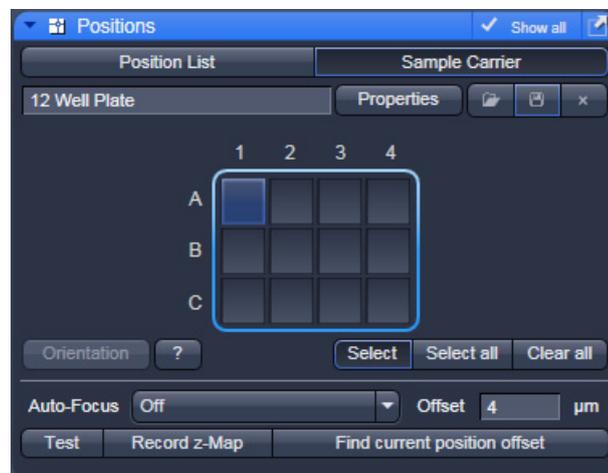


Fig. 5-54 Positions Tool, Sample Carrier tab

Using the second tab **Sample Carrier** (Fig. 5-54) 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. 5-55).

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.

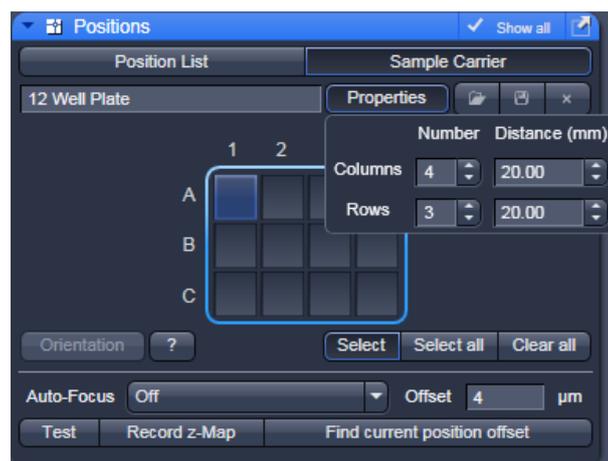


Fig. 5-55 Positions Tool, Sample Carrier tab, Properties interface

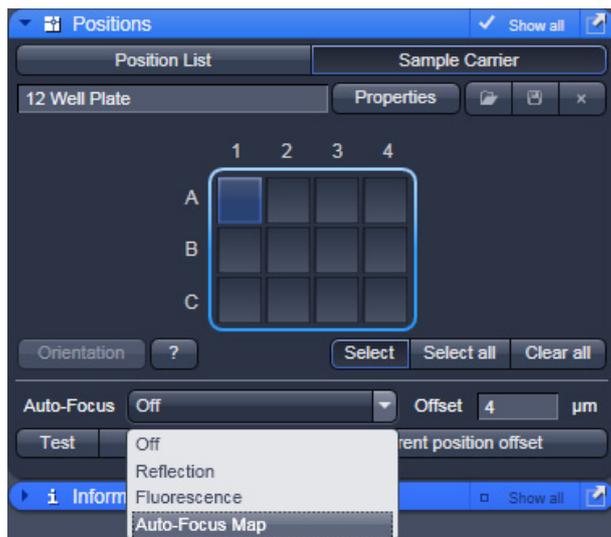


Fig. 5-56 Positions Tool, Sample Carrier tab, choice of methods for Auto-Focus

For this application and additional method for Auto-Focus is available: Auto-Focus Map (Fig. 5-56).

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.

5.2.18 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.

5.2.18.1 Requirements

- Position the specimen under the objective and focus onto the desired imaging plane.
- 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. 5-57).
- Switch on the Laser 561 nm.
- Open the **Light Path** tool.
- Click on the laser icon, select the 561 line, and set the transmission slider to about 3 % (this laser has an output power of 10 mW, other lasers have up to 100 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.

 Optimize the laser intensity subsequently via the **Transmission** slider when imaging.

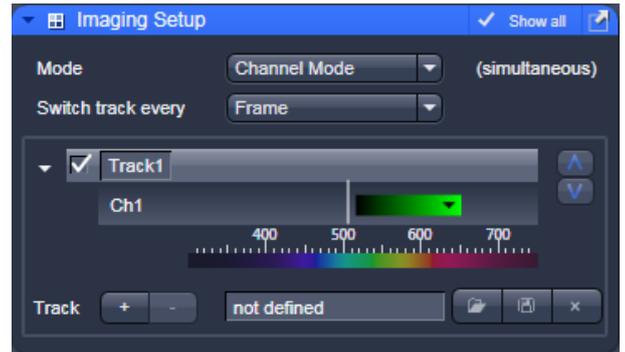


Fig. 5-57 Imaging Setup tool – imaging mode selection and track activation



Fig. 5-58 Laser line and laser power selection



Fig. 5-59 Channel color selection

- Activate channel 1 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. 5-59).
- Move on the slider to set a detection window above.
- If required, deactivate all the other channels (ChS, Ch2, 4, monitor diode, transmission, R1-2) via check box.
- Click on the icon of the main dichroic beam splitter and select **MBS 488/561** or any other MBS indicating the 561 line.



Fig. 5-60 Light Path tool window

The beam path in the **Light Path** tool now displays the way the potential emission signal takes. Channel 3 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. 5-61).

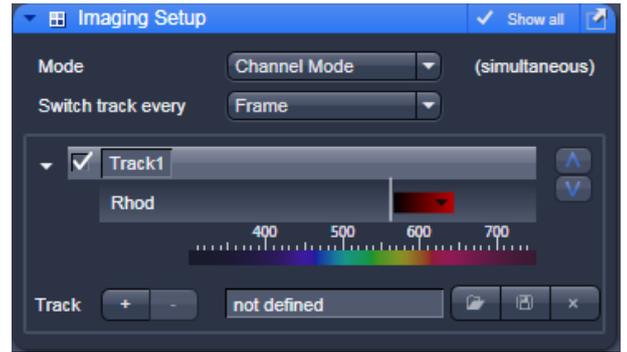


Fig. 5-61 Imaging Setup tool

5.2.18.2 Defining the Scan Parameters

- Open the **Acquisition Mode** tool (Fig. 5-62).
- 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.
- Start with the following settings on the **Averaging** panel:

Bit depth:	8 bits
Number:	1 (no averaging)

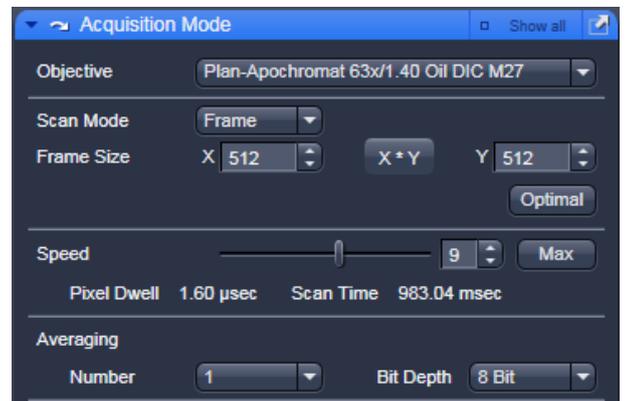


Fig. 5-62 Acquisition Mode tool

- ☞ 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.

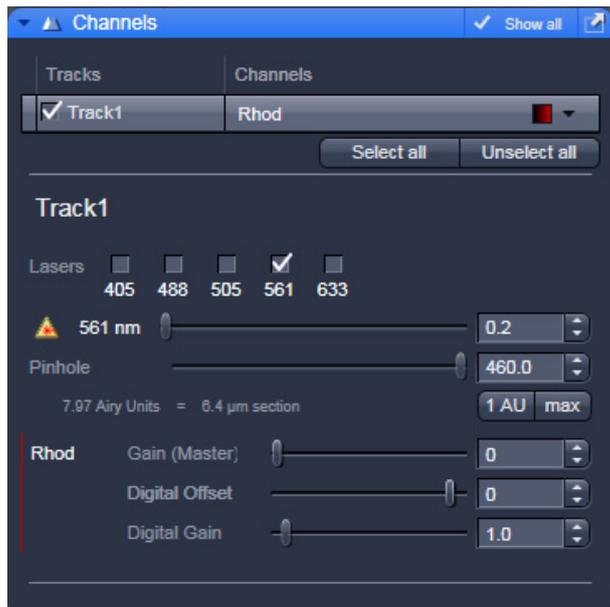


Fig. 5-63 Channels tool

5.2.18.3 Defining the Detection Parameters

- Activate the **Channels** tool (Fig. 5-63).
- 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.

5.2.18.4 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 **Dimensions**). 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.

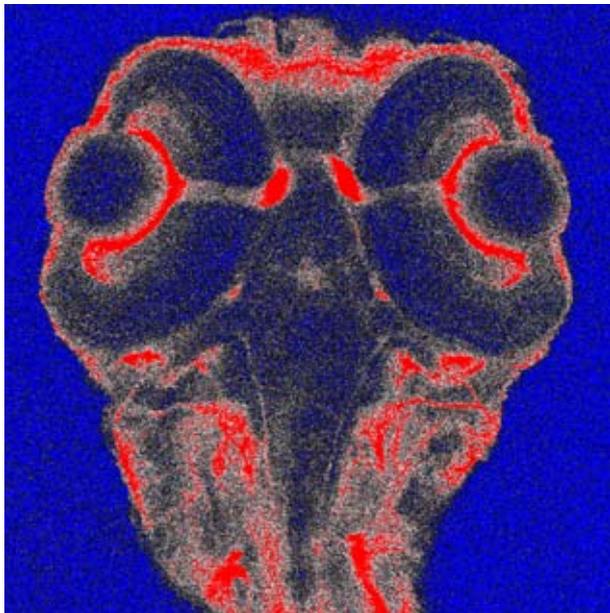


Fig. 5-64 Image displaying bad adjustment of gain and offset (blue regions are underexposed, red regions are overexposed)

- 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 **Digital 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.



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.

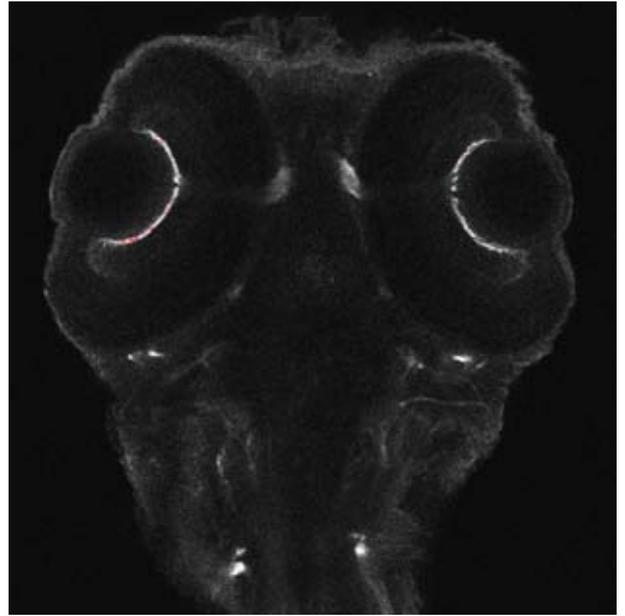


Fig. 5-65 Image displaying good adjustment of gain and offset

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 **Split View**) 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.

5.3 Processing Tab

5.3.1 General Structure of the Processing Tab

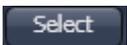
The functions of the **Processing** tab are designed to cover a large range of Image Processing methods to process and analyze already stored as well as just scanned images including mathematical operators and algorithms.



Fig. 5-66 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. 5-66), the list of available processing tools becomes available.

The **Processing** tool tab is structured as follows (see Fig. 5-67): On the top, under the **Main** tool

tab, is the **Processing** tab header with the  button. To process a loaded image, first select the required methods from the List of available processing methods (Fig. 5-67); select the active image as the input image with the  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  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. 5-67 The Processing tab structure



Fig. 5-68 Maximum Intensity Projection tool

5.3.2 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. 5-68).

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.

5.3.3 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. 5-69), click on the **Image Calculator** in the list of methods in the **Processing** 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** 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. 5-69).

By highlighting the selected operator a description of the function of this operator is displayed in the lower part of the operators list (Fig. 5-70).

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.



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.

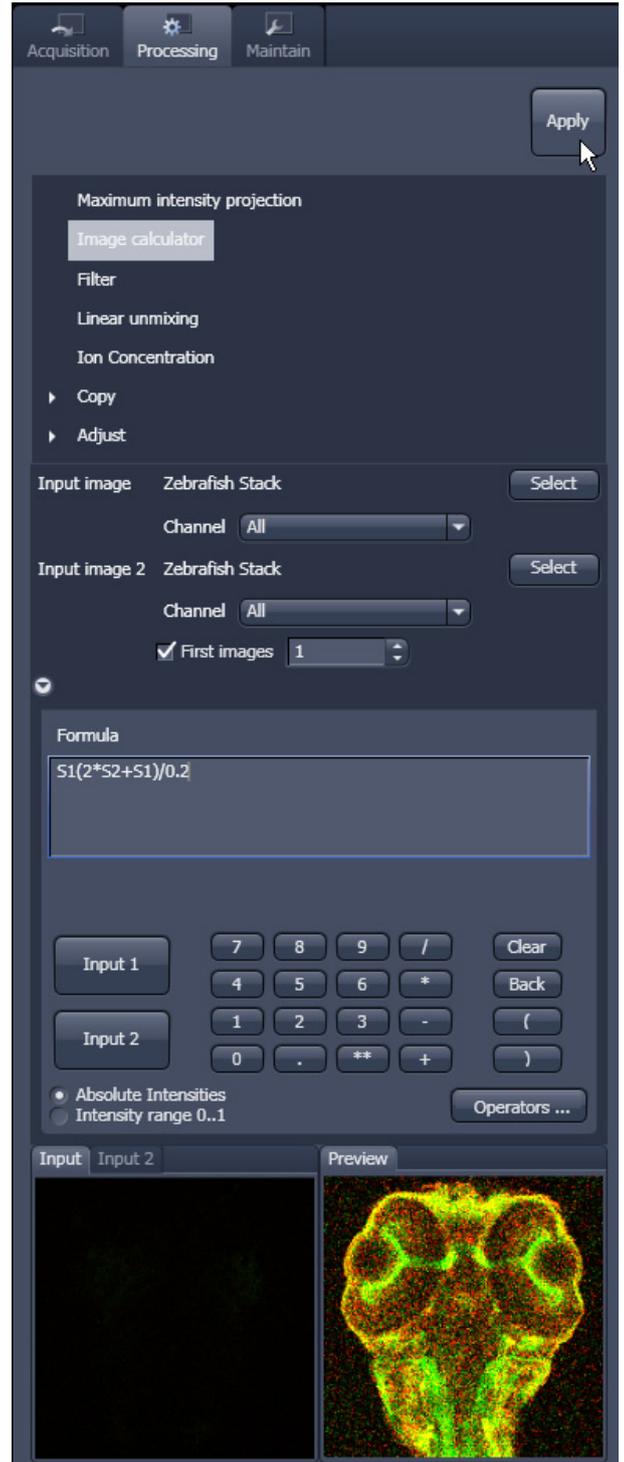


Fig. 5-69 The Image Calculator window

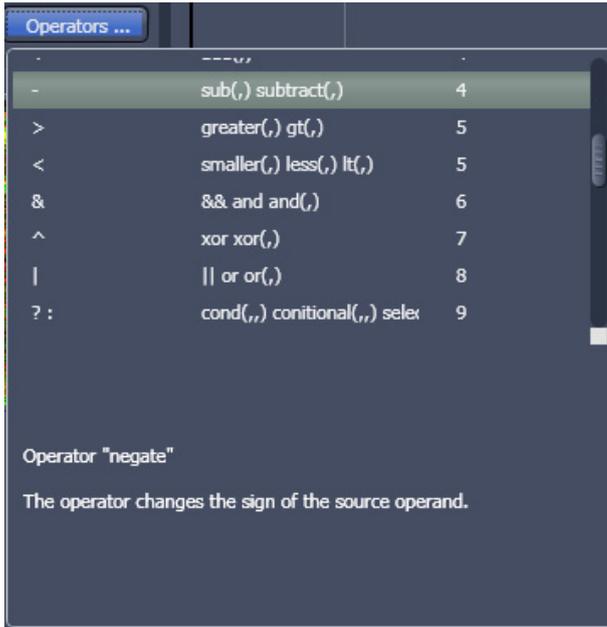


Fig. 5-70 Operators window

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 Fig. 5-71 that enhances the relative changes in the image with respect to the relatively bright signal level, for example:

$$S1-S2/2 = \text{source 1} - \frac{1}{2} * \text{source 2}.$$



Fig. 5-71 Image calculation window

5.3.4 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. 5-72). 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.

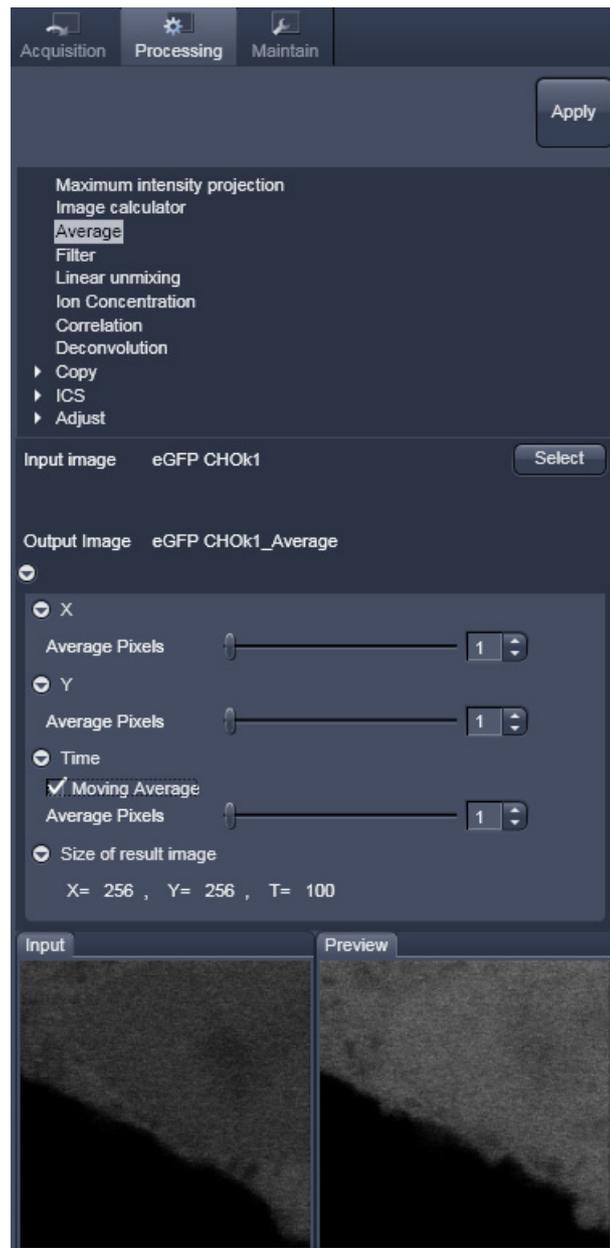


Fig. 5-72 Average window



Fig. 5-73 Filter window



Fig. 5-74 Median filter settings

5.3.5 Filter

The filter function permits the processing of scanned images with a **Median**, **Smooth**, **Sharpen** or **Bandpass** filter.

- To open the **Filter** tool, select it from the list in the **Processing** main tab (Fig. 5-73).
- 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. 5-74). 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. 5-75). 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. 5-75 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. 5-76). They are the same as in the **Smooth Filter**.



Fig. 5-76 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. 5-77 Band filter settings

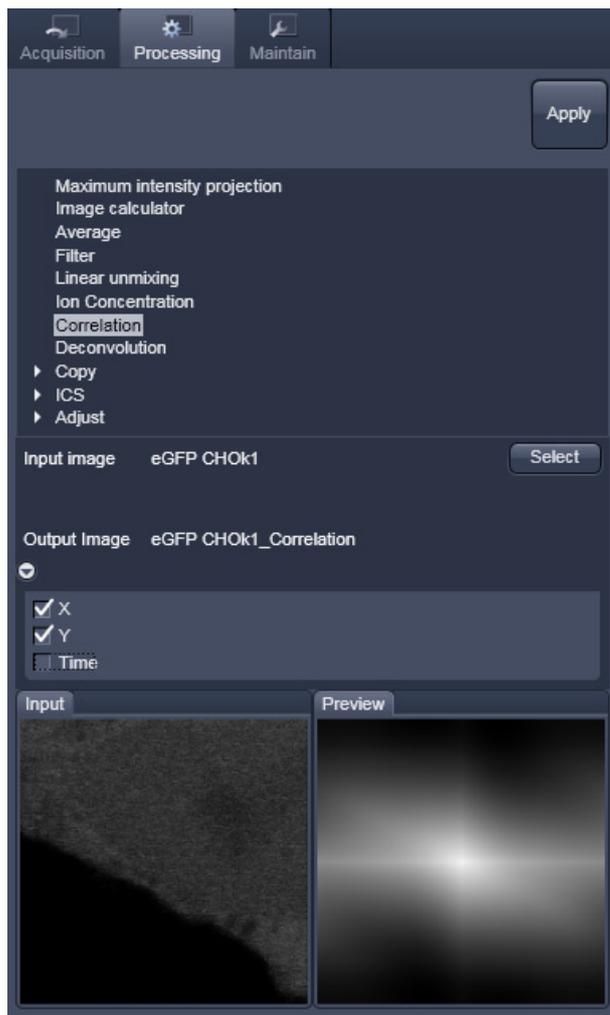


Fig. 5-78 Correlation window

5.3.6 Correlation

- Activate **Correlation** in the **Processing** tool. The **Correlation** menu will appear (Fig. 5-78).

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.

5.3.7 ICS

- Activate **ICS** in the **Processing** tool by clicking on the arrow.

The **ICS** menu will appear with three sub-processing functions: **Remove structures**, **ICS correlation** and **Map filter** (Fig. 5-79).

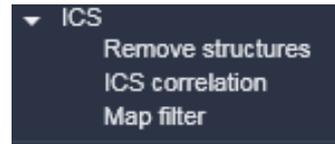


Fig. 5-79 ICS sub-processing functions

5.3.7.1 Remove Structures

- Activate **Remove structures** in the **ICS** tool. The **Remove structures** menu will appear (Fig. 5-80).
- 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 an image with the selected average subtracted is computed and the result presented in the **Preview** window.

- If the **Apply** button is pressed, the averaged subtracted image stack will be opened as a new image in the container.

You have four options that can be selected via the **Remove Structures** pull down menu (Fig. 5-81):

None, **Slowly moving structure**, **Immobile structures (+Stack)** and **Immobile structures (+Plane)**.

If **None** is selected, no average will be subtracted and the original image is kept. If **Slowly moving structure** is activated the **Average frames** input box becomes available. Type in the required number (minimum is 2) or use the slider or arrows for input. The number defines the number of frames that will be averaged and subtracted in a gliding way from the original stack in the following way:

- From a stack of n frames take successive m moving average frames with an overlap of $m-1$ and average these. The outcome is a new stack with $n-m+1$ gliding or moving average frames. Calculate from each of the average frames the overall average of all pixels, which is a scalar.
- Subtract the i -th of the gliding average frame from the $(i-1)+m/2$ original frame and add the i -th scalar. In this way, the first frame to be used from the original stack is $m/2$ and the last $m/2+(n-m+1)$. This creates a new stack of $n-m-1$ frames that is the output image.

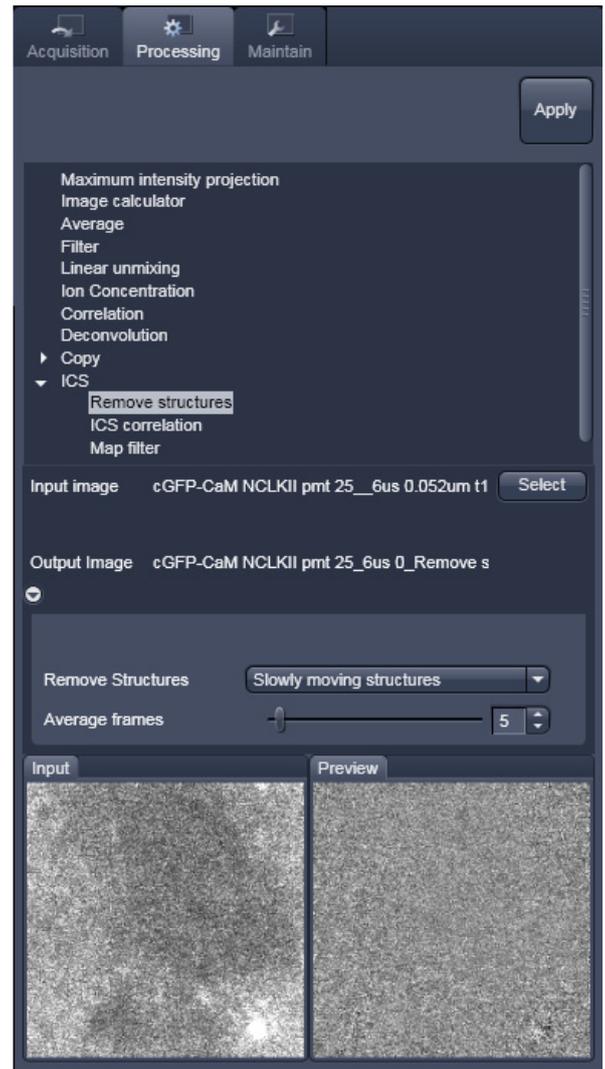


Fig. 5-80 Remove structures menu



Fig. 5-81 Remove Structures pull down menu

If **Immobile structures (+Stack)** is selected, the average frame of the stack will be computed and subtracted from each original frame. This creates a new average subtracted stack of the same size. An average value of all pixels from all frames is computed, which is a scalar, and added to each pixel of the single frames of the average subtracted stack to avoid negative numbers. The resulting stack is the output image.

If **Immobile structures (+Plane)** is selected, the average frame of the stack will be computed and subtracted from each original frame. This creates a new average subtracted stack of the same size. Average values of all pixels from each of the original frames is computed, which is a scalar, and added to each pixel of each corresponding single frame of the average subtracted stack to avoid negative numbers. The resulting stack is the output image.

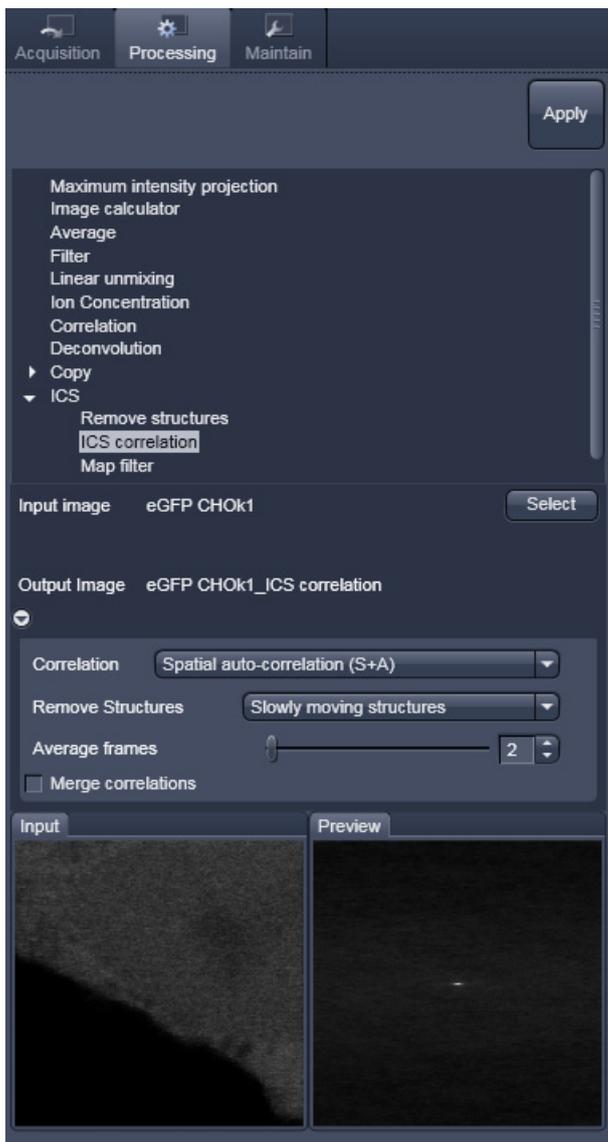


Fig. 5-82 ICS correlation menu

5.3.7.2 ICS Correlation

- Activate **ICS correlation** in the **ICS** tool. The **ICS correlation** menu will appear (Fig. 5-82).
- To select an image press the **Select** button and click into the image in the container. This will be your **Input** image.

A correlation image will be computed based on the settings and displayed in the **Preview** window. As an output image an image with the selected average subtracted is computed and the result presented in the **Preview** window.

- The correlation to be computed can be selected from the **Correlation** pull down menu (Fig. 5-83). For one channel recordings, only **Spatial auto-correlation (S+A)** is available, for a two channel recording in addition **Spatial cross-correlation (S+C)**.

The way of average subtraction can be selected from the **Remove structures** pull down menu (Fig. 5-84).

- You can select between **None** (no average subtraction), **Slowly moving structures** (subtraction of moving average, the frame size of which can be selected in the **Average frames** input box), **Immobile structures (+Stack)** and **Immobile structures (+Plane)** (subtraction of total average and adding average scalar of all pixels from one frame to each corresponding frame).

The output image will be a correlation stack.

- If the **Merge correlations** check box is ticked, the output will be an average correlation function image computed from the correlation image stack.

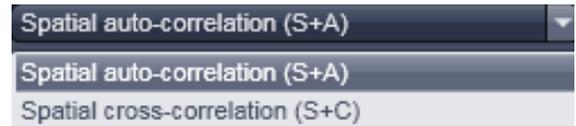


Fig. 5-83 Correlation pull down menu

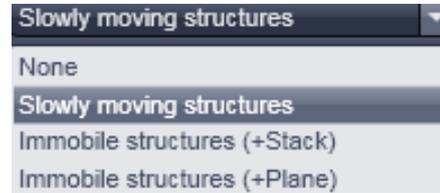


Fig. 5-84 Remove Structures pull down menu

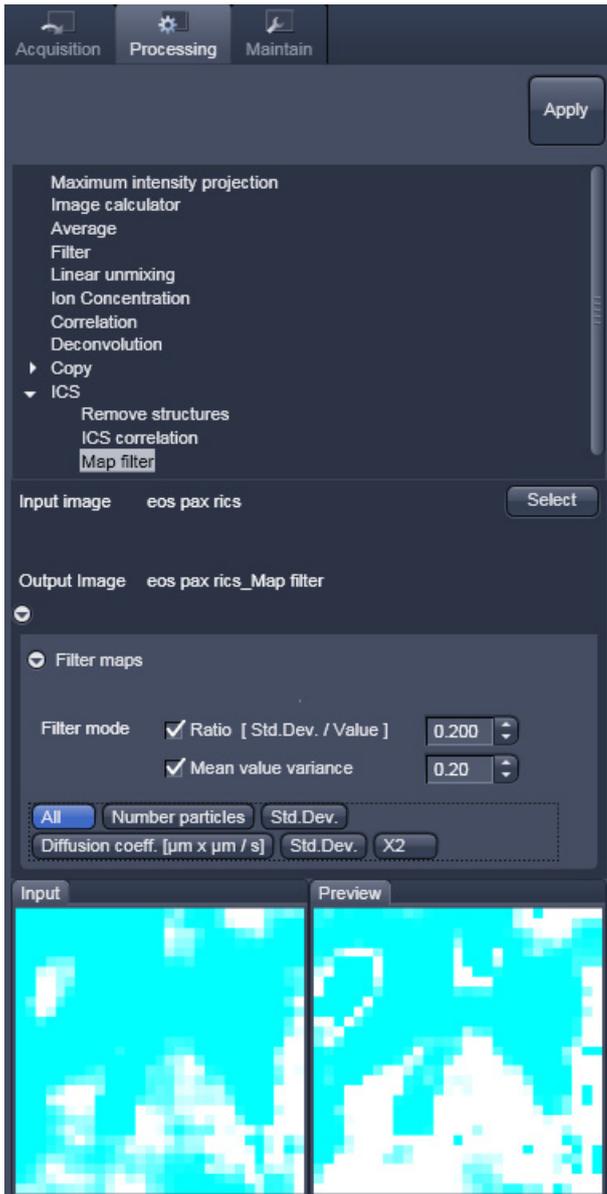


Fig. 5-85 Map filter menu

5.3.7.3 Map Filter

- Activate **Map filter** in the **ICS** tool. The **Map filter** menu will appear (Fig. 5-85).
- To select an image map press the **Select** button and click into the image map in the container. This will be your **Input** image map.

As an output image map an image map with the selected filters applied is computed and the result filtered image maps will be opened as a new image maps in the container.

The way of filters to be applied can be selected in the **Filter maps** menu, which can be opened by the arrow, by checking the **Ratio [Std.Dev./value]** and the **Mean value variance** check boxes.



Note that both filters can be applied together by checking both boxes.

- Thresholds for the filters can be entered in the corresponding input boxes by typing or by using the arrows. Pixels with values exceeding the threshold will be displayed in black and will be discarded for the scaling of the map.

You can define if all maps should be filtered by pressing **All** button in the available filter display area.

- If you want to apply filtering only to a specific map (**Number of particles**, its standard deviation **Std.Dev.**; **Diffusion coeff. [µm x µm /s]**, its standard deviation **Std.Dev.** and **X²**) the **Filter all maps** box must not be ticked and the corresponding map button pressed, which is indicated by highlighting in blue.



Note that filtering is never applied to the **X²** map, even if it was selected.

5.3.8 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 multi-channel images and so-called "Lambda stacks" (see section **Imaging in Lambda Mode**).

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 **Unmixing View**) as an external reference. Then a multi-channel image or 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 **Unmixing View**))
- b) Automatically by software analyses of what the individual spectral signatures are. This processing function is called "Multi Channel Unmixing" or "Automatic Component Extraction" (ACE). Note: in some cases, spectrally acquired images are not appropriate for ACE or "Multi Channel Unmixing" and linear unmixing can lead to wrong results.

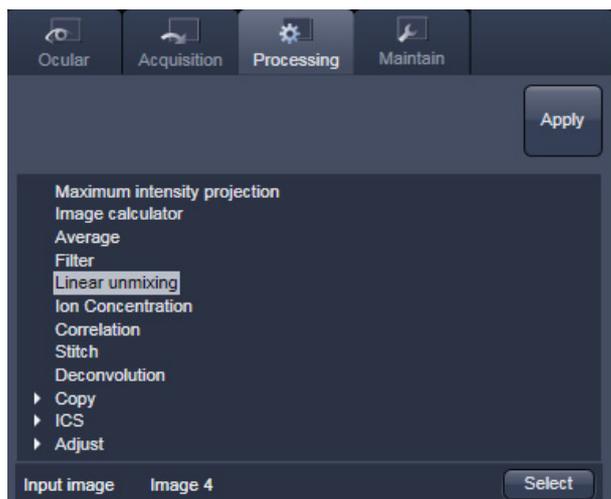


Fig. 5-86 Linear Unmixing in the Processing tab



Fig. 5-87 Linear Unmixing panel

- To open the **Linear Unmixing** tool, click on **Linear Unmixing** in the **Processing** tool list in the **Processing** tab (Fig. 5-86).
- Pressing the **Select** button loads the active image from the **Image Display** and displays it as Input Image. Only Lambda stacks or multi-channel images can be loaded.
- In the **Linear Unmixing** panel (Fig. 5-87), the number of spectrally distinguishable fluorescent components of the imaged sample can be selected from the **Components** 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. 5-88).
- Assign appropriate colors to the fluorescence channels by clicking on the color flag and choose from the available lookup tables (LUTs) (Fig. 5-88).
- 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 **Unmixing View**).
 - 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. 5-89 and Fig. 5-90).

The following additional settings and functions are available for linear unmixing (see Fig. 5-87):

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 **Unmixing View**). Even small background contributions to the image should be indicated to the unmixing algorithm to ensure best unmixing results.

Weighted unmixing: When this option is checked, spectral channels with high noise do contribute less to the unmixing result. This option includes a statistical analysis of the signal-related (Poisson-) noise and weighs the respective contribution to the fitting of the combination of reference spectra to the experimental data. Note: this option involves a more sophisticated unmixing algorithm and therefore takes longer than the basic unmixing analysis. Weighted unmixing generates improved unmixing results when acquisition channels are not so well balanced but still have a good signal-to-noise ratio.



Fig. 5-88 Linear Unmixing: Spectra and LUT assignment

Display channels with statistical confidence: This option displays an additional channel per unmixed component which shows the relative statistical error in each unmixed component channel. This statistical uncertainty of the pixel intensity in the unmixed channel is calculated based on the (Poisson-) noise of the acquired (input-) channels, the bandwidth and –position and the quality of the reference spectra. The display can be interpreted best when an appropriate false color LUT is chosen. The error is displayed as percentage of unmixed channel pixel intensity. For example: A "confidence value", i.e., pixel intensity of 50 represents a 50% statistical uncertainty of the corresponding intensity in the unmixed channel. The higher the statistical error of the acquired pixel intensity (the shot noise), the higher is the intensity value of this pixel.

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 **Automatic Component Extraction**.

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.

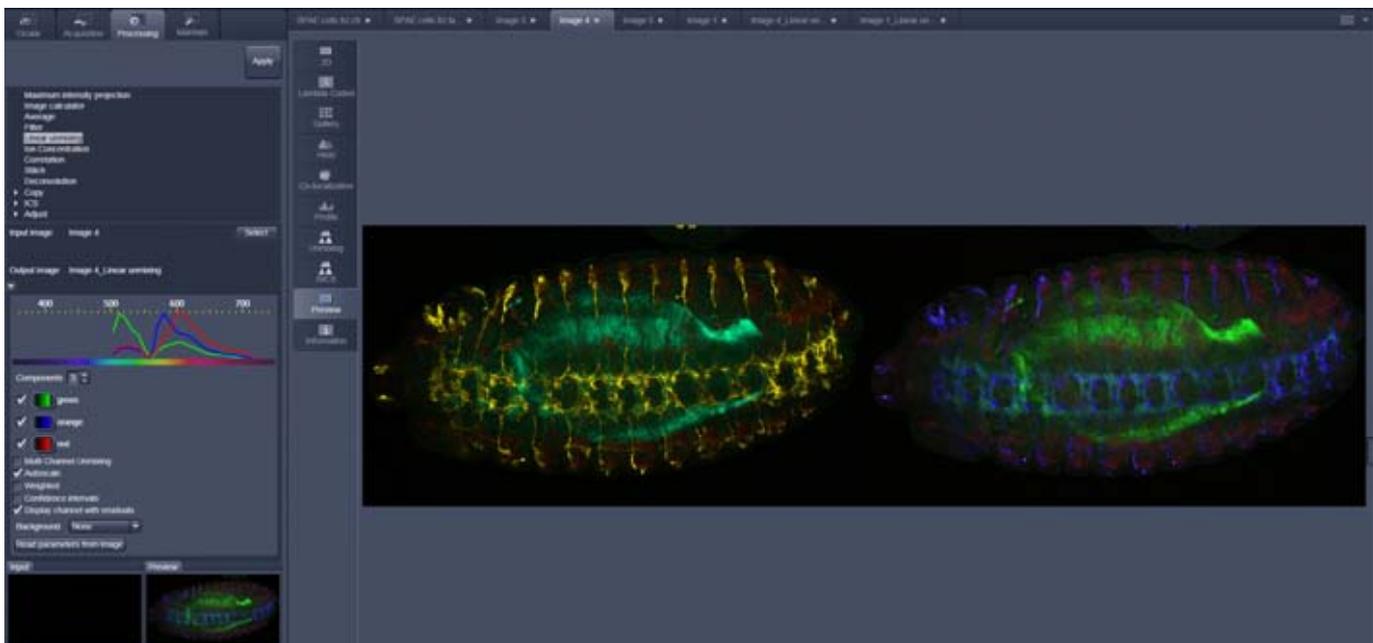


Fig. 5-89 Image Display window before unmixing in Preview

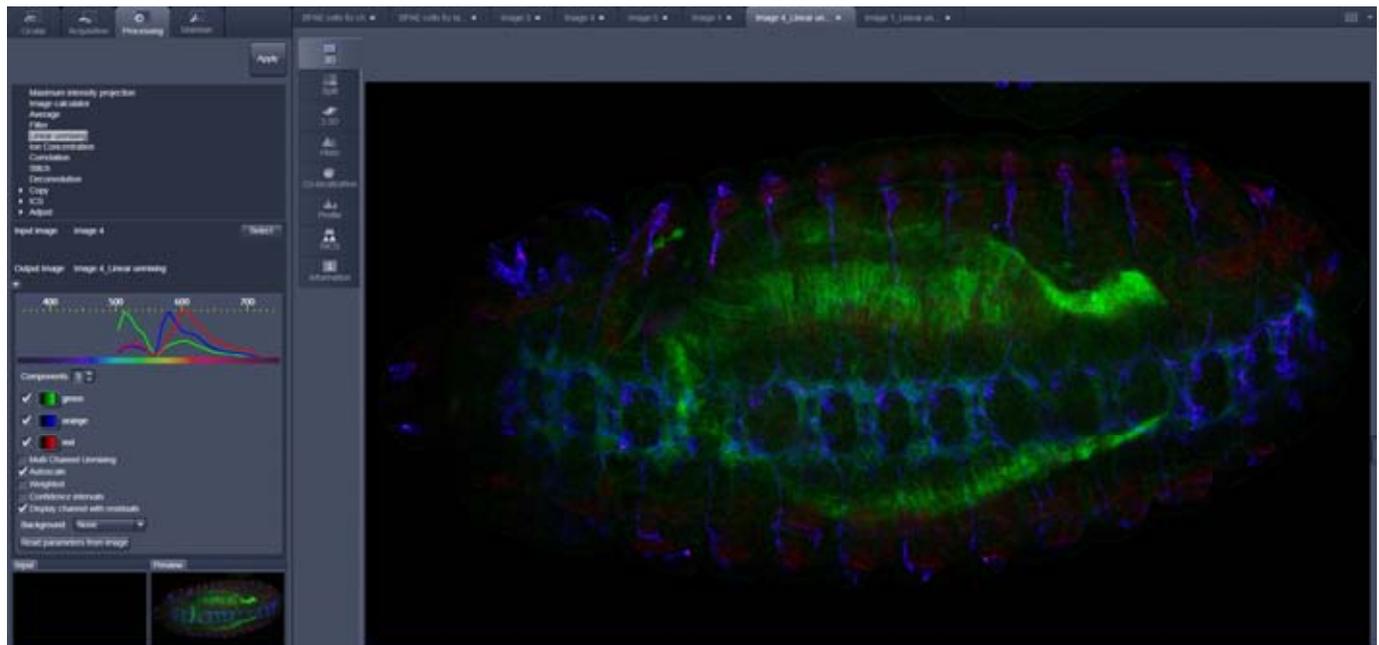


Fig. 5-90 Image Display window after unmixing

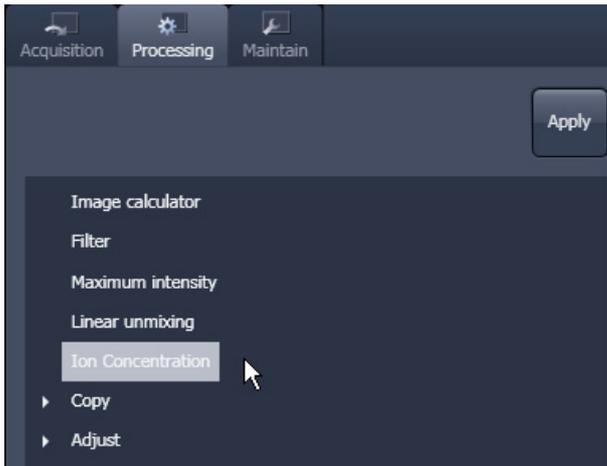


Fig. 5-91 Ion Concentration in the Processing tab

5.3.9 Ion Concentration

This Processing function permits the calibration of ion concentrations in physiological experiments.

To open the **Ion Concentration** tools click **Ion concentration** (Fig. 5-91).

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. 5-92), the full tool window opens and the following functions are available (Fig. 5-93).

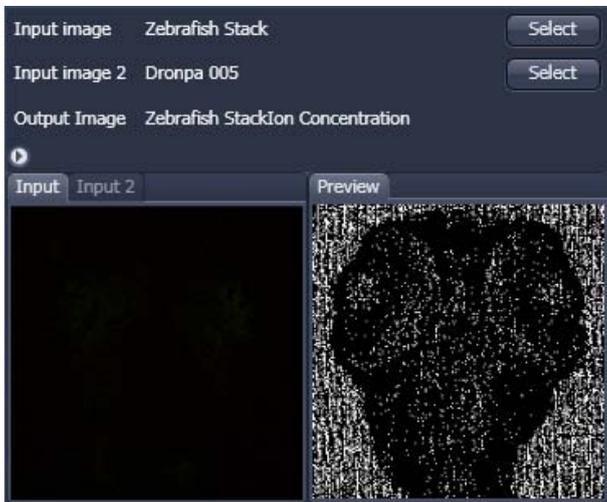
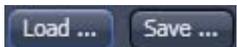


Fig. 5-92 Ion Concentration tool window



Opens the display for the resulting calibration curve.



To load & save calibration curves from / to files.

buttons



To select Input Image 2 as background image.

tick box



Spin boxes to set the Min and Max in the Output Image

Image scaling

Pull down menus for



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**.

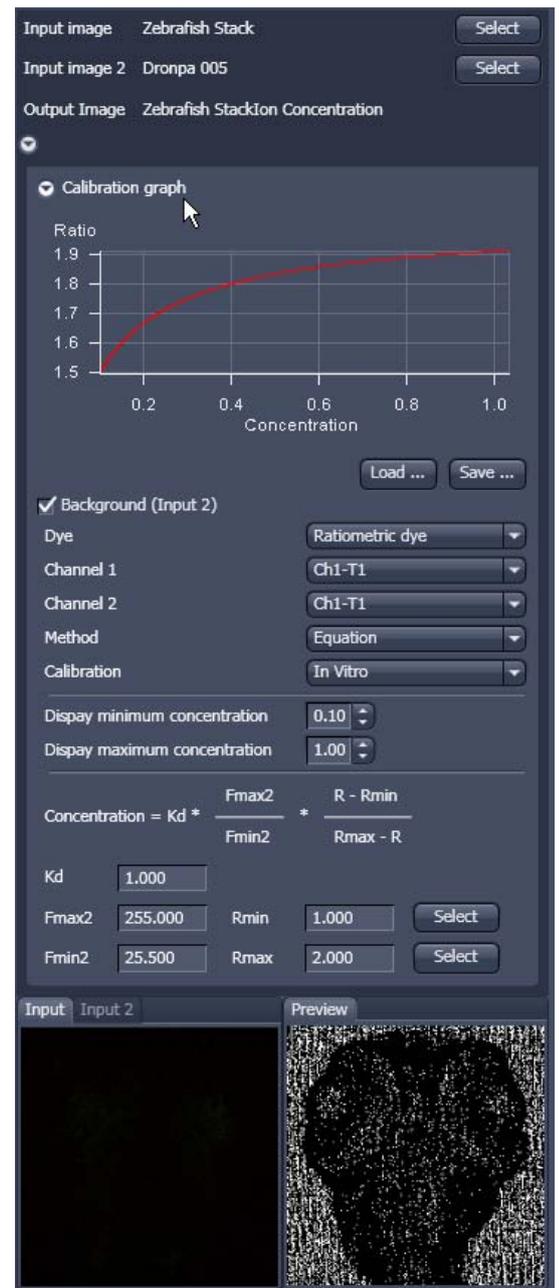


Fig. 5-93 Fully opened Ion Concentration tool window - Ratiometric dye in equation mode

5.3.9.1 Single Wavelength Dyes – Offline Calibration

- Subtract background/autofluorescence image from raw images to obtain better raw data to start with (Fig. 5-94).
- Perform equation or titration calibration (compare F with a calibration curve → titration calibration or put F values in calibration formula).

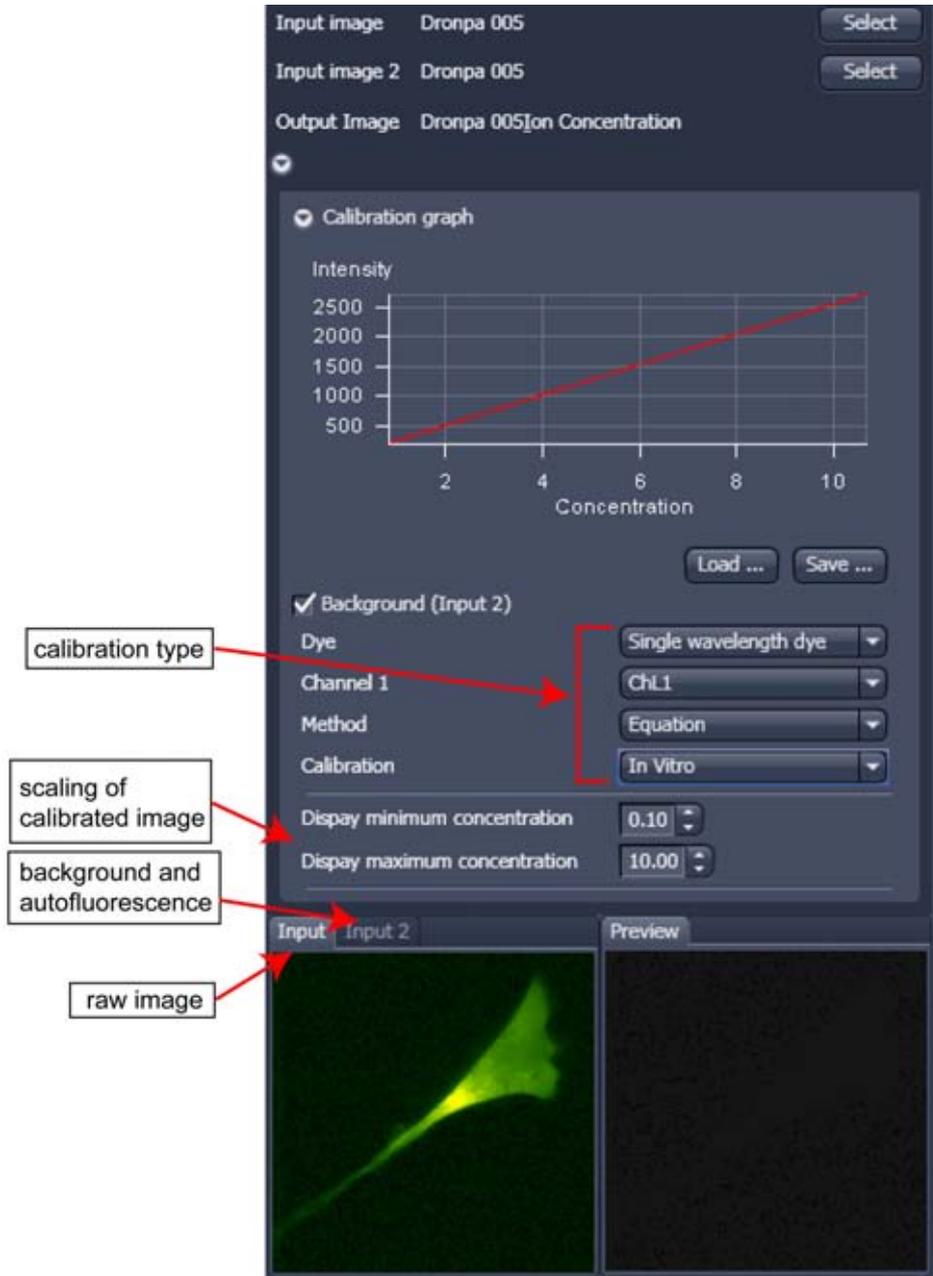


Fig. 5-94 Ion Concentration panel

5.3.9.2 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. 5-95)

$$R(t1) = F1(t1) / F2(t1), R(t2) = F1(t2) / F2(t2) \dots$$

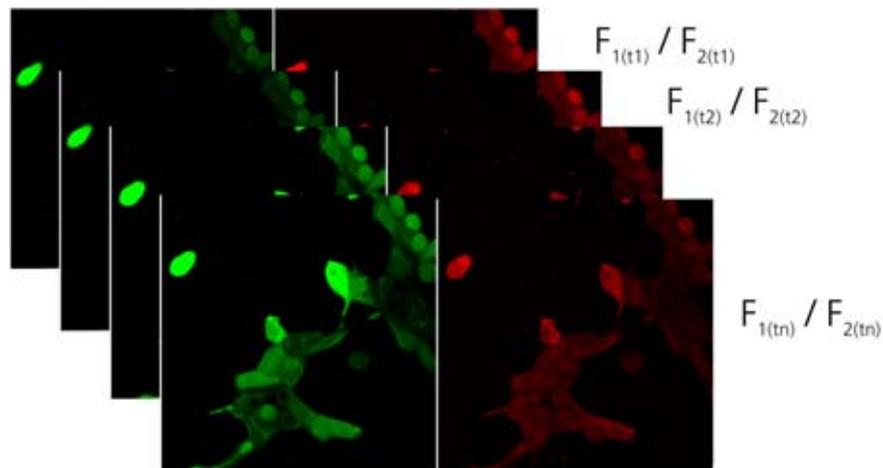


Fig. 5-95 Ratiometric Dyes – Online ratio

Ratiometric Dyes - Calibration (Fig. 5-96)

- Subtract background/autofluorescence images from raw images to obtain

$$R_{\text{korr}} [= (F1 - F1_{\text{Background}}) / (F2 - F2_{\text{Background}})]$$

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).

The figure displays two screenshots of the software interface for ratiometric dye calibration. Both screenshots show the same input images (Dronpa 005) and output image (Dronpa 005Ion Concentration). The left screenshot shows the 'Titration' method selected, and the right screenshot shows the 'Equation' method selected. Both screenshots show a calibration graph with 'Ratio' on the y-axis and 'Concentration' on the x-axis. The left graph shows a curve starting at (0,0) and leveling off at a ratio of approximately 300. The right graph shows a curve starting at a ratio of approximately 1.5 and leveling off at a ratio of approximately 1.9. The right screenshot also shows a table of parameters for the equation method, which is highlighted with a red box. The equation is $Concentration = Kd * \frac{Fmax2}{Fmin2} * \frac{R - Rmin}{Rmax - R}$. The parameters are: Kd = 1.000, Fmax2 = 255.000, Fmin2 = 25.500, Rmin = 1.000, and Rmax = 2.000. A red arrow points from the 'Equation' method's formula to the 'Input 2' image in the preview area, with a label 'calibration type'.

Fig. 5-96 Ratiometric Dyes - Calibration

Ratiometric Dyes - Equation Calibration (Grynkiewicz) (Fig. 5-97)

Fura-2, Indo-1,..

K_D (dissociation constant) taken from literature

R_{min}: derived from ion-free state of the dye (e.g. 0 Ca²⁺)

R_{max}: derived from ion-bound state of the dye (e.g. saturated with Ca²⁺)

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)

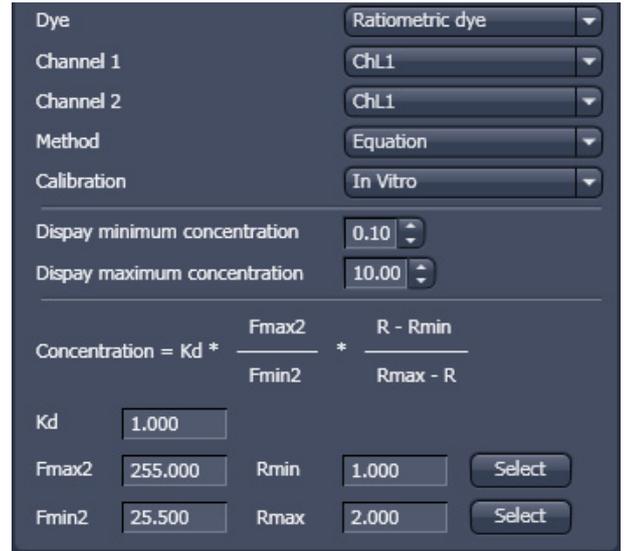


Fig. 5-97 Ion Concentration - Ratiometric dye, Equation Calibration

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)

5.3.10 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.

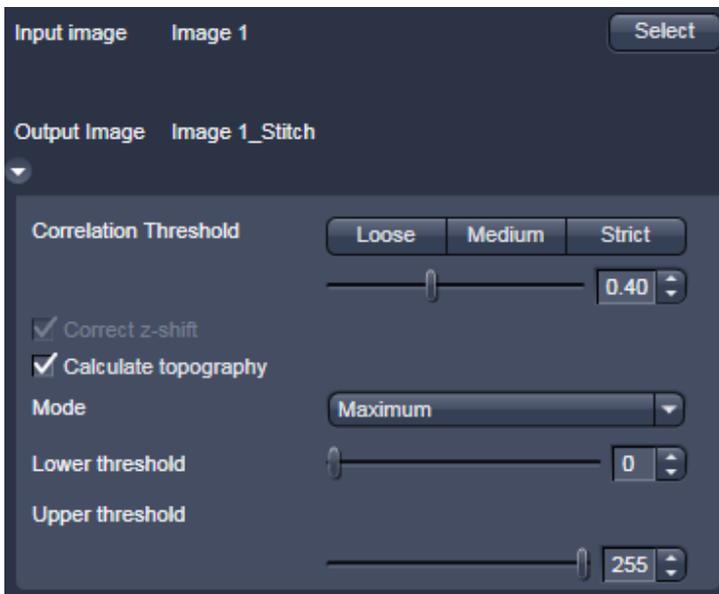


Fig. 5-98 Stitch function

Calculate Topography provides a topographic image of the 3D stitched image data. This calculation can be done in several modes (see also section **Topography View**).

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.

5.3.11 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)**.

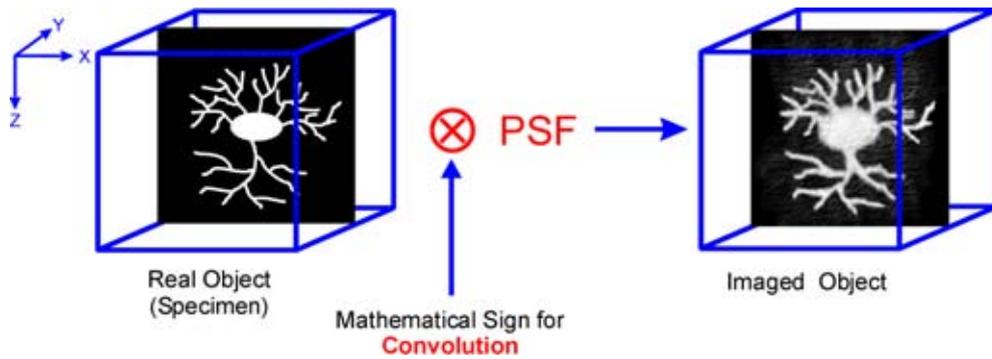


Fig. 5-99 Point Spread Function (PSF)

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:

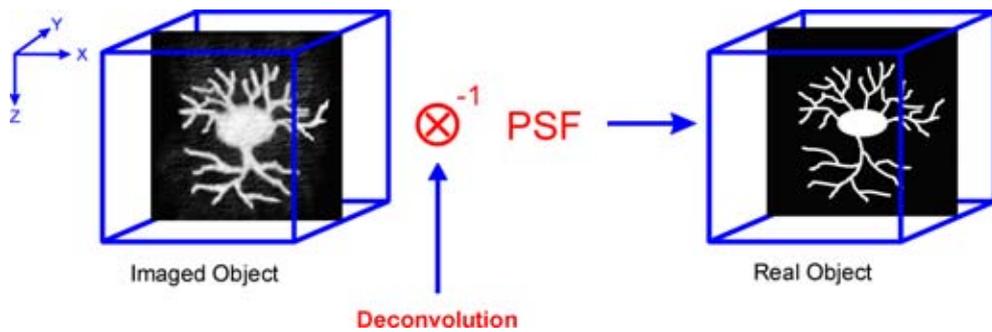


Fig. 5-100 Point Spread Function (PSF)

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 .

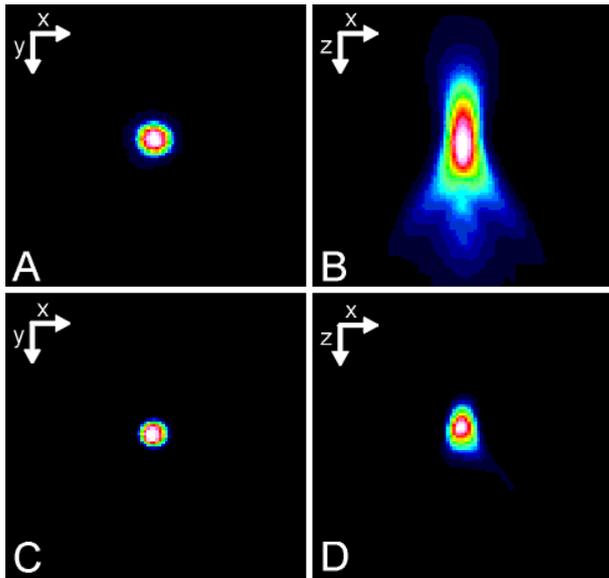


Fig. 5-101 Image of a fluorescent bead with a diameter of $1\mu\text{m}$ before deconvolution (A,B) and after deconvolution (C,D)

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.

To open the **Deconvolution** tools click **Deconvolution** (Fig. 5-102).

5.3.11.1 Methods

The **Method** pull down menu (Fig. 5-102) 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**.



Fig. 5-102 Deconvolution window

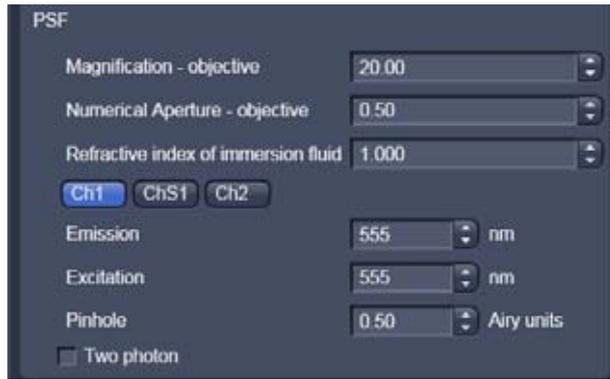


Fig. 5-103 PSF data input fields

5.3.11.2 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.

5.3.12 Copy

The **Copy** function provides means to copy or duplicate images.

Copy Channel (Fig. 5-104) 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.

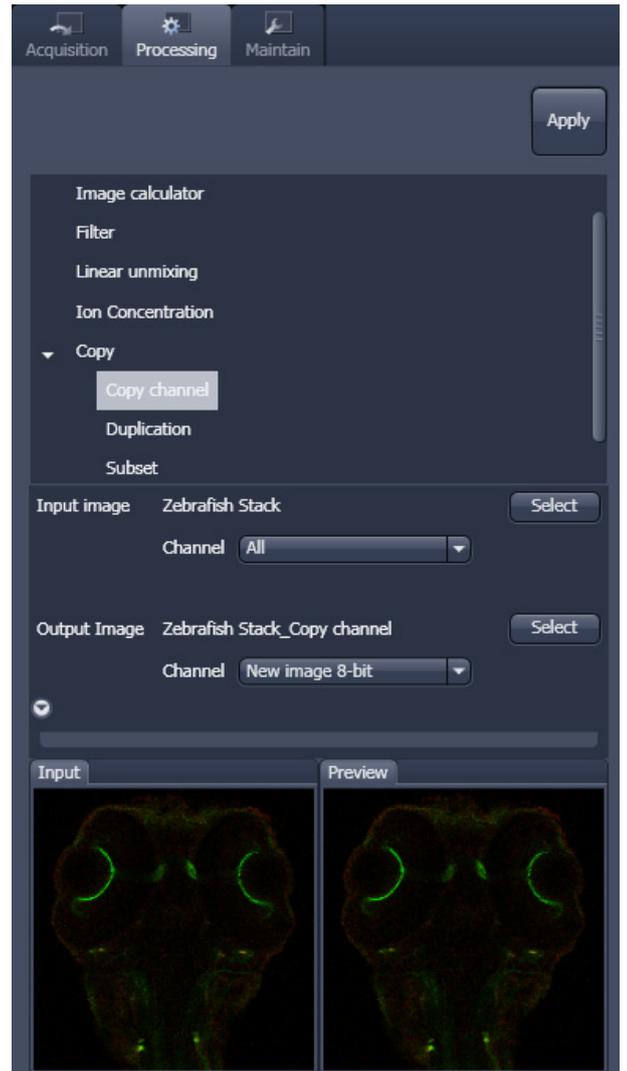


Fig. 5-104 Copy window

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.



Fig. 5-105 Subset tool with loaded Z-Stack

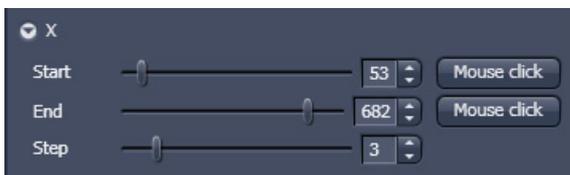


Fig. 5-106 Subset panel for the x coordinate

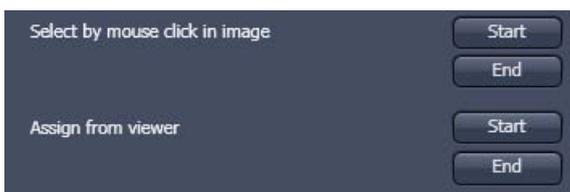


Fig. 5-107 Subset panel for "visible"

Subset

The **Subset** function is a set of tools to truncate a multidimensional data set in all available coordinates to the desired size (Fig. 5-105).

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. 5-106) brings up three sliders to set the **Start** and **End** coordinates of the new data set.

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, etc). The panel **Selection for all visible coordinates** is shown in Fig. 5-107. 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 in the 2D view, z 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.

5.3.13 Adjust

5.3.13.1 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 **Center Screen Area / Image Containers - Display and Image Analysis**) are permanently written to the image file (Fig. 5-108).

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.

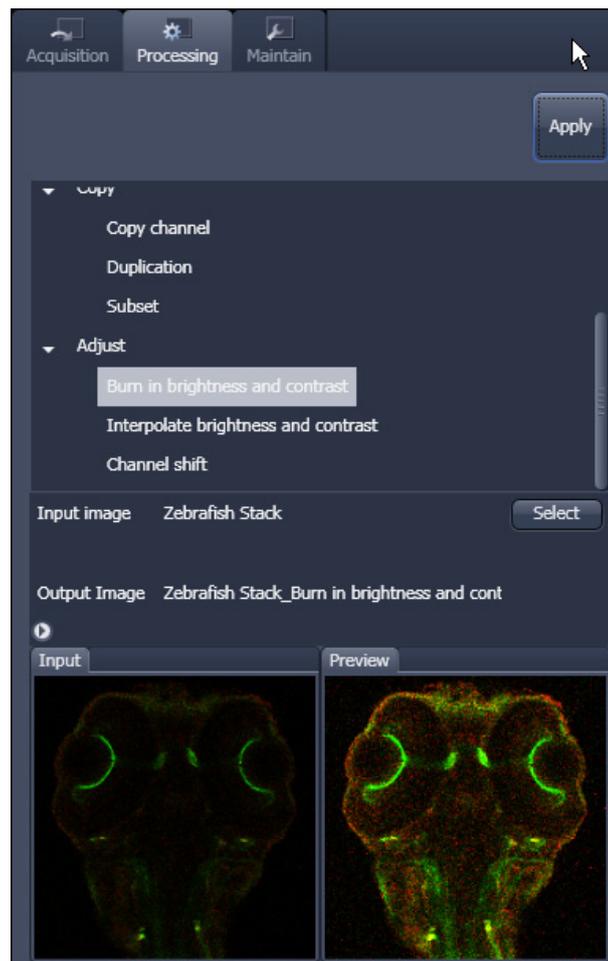


Fig. 5-108 Burn in Brightness and contrast tool

5.3.13.2 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. 5-109).

The **Select** button selects the active image in the **Image Display** as the Input Image to be processed.

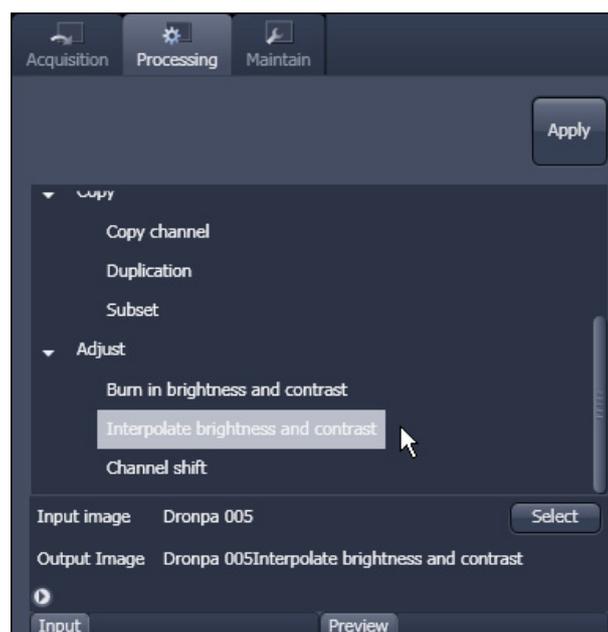


Fig. 5-109 Interpolate brightness and contrast tool

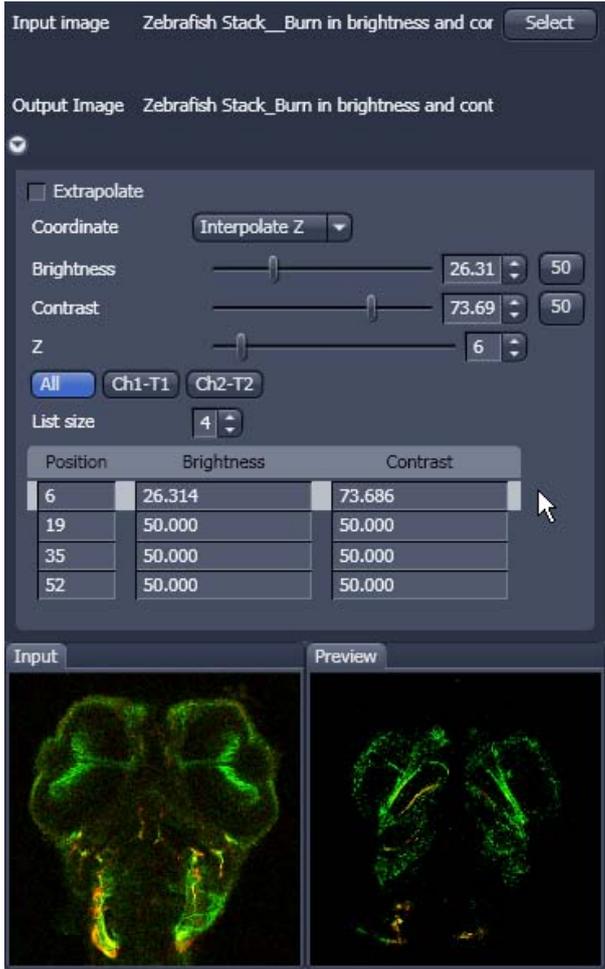


Fig. 5-110 Interpolation brightness and contrast tool with loaded Z-Stack

In the **Interpolation** panel (Fig. 5-110) 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** 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.

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.

5.3.13.3 Channel Shift

Channel Shift is used to produce a congruent image with relation to the pixels of the various channels (Fig. 5-111).

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.

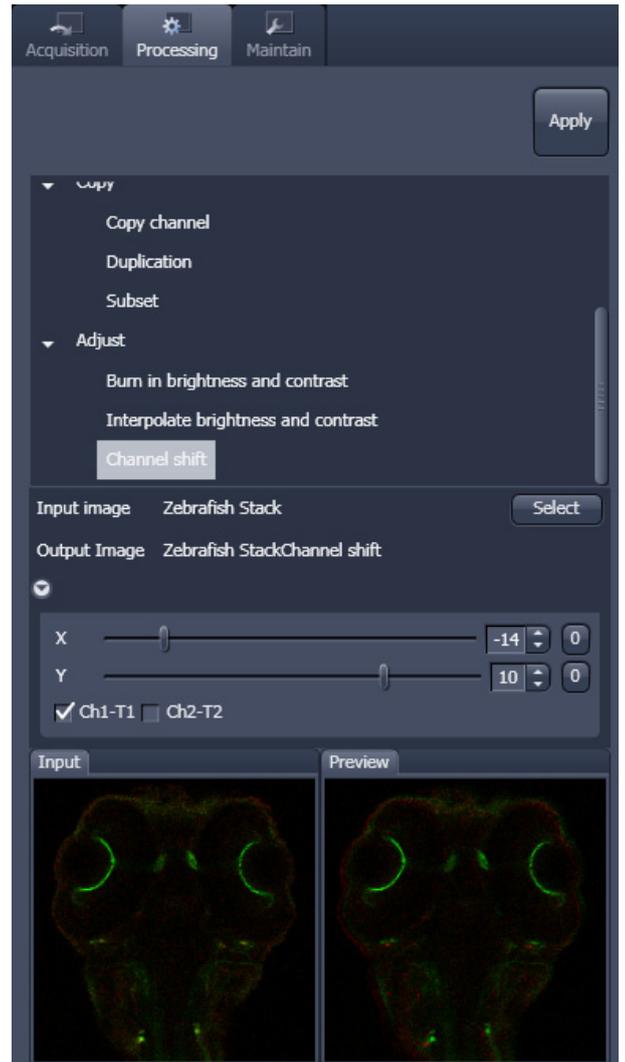


Fig. 5-111 Channel Shift window

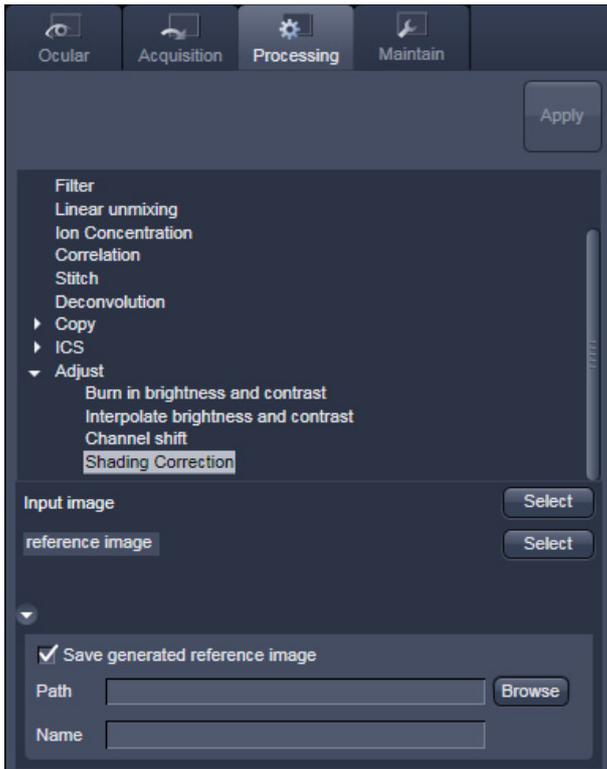


Fig. 5-112 Shading Correction interface

5.3.13.4 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 must 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.

5.4 Maintain

The **Maintain** tool group on the **Maintain** tab provides functions to adjust hardware components of the system.

5.4.1 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. 5-113).

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. 5-113 Adjust Pinhole and Collimator tool

5.4.1.1 Pinholes Section Panel

The **Pinholes Section** panel has the following options:

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 arrow buttons
Position X Vis [μm] slider	
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 Automatically button:	Automatic pinhole adjustment (not available in some systems).
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.

5.4.1.2 Collimators Section Panel

The **Collimators Section** panel has the following options (Fig. 5-114):

- 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. 5-114 Collimator section panel

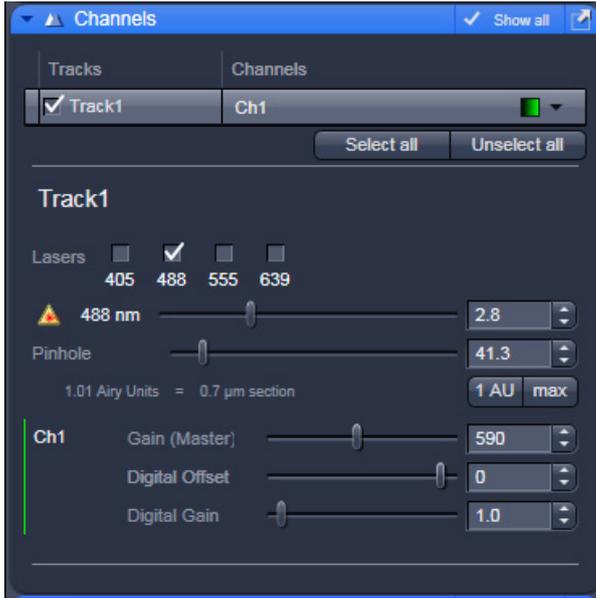


Fig. 5-115 Channels tool

5.4.1.3 How to Adjust the Pinhole

Adjustment of pinholes can be performed manually or automatically.

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. 5-115).

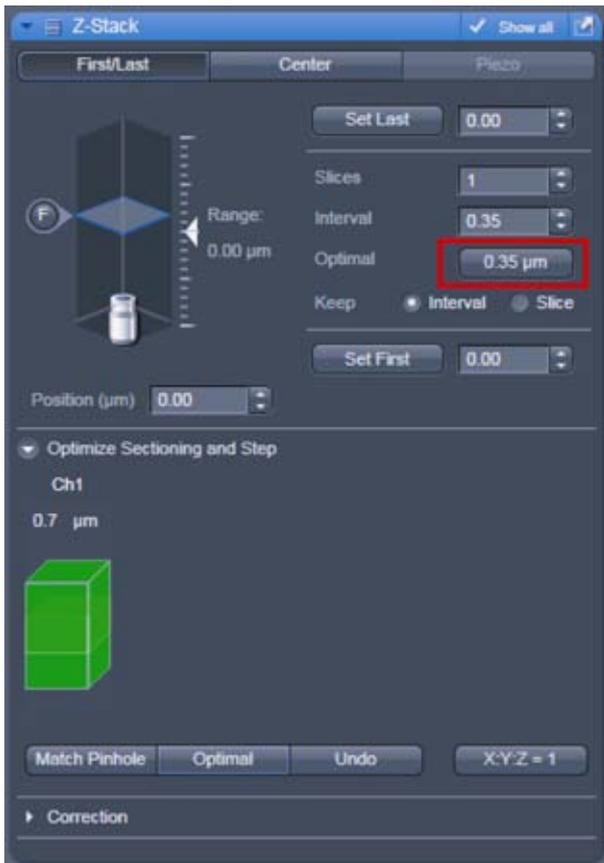


Fig. 5-116 Z-Stack tool

- 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. 5-116).



Image optimization can be effected with the **Range Indicator** or in the **Line-Scan** mode.

- 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. 5-117).
- 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.

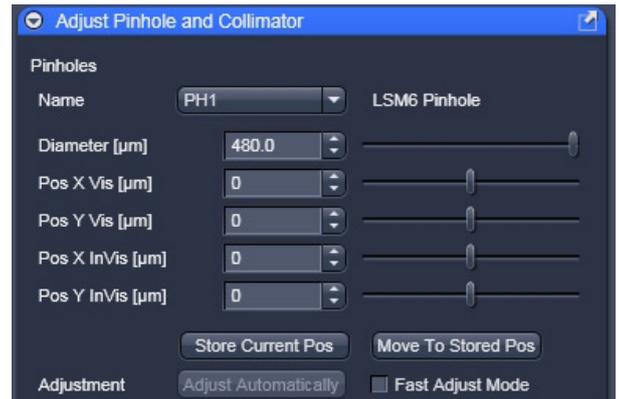


Fig. 5-117 Manual pinhole adjustment

Automatic pinhole adjustment:

The automatic adjustment allows the LSM 710 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 **Adjustment Adjust Automatically** button.
 - The **Requirements for Adjustment** window will then appear.
- Meet the requirements listed in the **Requirements for Adjustment** window (Fig. 5-118). 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. 5-119). 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.

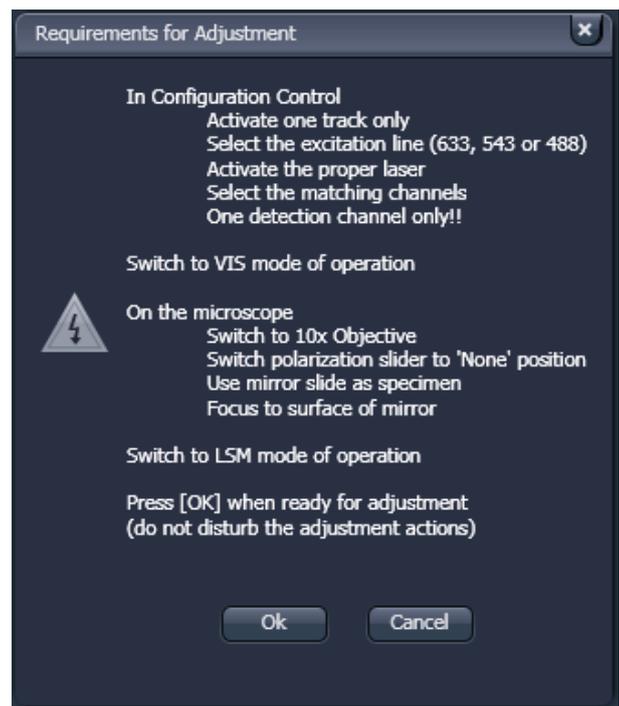


Fig. 5-118 Requirements for Adjustment window

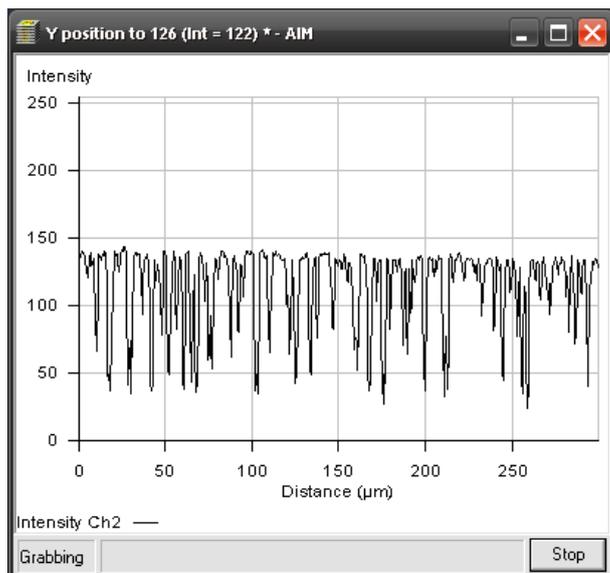


Fig. 5-119 Pinhole adjustment scan window



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).

5.4.2 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. 5-120).

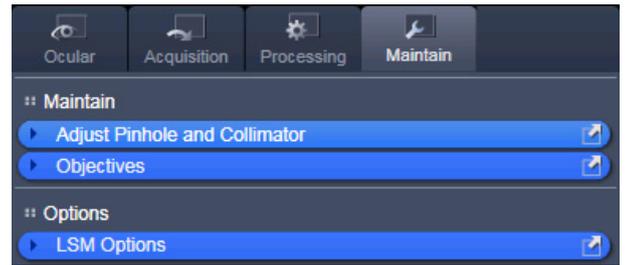


Fig. 5-120 Maintain and Options tool groups

5.4.2.1 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. 5-121).
- 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. 5-122).
- 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.

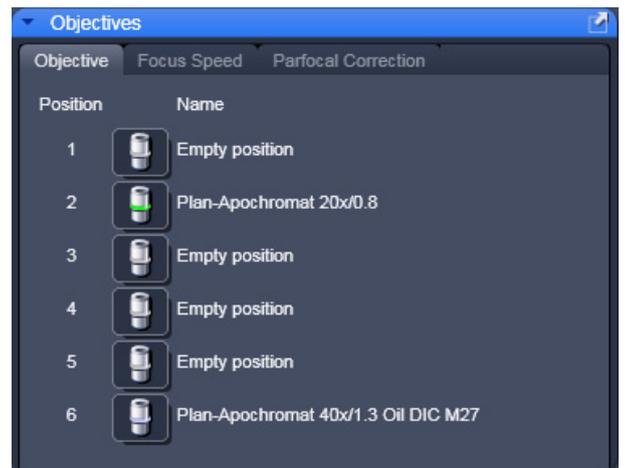


Fig. 5-121 Objectives tool

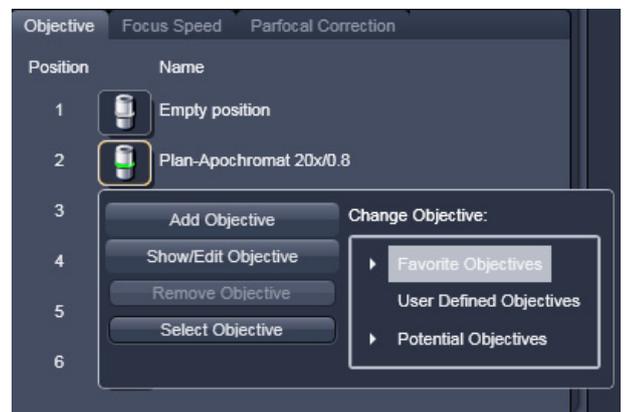


Fig. 5-122 Change objective window

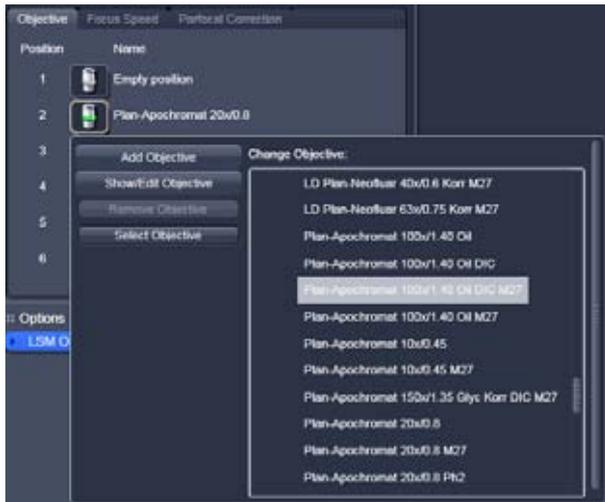


Fig. 5-123 Add Objective window – Potential Objectives

5.4.2.2 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. 5-123).
 - An expansion (**Create new Objective**) window is opened (Fig. 5-124).
- 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**.

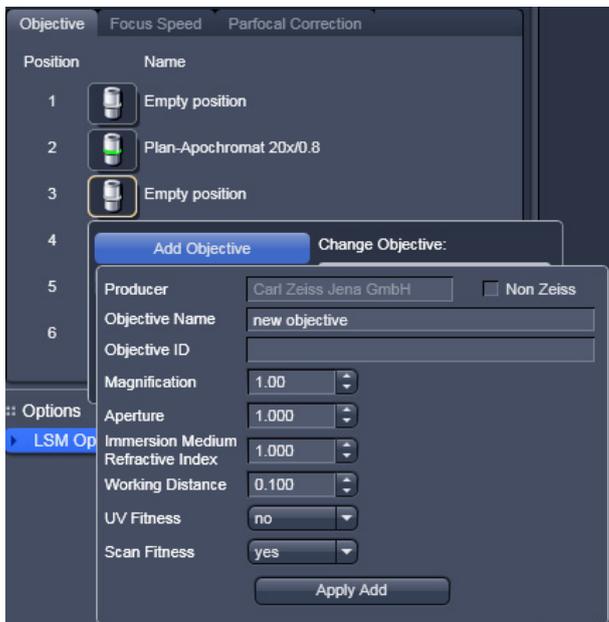


Fig. 5-124 Editing a new objective



If you have activated the **Non Zeiss** check box, objectives from other manufacturers can also be included in the database.

5.4.2.3 Remove Objective

You can remove user-defined objectives. Hence, all objectives in the **User Defined Objectives** directory can be deleted (see Fig. 5-125).

- 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**.

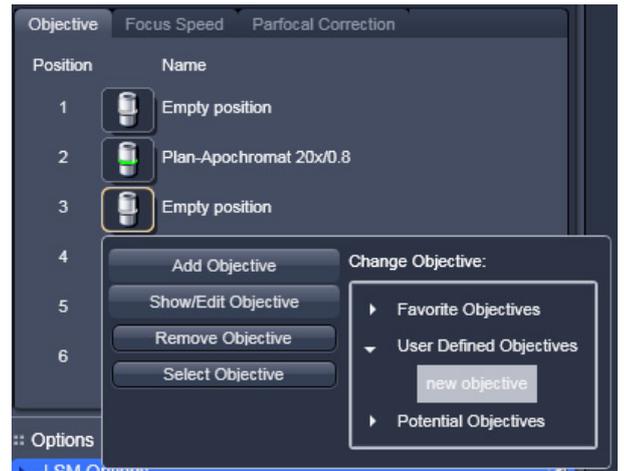


Fig. 5-125 Remove an objective

5.4.2.4 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.

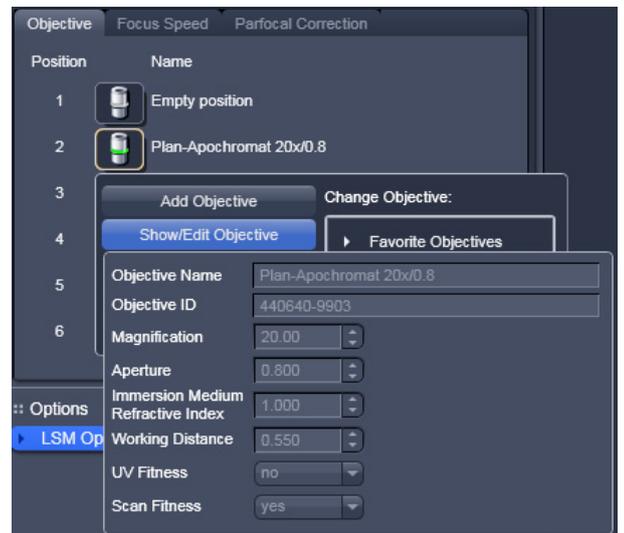


Fig. 5-126 Edit an objective

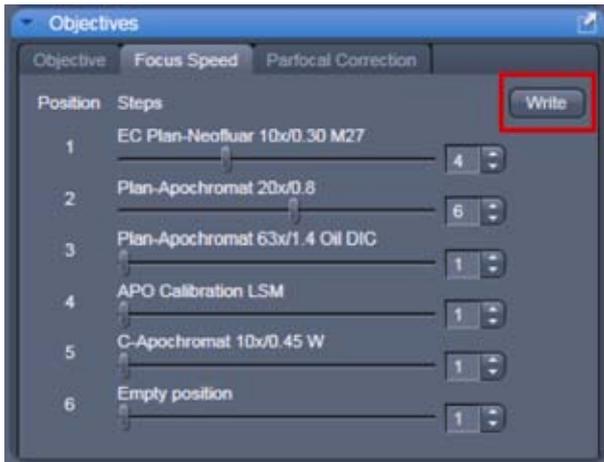


Fig. 5-127 Focus speed setting

5.4.2.5 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.

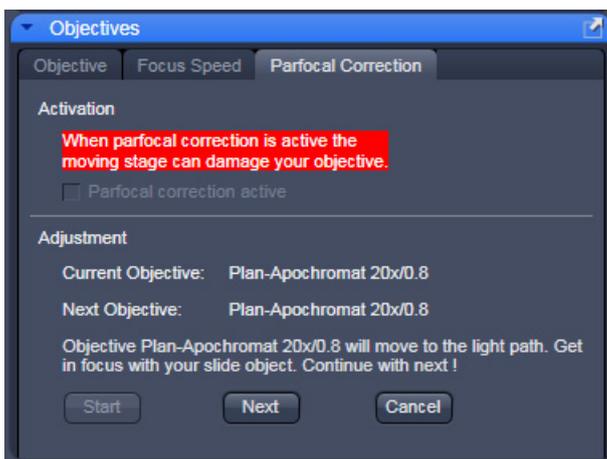


Fig. 5-128 Parfocal correction

5.4.2.6 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!

5.4.3 Maintain Tab: LSM Options

In the **LSM Options** tool the following user-accessible program **Settings** of the ZEN software are available (see Fig. 5-129):

- **Load configuration**
 - **Re-use**
 - **Hardware**
 - **Image display**
- Click on the respective register to display the panel.

(1) Load configuration

Load configuration (see Fig. 5-129) lists parameters to be taken into consideration (if checked active) for loading an imaging configuration.

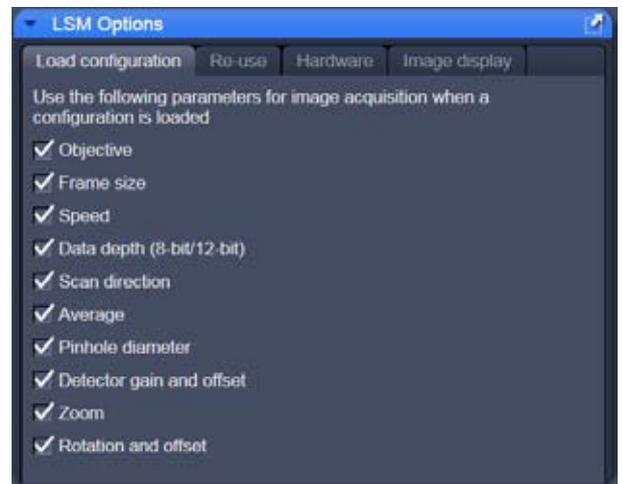


Fig. 5-129 Options tool with load configurations panel opened

(2) Re-use

Within the **Re-Use** panel (see Fig. 5-130) you can determine whether the objective setting and/or the collimator setting stored with the image should be taken over when the **Reuse** function is used.

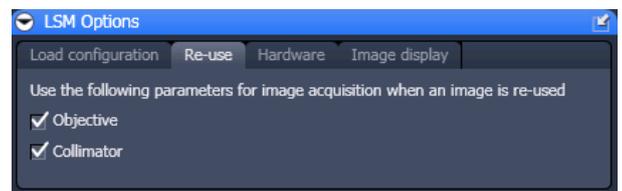


Fig. 5-130 Options tool with re-use panel opened



Fig. 5-131 Options tool with hardware panel opened

(3) Hardware

Press the **Load configuration** register to open the panel.

The **Hardware** tab (see Fig. 5-131) allows you to set several 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.

Online scanner calibration is on as default setting. This function assures the calibration of the scanners for bidirectional scanning. If that function is not checked it might be required to calibrate the scanners in x and y manually when bidirectional scanning is used for image acquisition.

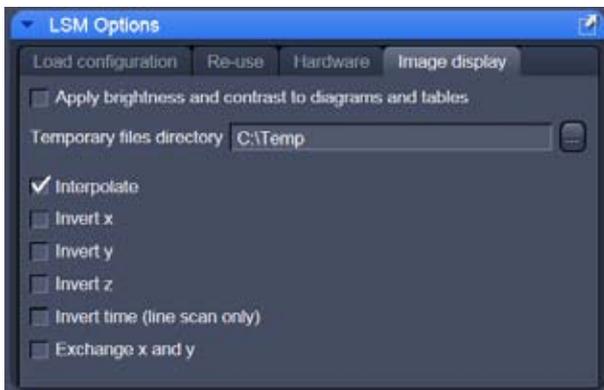


Fig. 5-132 Options tool with image display panel opened

(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. 5-132) 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.

5.4.4 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 710. In the **Menu bar**, click on **Maintain**. This opens the Maintain list (Fig. 5-133).



Fig. 5-133 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.

5.4.4.1 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. 5-134).
- 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.

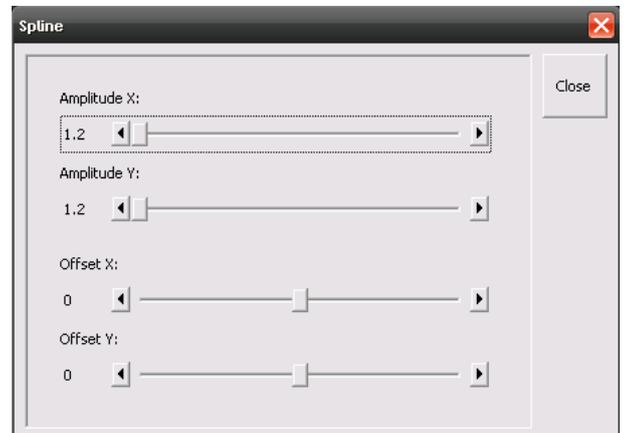


Fig. 5-134 Spline window

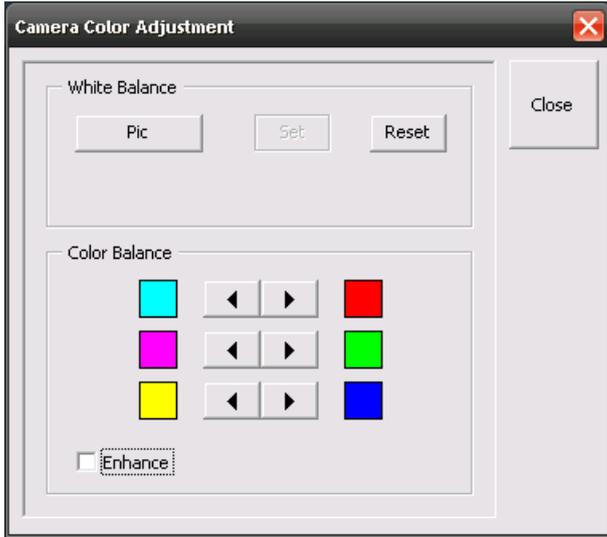


Fig. 5-135 Camera Color Adjustment window

5.4.4.2 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. 5-135).

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.

5.4.4.3 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.

5.4.4.4 Test Grid

The **TestGrid** function is for servicing purposes only and may only be performed by authorized personnel. Its access is therefore password-protected.

6 Center Screen Area / Image Containers - Display and Image Analysis

6.1 Structure and Functional Concept of the Central Screen Area and the Image Display Container

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. 6-1 shows the layout with one or two Image Containers.

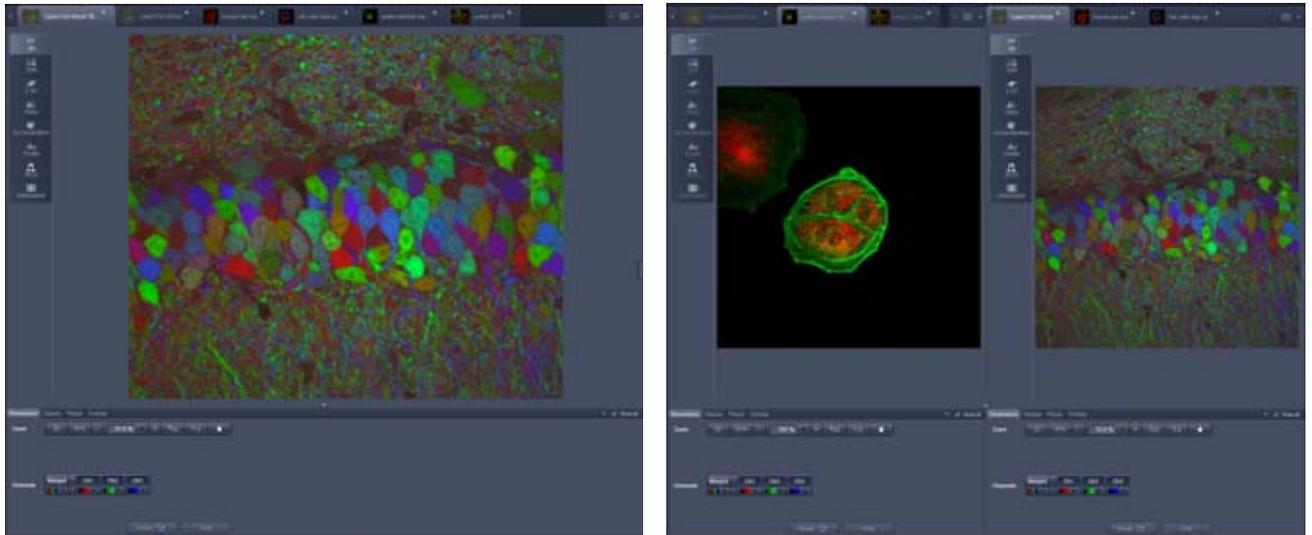


Fig. 6-1 The Centre Screen Area of the ZEN Main Application window – Left: 1 Image Container, Right: 2 Image Containers

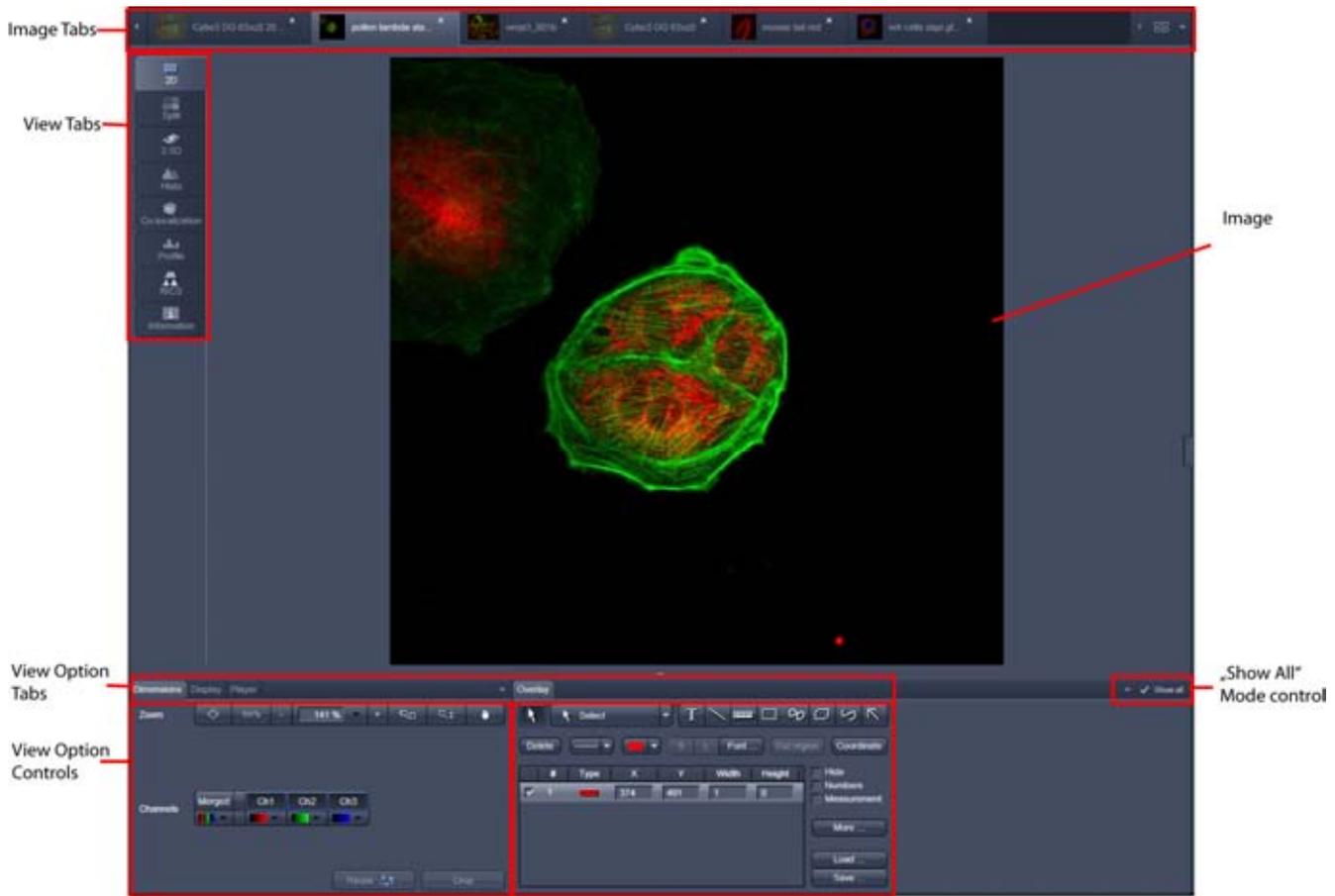


Fig. 6-2 Image Display window; Select - Overlay

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. 6-3/a), the name and a small image thumbnail (Fig. 6-3/b) or the file name and a larger image thumbnail (Fig. 6-3/c). This can be configured in the Container Context menu (see Fig. 6-5).



Fig. 6-3 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. **Histogram** 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 underlying area is displayed in blue:

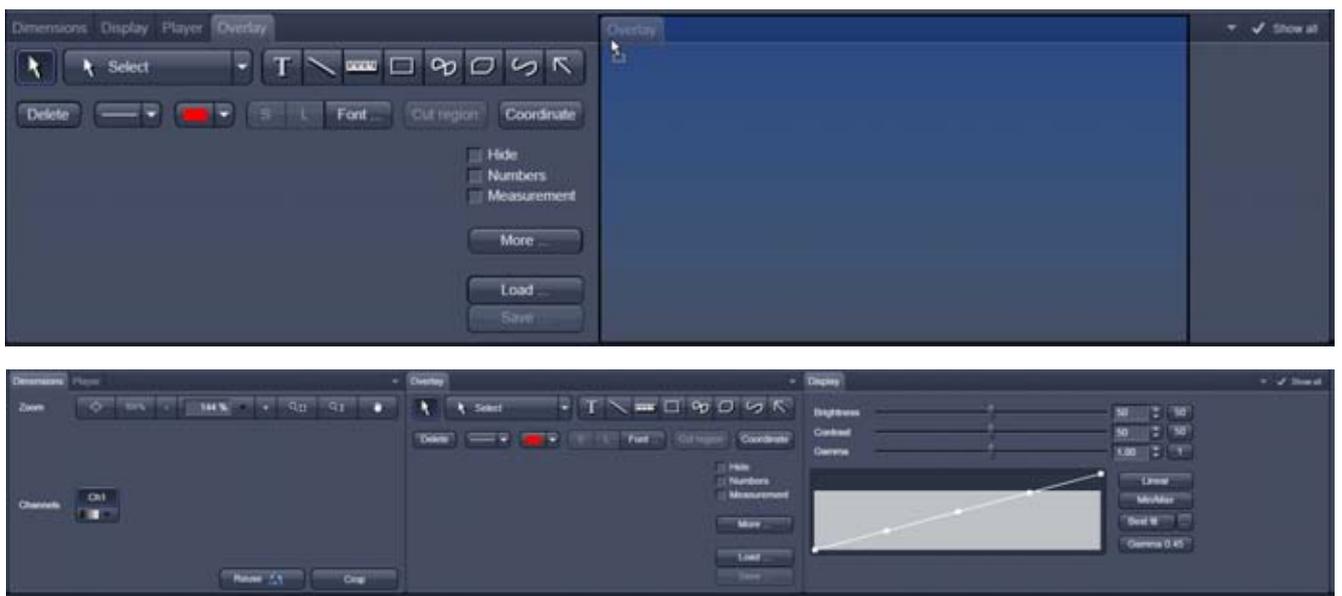


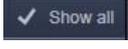
Fig. 6-4 General View Options control blocks

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 **Subset** and/or **Maintain Tab: LSM Options**.

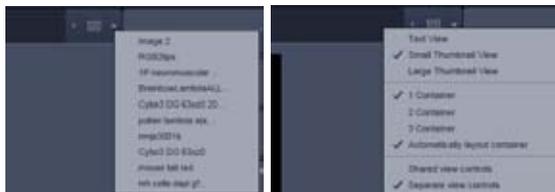


Fig. 6-5 Open Image pull down and context menu of the Central Screen Area

Image Handling and Container Layout Configuration:

This little control area (Fig. 6-5) 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 **Right Tool Area: Data Management and Storage**). 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  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. 6-6).

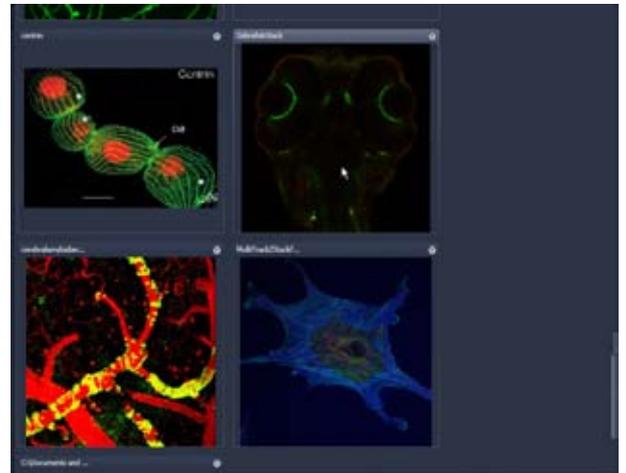


Fig. 6-6 Expose Mode Display

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.

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.

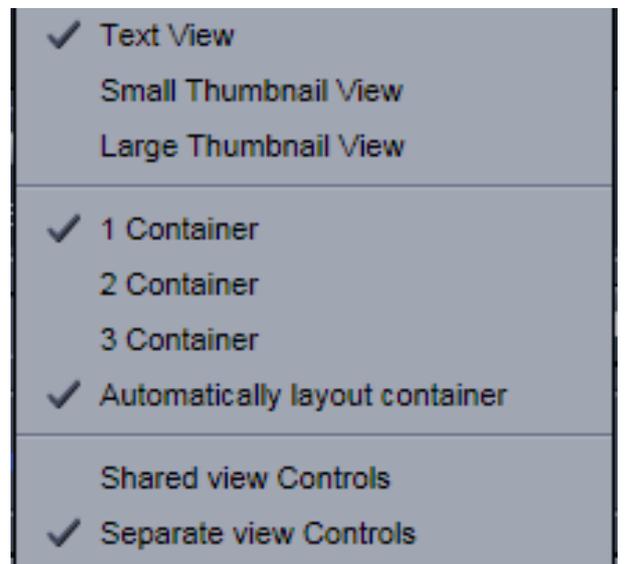


Fig. 6-7 Container Context Menu

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. 6-8 View Options control block - Dimensions

6.2.1 Dimensions

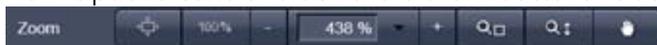
The **Dimensions View Options** control block holds the tools to modify the image display with respect to the (multiple) dimensions of the image data set.

The sections (slices) in the dimensions can be scrolled with sliders (z-position and Time in Fig. 6-8 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 8 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.



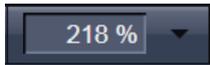
Zoom-Mouse allows you to enlarge / reduce the zoom factor of an image using the left / right mouse button, provided that the cursor is inside the image.



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.

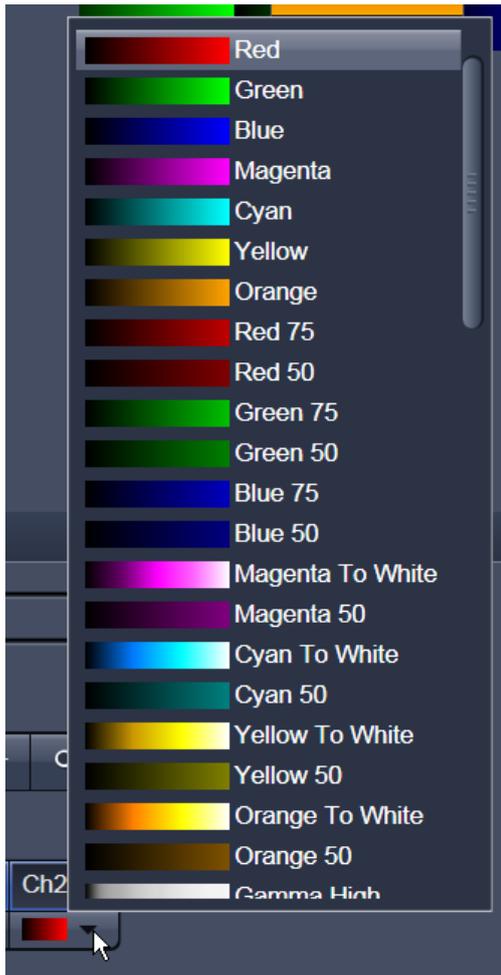


Display box: type in your magnification value in percent directly.

Slider



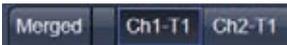
The zoom factor can be set by moving the slider. The display box displays the current zoom factor in %. 100% corresponds to the original size.



(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:

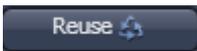


Clicking on the  buttons switches the respective channels on/off in the display.

Clicking on the  buttons switches forth and back between the assigned LUT and the **Range Indicator**. Clicking on the little black arrow on the  buttons opens a selection of color look-up tables to assign a color or LUT to the respective channel (see Fig. 6-9). Any changes on these buttons affect the displayed image immediately.

Fig. 6-9 Image Display - Dimensions - Channels

(3) Reuse

Clicking the  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.

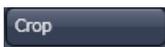
The acquisition parameters of an image are displayed in the Information View (section **Information View**).

In the **Maintain** tab (section **Maintain Tab: LSM Options**) (**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 **Tool Group Online Acquisition: Acquisition Mode Tool**).

Click on  will display the **Crop Rectangle** in the **Image Display** (Fig. 6-10). 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  button is de-selected, the scan parameters are reset as well.

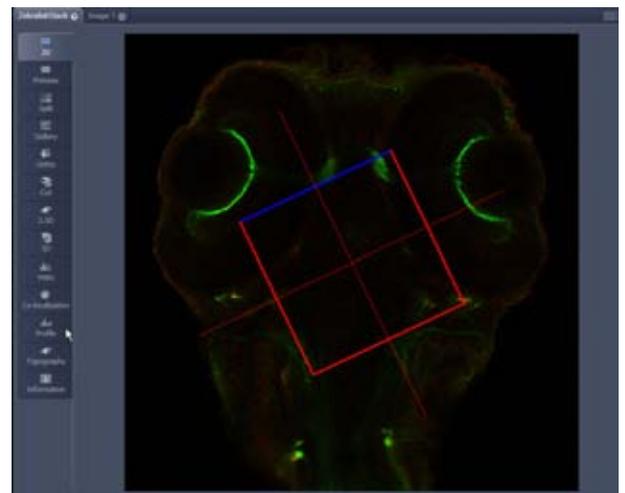


Fig. 6-10 Image Display - Dimensions - Crop

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

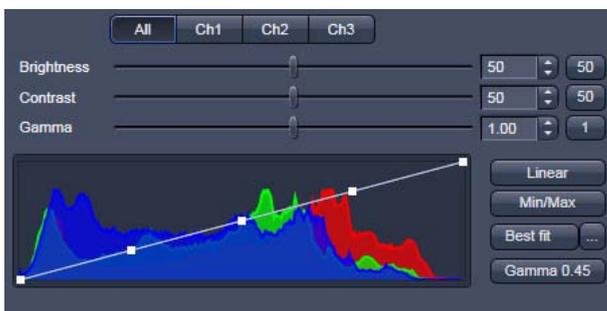


Fig. 6-11 View Options control block - Display

6.2.2 Display

In the **Display** View Option control block, **Brightness**, **Contrast** and **Gamma** of the displayed image can be adjusted (Fig. 6-11). With the **All**, **Ch1**, **Ch2**, **Ch3** 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 block 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 **Processing Tab**).

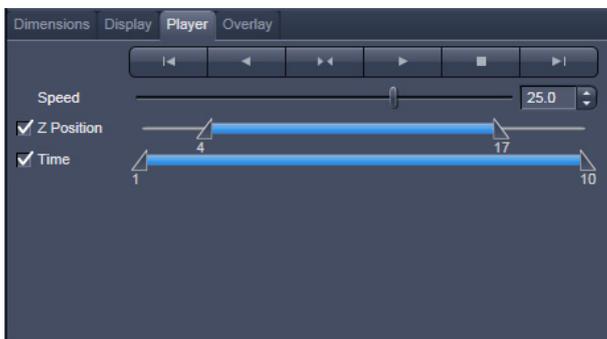


Fig. 6-12 View Options control block - Player

6.2.3 Player

This function allows to

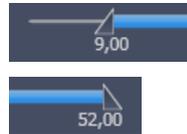
- animate frames of a Z-Stack or a time series
- specify animation parameters such as range and animation speed

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.

The **Player View Option** control block is controlled by the following elements:

Z Position

The **current position** slider is displayed in the View Option control block **Dimensions** and can also be 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.



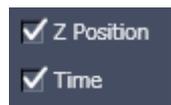
Move to the first slice.



Move to the last slice.

Speed

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.

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).



Fig. 6-13 View Options control block - Overlay

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.

Available functions in the Overlay View Control Block

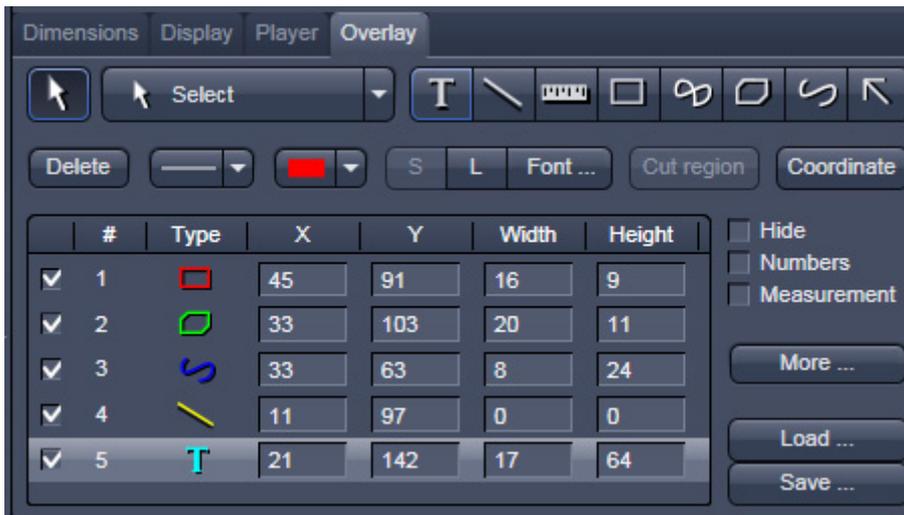


Fig. 6-14 Overlay view control block

The following list describes the most common functions in the **Overlay** view control block. More can be found in the  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.



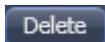
Scale bar: Creation of a horizontal or vertical scale bar with default increments in the Image Display. Click and hold the mouse button for the starting point, drag horizontal or vertical scale, release the mouse button to end the procedure.



Gray tones / color shades button: Generates a rectangle with a display of gray tones or color shades in the image.



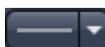
Text (Annotation) tool: Creation of a text box in the Image Display. After clicking on T, the Text window will be displayed, and text can be entered via the keyboard. The Font ... button enables you to select the font style and size in the Font window. The entered text will be displayed in the left upper 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

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.

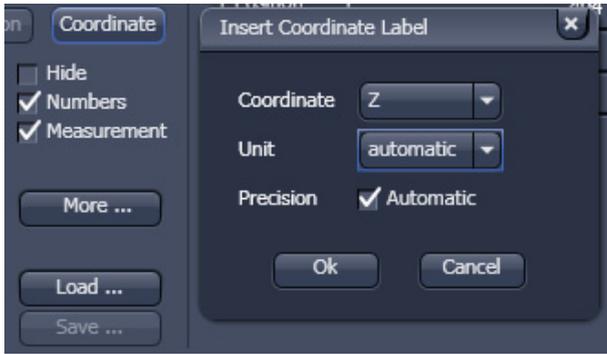
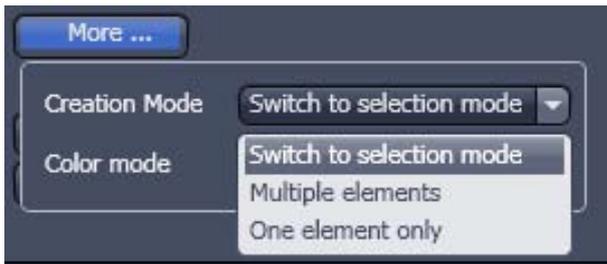


Fig. 6-15 View Options Overlay - Coordinate

To insert a **Coordinate Label** in the image display, click the **Coordinate** button. The coordinate settings dialog (Fig. 6-15) 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. 6-14).



More ...: The **More** options (Fig. 6-16) 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.



Fig. 6-16 View Options Overlay - More

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.

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.

6.2.5 Preview View

The Preview View Type is an enlarged copy of the thumbnail Previews described in the section **Processing Tab**.

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.

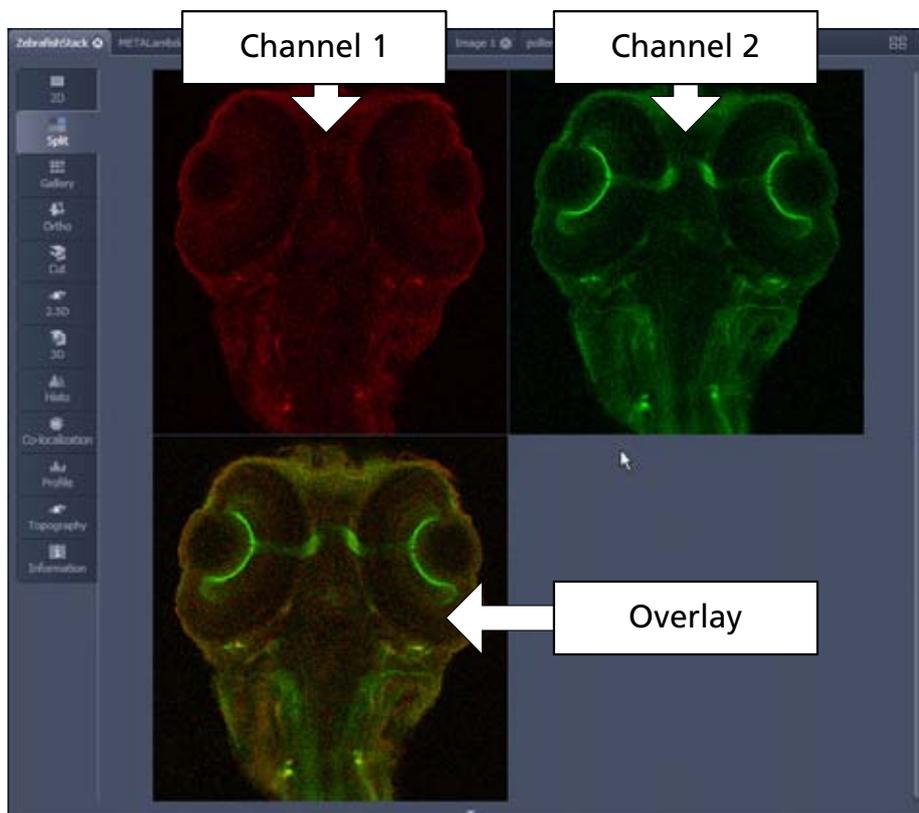


Fig. 6-17 Image Display, Split view type

 This function is useful to optimize the individual channels in a multi channel image acquisition together with the **Range Indicator** palette.

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 **Subset**).

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. 6-18).

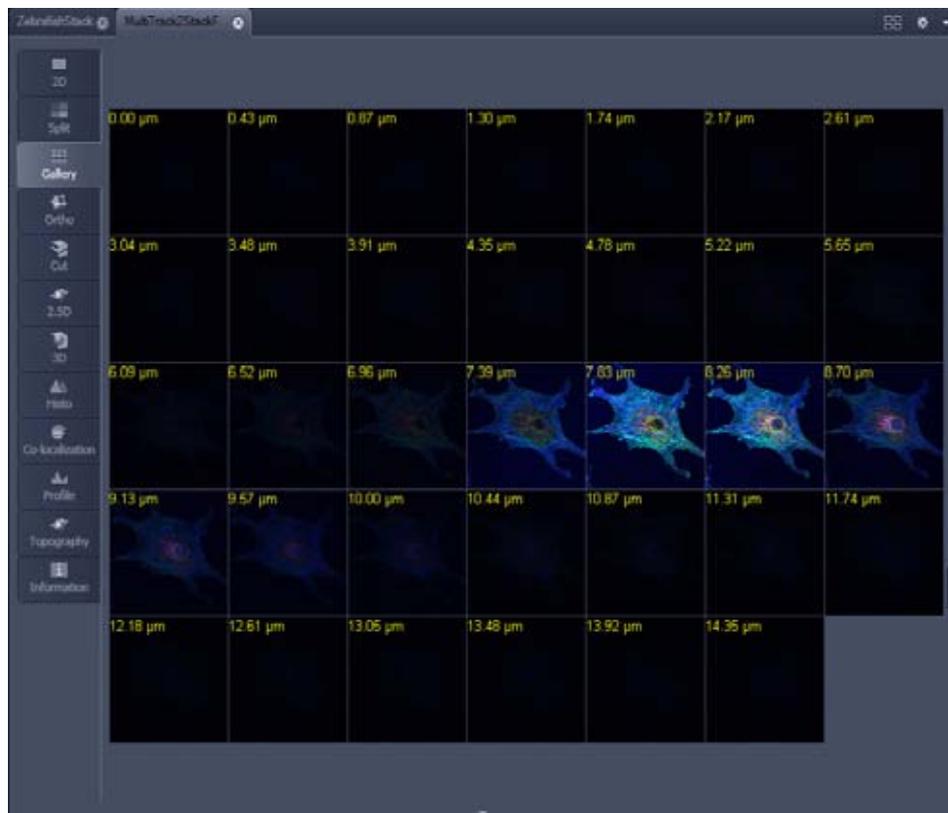


Fig. 6-18 Image Display window - Gallery display

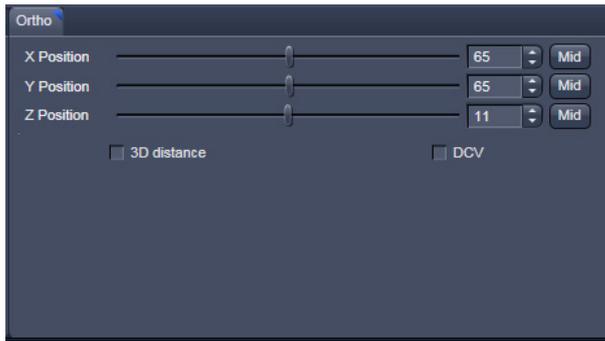


Fig. 6-19 View Options control block - Ortho

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. 6-19).

In the **Ortho** View, section sliders appear in the **Ortho View Option** control block together with orthogonal projections in the image (Fig. 6-20).

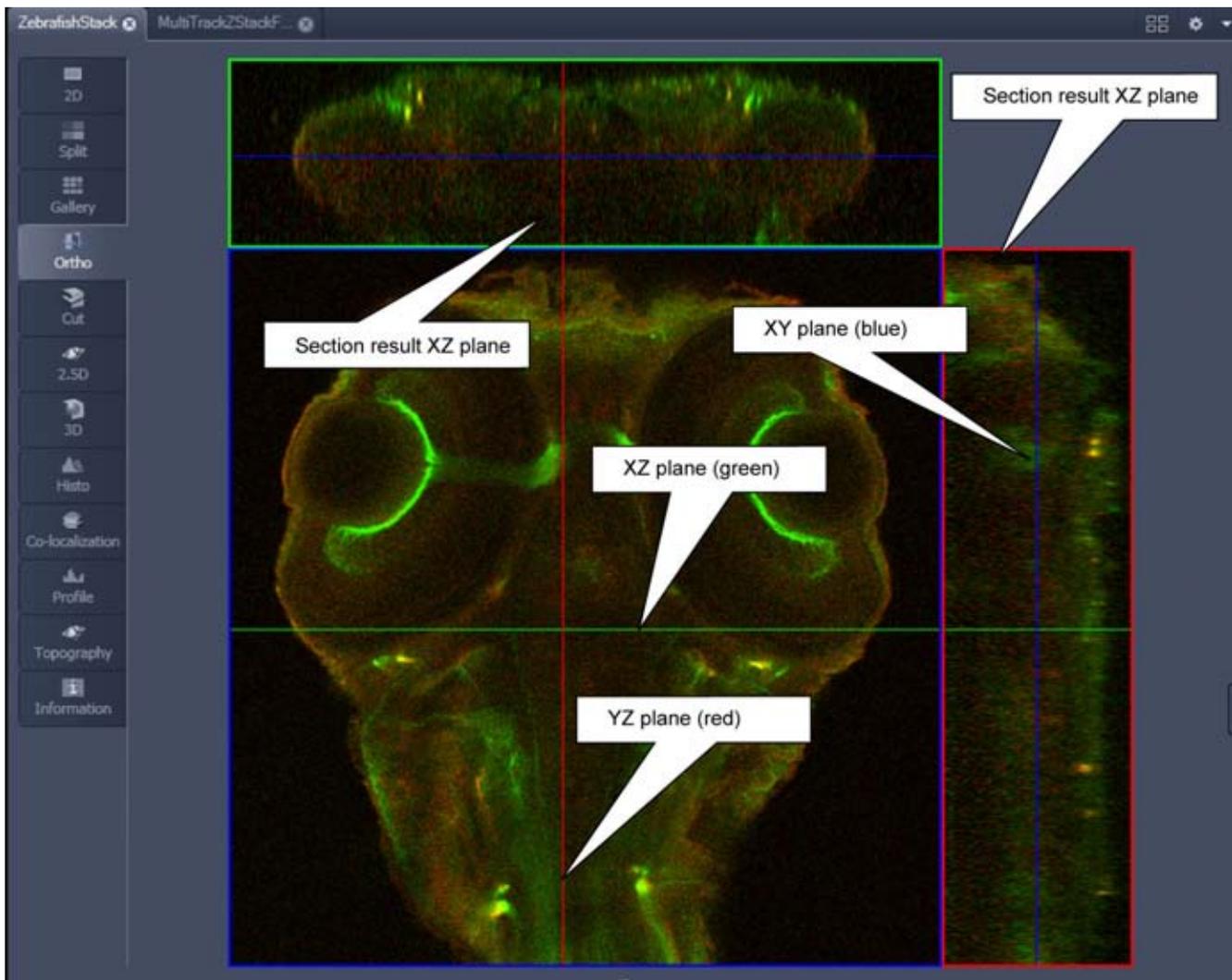


Fig. 6-20 Image Display - Ortho View

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: \leftrightarrow 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 \updownarrow 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 $\leftarrow\rightarrow$ 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.

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. 6-21).
The actual spatial distance is calculated and shown in μm next to the **Mark** button.

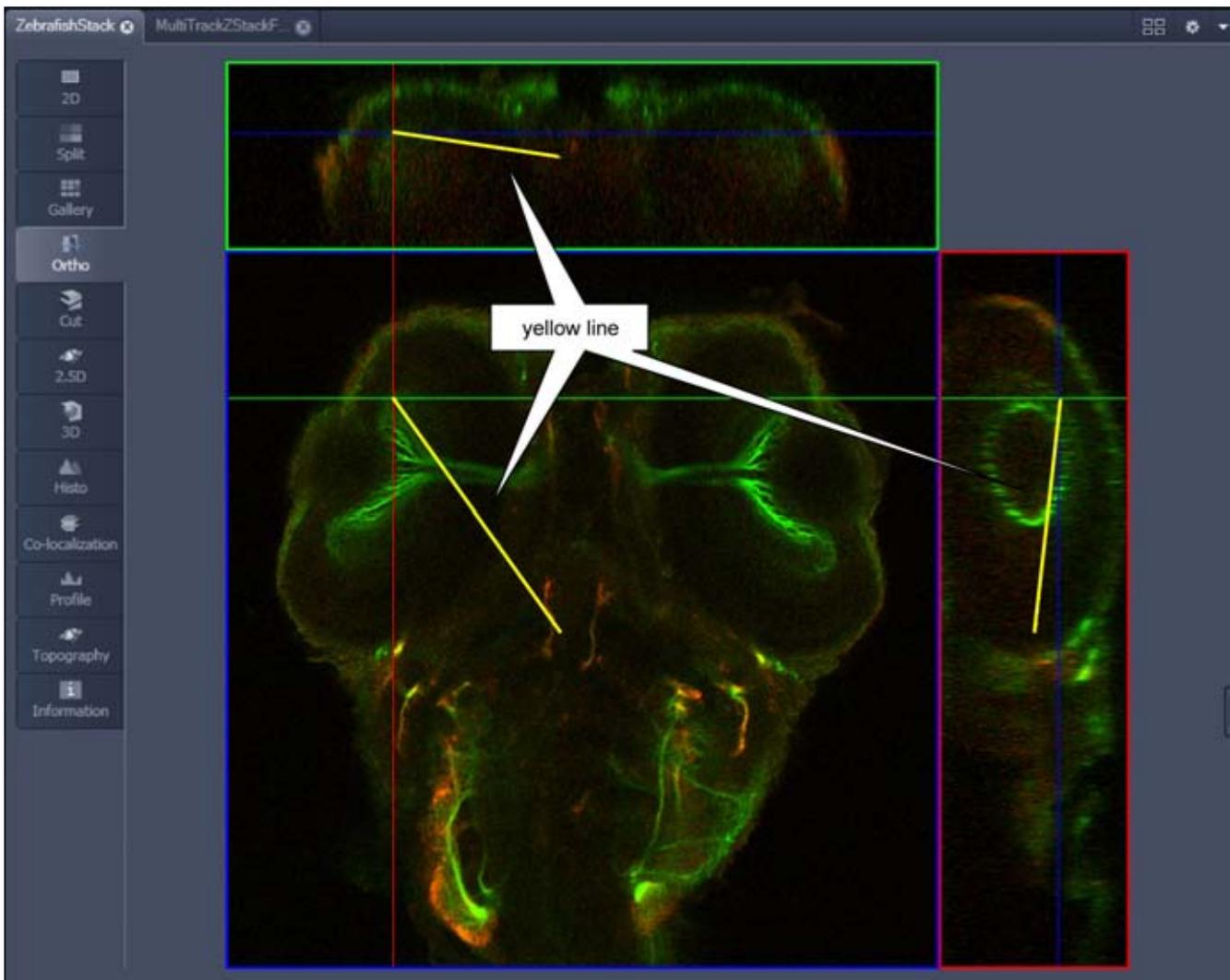


Fig. 6-21 Image Display – Ortho View - 3D Distance Measurement display

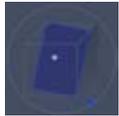
6.6 Cut View

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.

- 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.



Fig. 6-22 Cut View – control block

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 (VisArtplus)** View Type. For a description of the controls in the display window see section **3D Rendering Settings in VisArtplus**.

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.

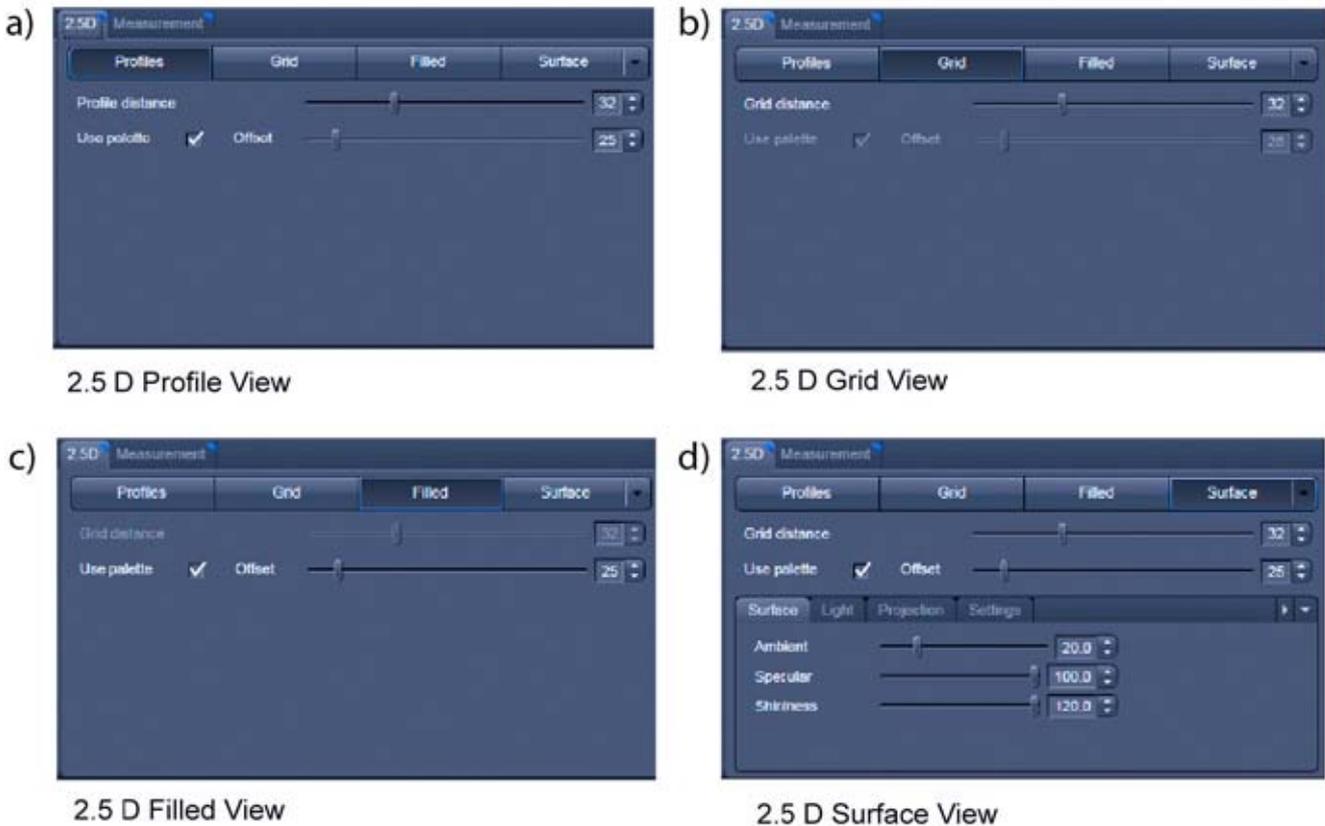


Fig. 6-23 2.5 D View Option control tabs

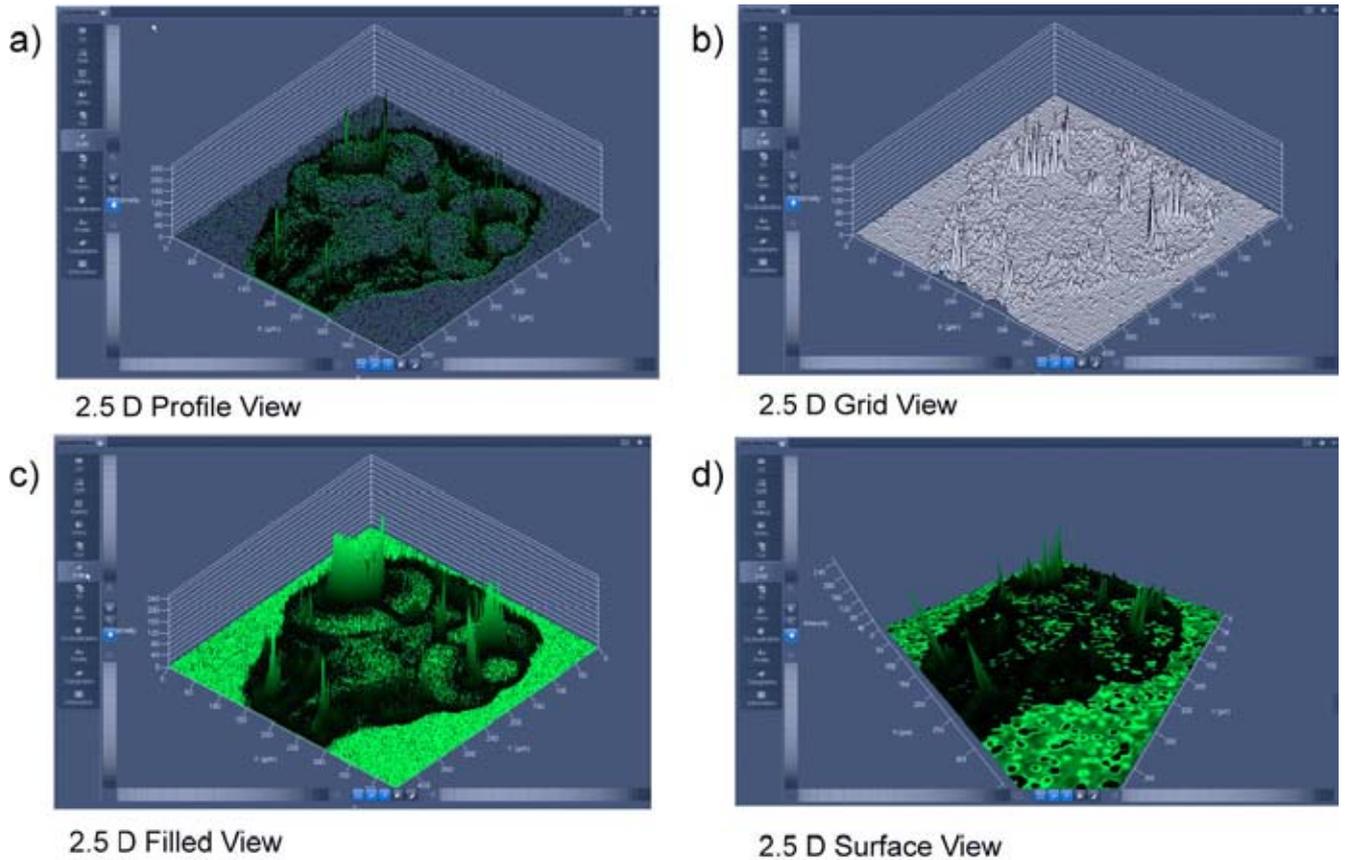


Fig. 6-24 2.5 D Image Display

The 2.5 D view control tab 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. |

6.8 3D View (Image VisArtplus)

With Image VisArtplus 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.



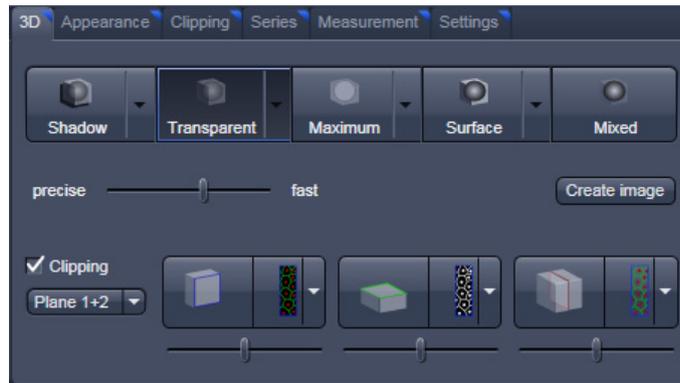
Fig. 6-25 Image Display window - 3D display

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:

VisArtplus 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 *VisArtplus* 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 *VisArtplus*.

Settings:

In the **Settings** panel global settings for the *VisArtplus* Module can be found.



Fig. 6-26 3D panel - Shadow button

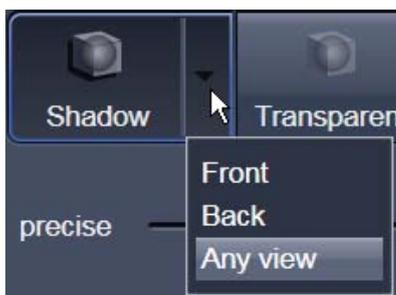


Fig. 6-27 Shadow pull down menu

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. 6-27).

- **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.

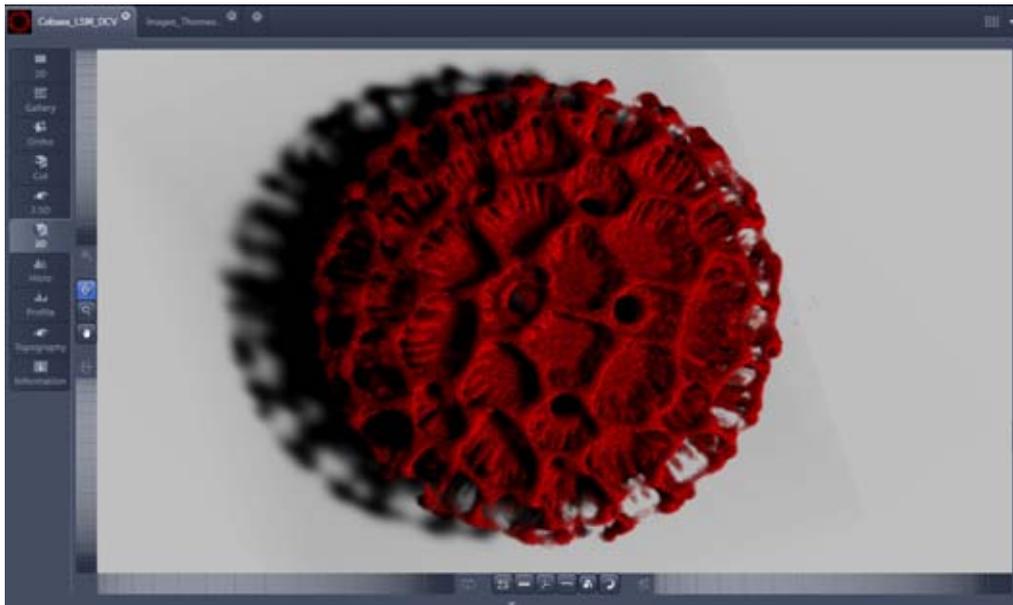


Fig. 6-28 Image Display window, 3D display, Shadow projection, Front view

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.

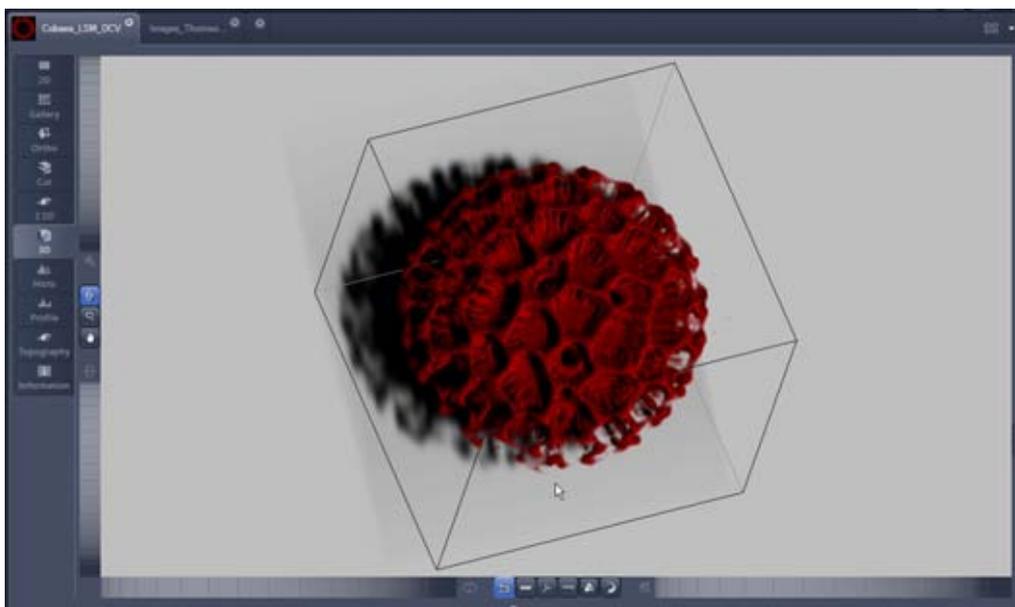


Fig. 6-29 Image Display window, 3D display, Shadow projection, Any View

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.

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. 6-30 3D View Control Panel, Transparent

Basic Transparency rendering (voxel based) (CPU)

Advanced Transparency rendering (voxel based) with textures (GPU)

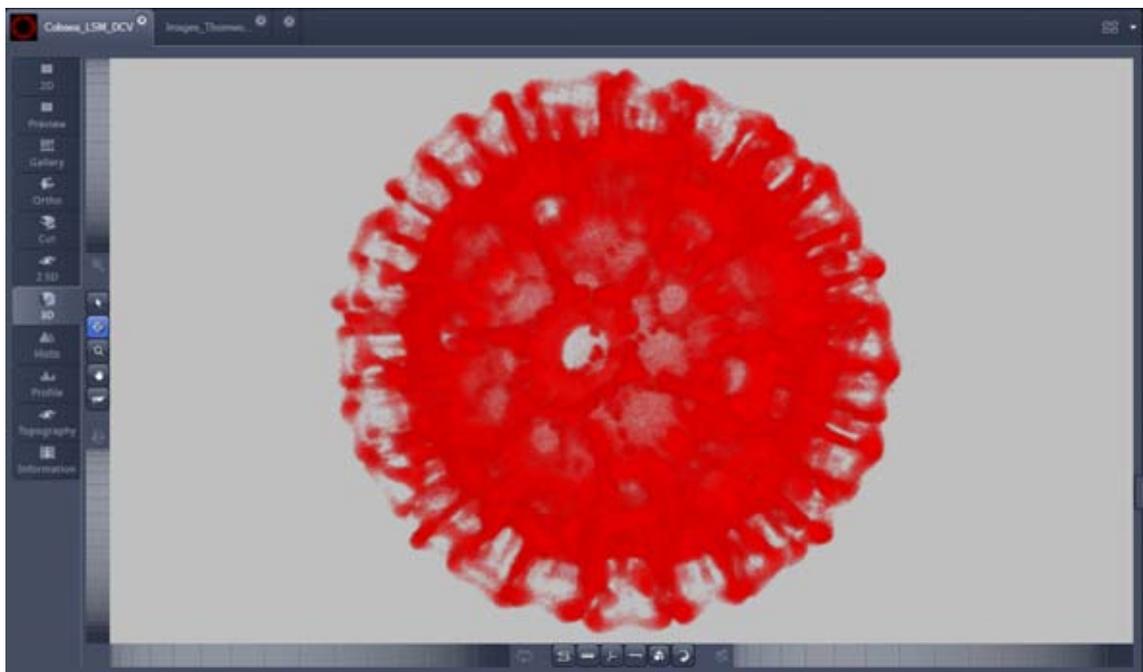


Fig. 6-31 Image Display window, 3D display, Transparency projection, Advanced

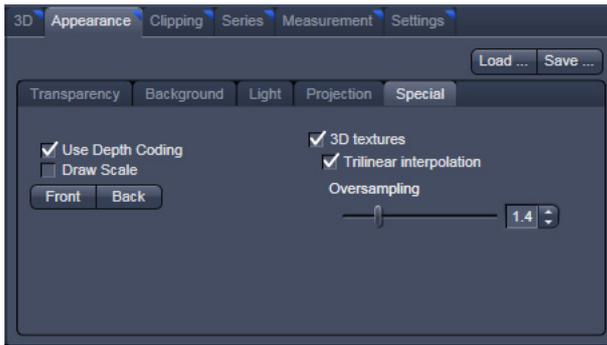


Fig. 6-32 3D toolbar – Transparent – Depth Coding

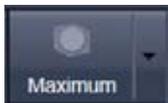
By means of the **Depth Coding** function (Fig. 6-32), the Z-information contained in a sequence can be colored with the colors of the rainbow, in which case "blue" stands for higher values and "red" stands for lower values.

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.

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 **Maximum Intensity Projection**). With these Images exact Intensity Measurements can be performed.

Maximum Basic Maximum Intensity CPU based

Maximum Advanced Maximum Intensity GPU based



Fig. 6-33 3D toolbar – Surface

6.8.4 Surface Render Mode

In the **Surface** mode (Fig. 6-33) grey values in the image are computed as solid surfaces (also known as Iso surfaces) 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.

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)

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.

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. 6-34 3D toolbar – Mixed

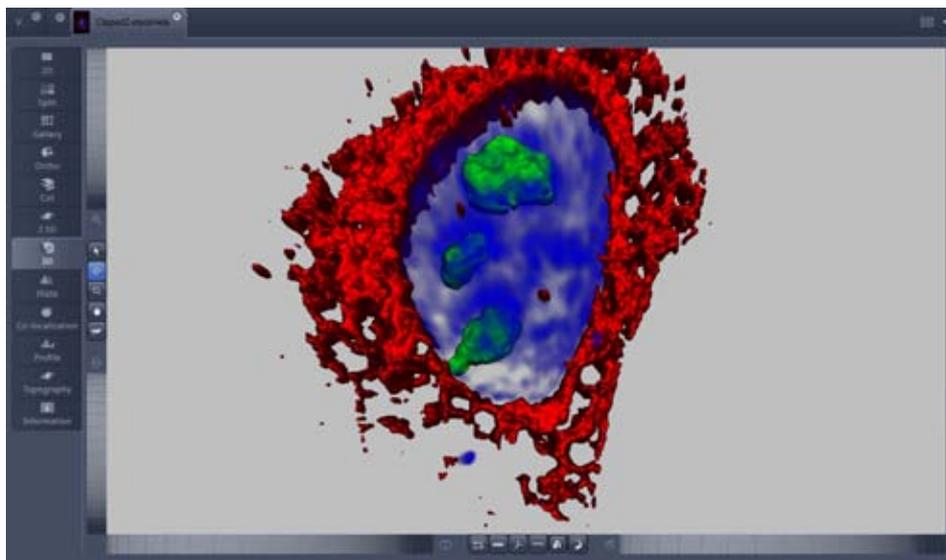


Fig. 6-35 Image Display window, 3D display, Mixed projection

6.8.6 Clipping Planes

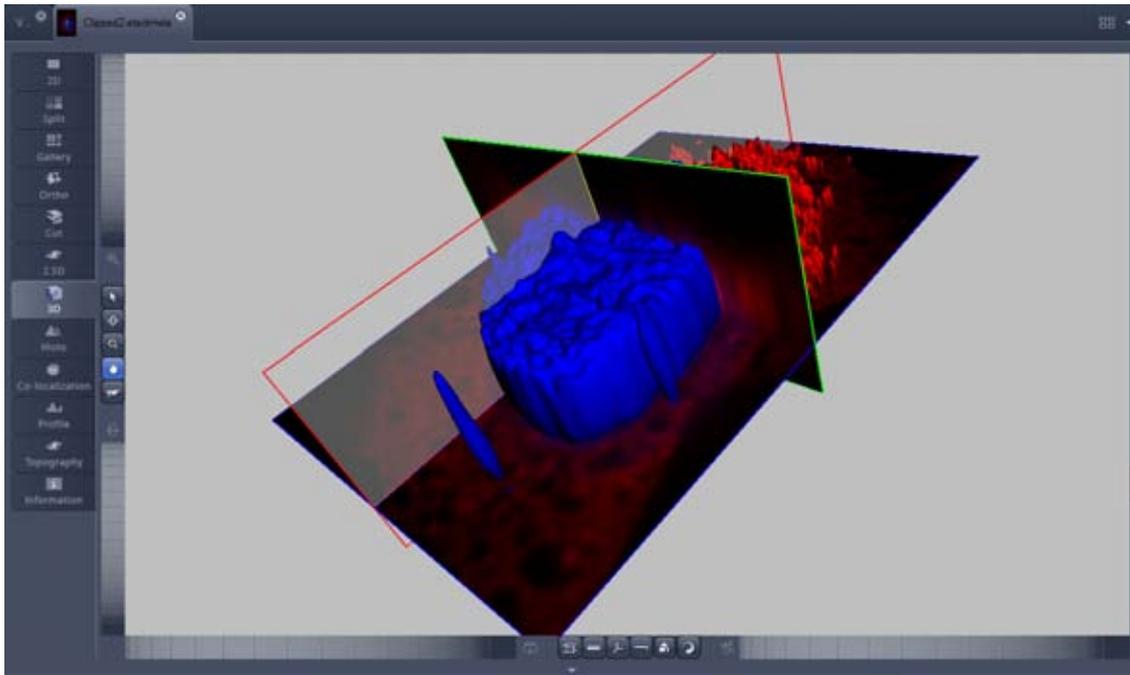
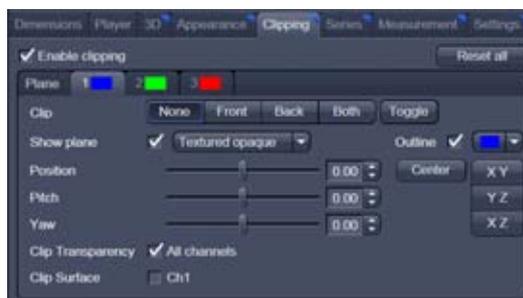


Fig. 6-36 Image Display window, 3D display, Clipping planes

VisArtplus 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. 

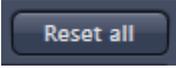


In **Surface** mode clipping planes can be used in a channel specific manner. This means, that in a multichannel image one can selectively cut away portions of the volume in one channel in order to unveil other structures from other channels. This technique is also applicable in **Mixed** mode.

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!

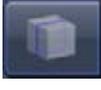


Fig. 6-37 Toggle buttons

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. 6-38 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

Transparent:

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.

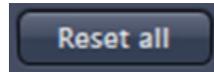
Interactive positioning of the clipping planes

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.

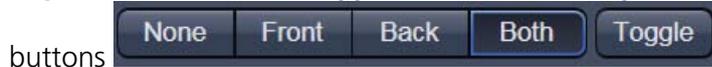
Show Plane hides or displays the plane.

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. 6-38).

Clip names a row of toggle buttons switching between different clipping modes as named on the



Using the **Pitch**, **Yaw**, and **Position** sliders each plane can be freely orientated in three-dimensional space.



Fig. 6-39 Clipping planes settings



Fig. 6-40 Sliders Pitch, Yaw and Position

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 flying direction. 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.

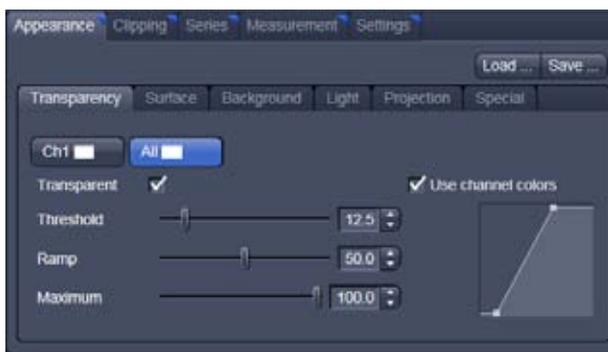


Fig. 6-41 3D Rendering window (e.g. Surface Advanced rendering mode)

6.8.8 3D Rendering Settings in VisArtplus

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  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. 6-42 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 / Z – scaling:** 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. 6-43 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. 6-44 Background



Fig. 6-45 Series tab



Fig. 6-46 Render Series window (e.g. Turn around X mode)



Fig. 6-47 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).



Fig. 6-48 Start panel in the Render View control block

6.8.9 Series

The **Series** tab (Fig. 6-45) 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 *VisArtplus*.

There are four different modes for producing render series (see Fig. 6-47).

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).

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.

(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. 6-48).

(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.

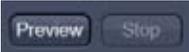
The rotation angles for X, Y and Z and the zoom can be determined directly in the image.

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.



Fig. 6-49 Render Series window - Position List mode

6.8.10 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 .



Fig. 6-50 3D Measurement View control block

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.

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.

6.8.11 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.

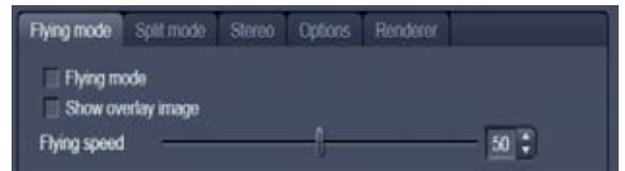


Fig. 6-51 3D Renderer Settings – Flying mode

Split Mode

Ticking this tick box activates a channel wise 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**.

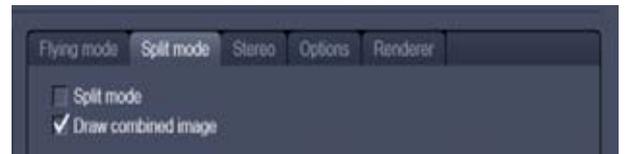


Fig. 6-52 3D Renderer Settings – Split mode

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.



Fig. 6-53 3D Renderer Settings – Stereo

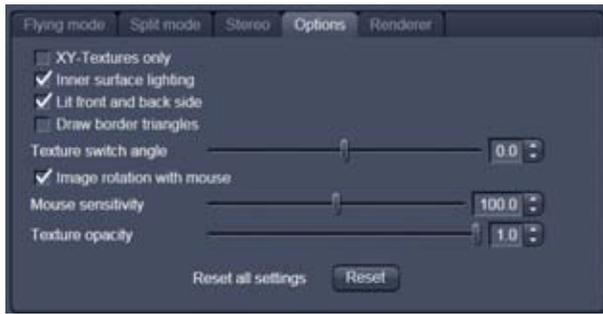


Fig. 6-54 3D Renderer Settings – Options

6.8.12 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.

Lit front and back side: With activated **Inner surface lighting**, this check box 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.



Fig. 6-55 3D OpenGL Renderer 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.

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 **Series**) 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.

6.8.13 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 **3D View (Image VisArtplus)**. 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.

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.

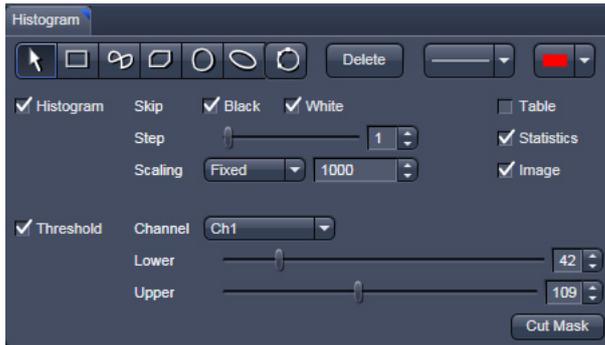


Fig. 6-56 View Option control block - Histo View

The settings of the **Dimensions**, **Display**, **Player** and **Overlay** view options control blocks apply. The additional view-specific **Histo View Option** control block is shown in Fig. 6-56.

Any changes done with this View Option control block are effective immediately.

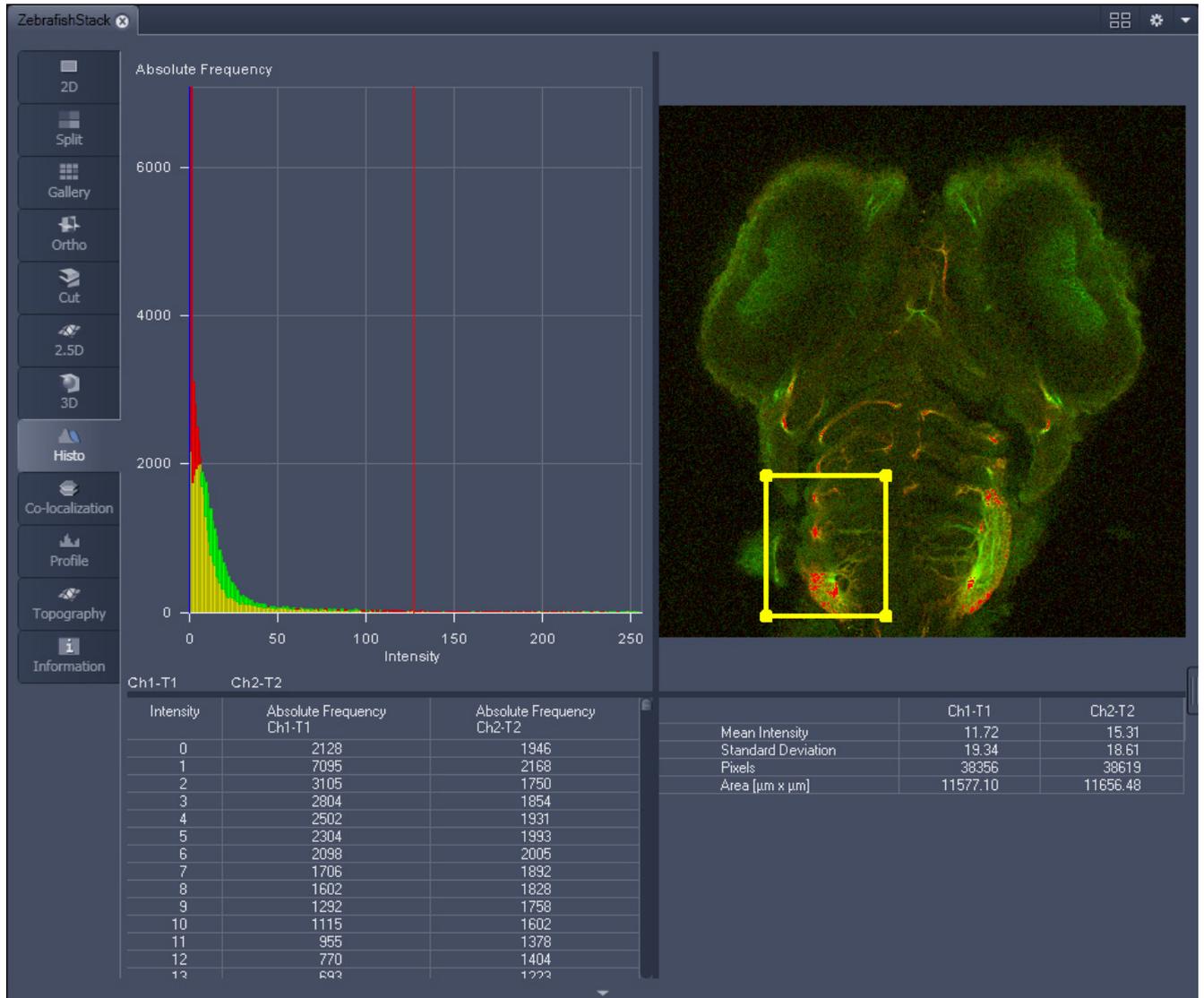
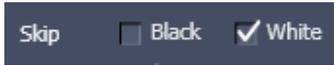


Fig. 6-57 Image Display, Histogram view

 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. 6-58 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. 6-58). The threshold can be set for each channel (channel selection pull down menu).

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. 6-59.

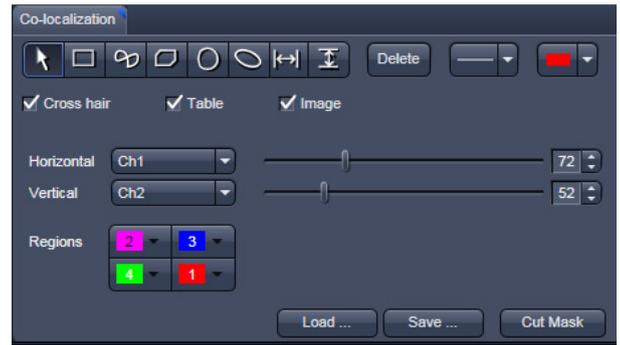


Fig. 6-59 Colocalization View Option control block

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. 6-60 and Fig. 6-61).



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.

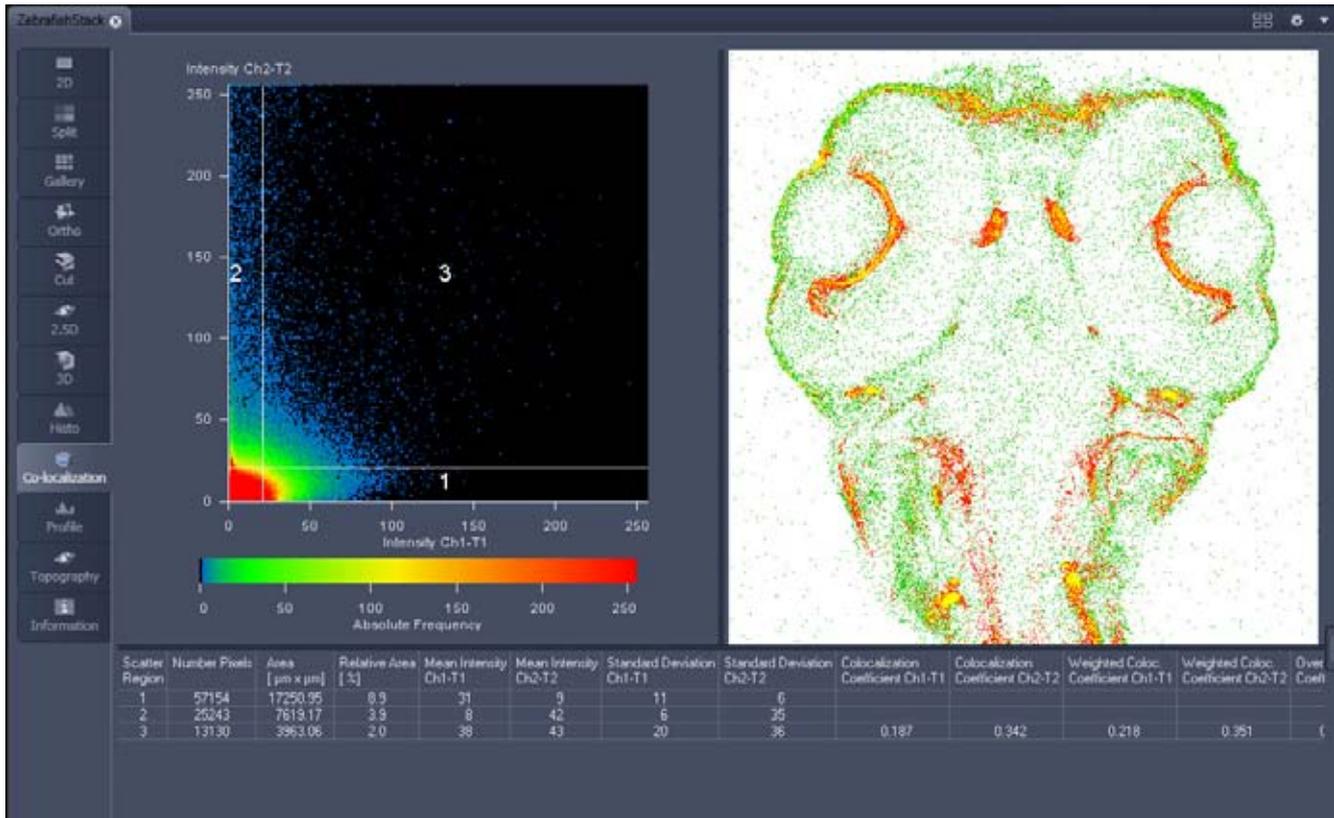


Fig. 6-60 Image Display, Colocalization view

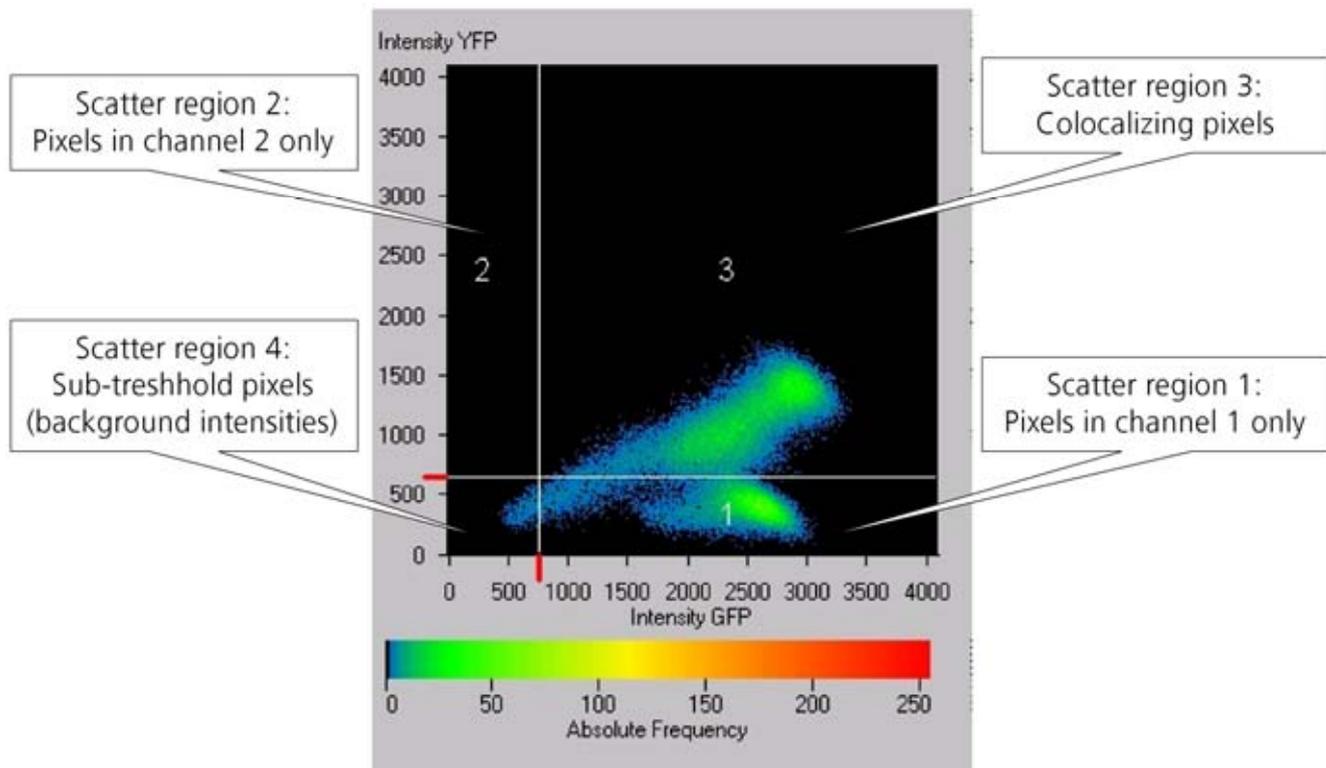


Fig. 6-61 Scatter diagram and threshold with crosshair

6.10.1 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



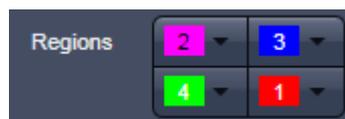
With the drawing tools the colocalization analysis can be restricted to a region of the image. Tools in the Colocalization View Option control block and in the Overlay View Option control block work the same and can be combined.



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.



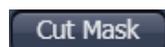
Scaling pull down menu: Sets the histogram graph scaling to either dynamic (adjusting to the maximum value) or fixed



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!

6.10.2 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 R²)

6.10.3 Colocalization Coefficients

$$c_1 = \frac{\text{pixels}_{Ch1,coloc}}{\text{pixels}_{Ch1,total}} \qquad c_2 = \frac{\text{pixels}_{Ch2,coloc}}{\text{pixels}_{Ch2,total}}$$

- 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

6.10.4 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)

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.

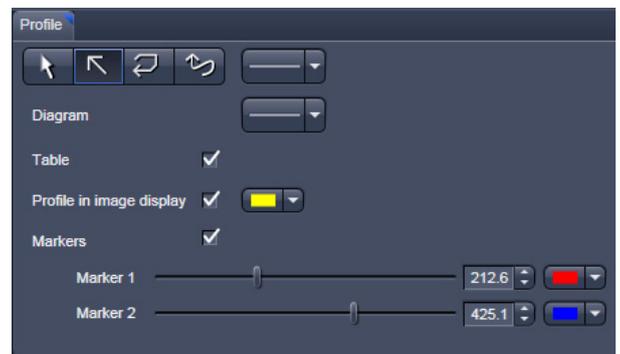


Fig. 6-62 View Option control block - Profile View

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. 6-62.

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. 6-63).
- 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. 6-64).

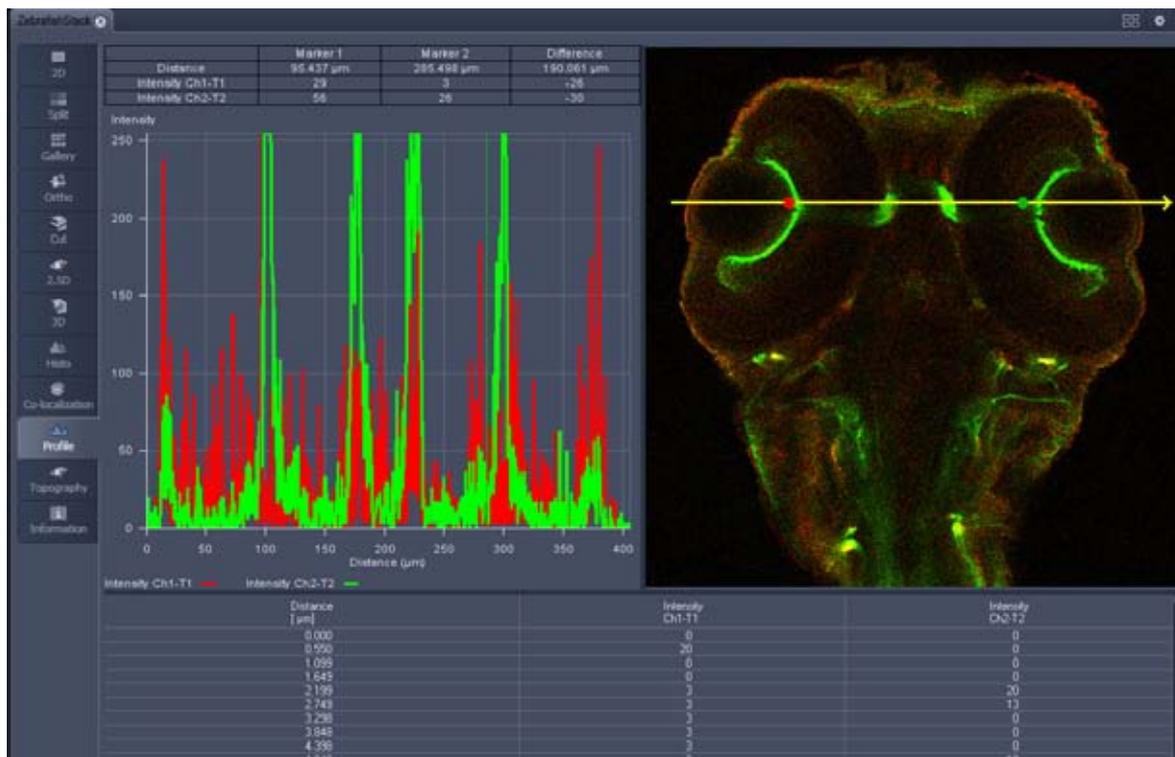


Fig. 6-63 Image Display - Profile View, Line Profile with markers

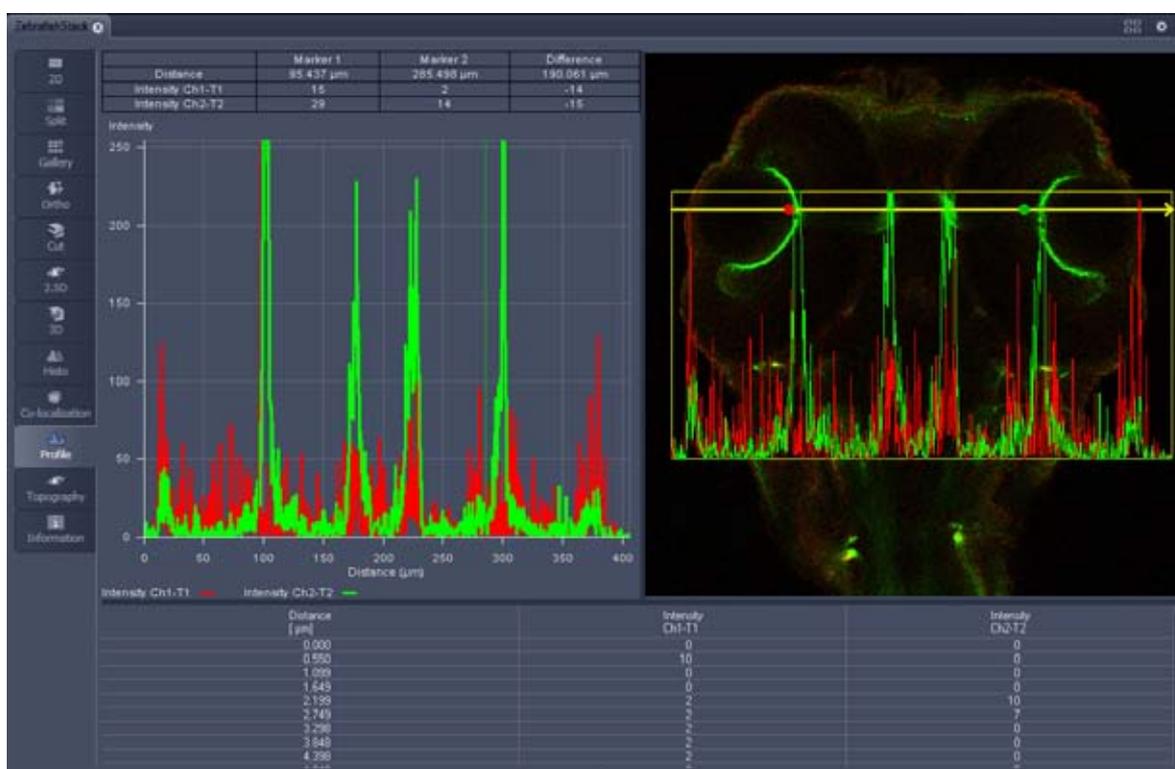


Fig. 6-64 Image Display - Profile View, Profile displayed in Image

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!

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.

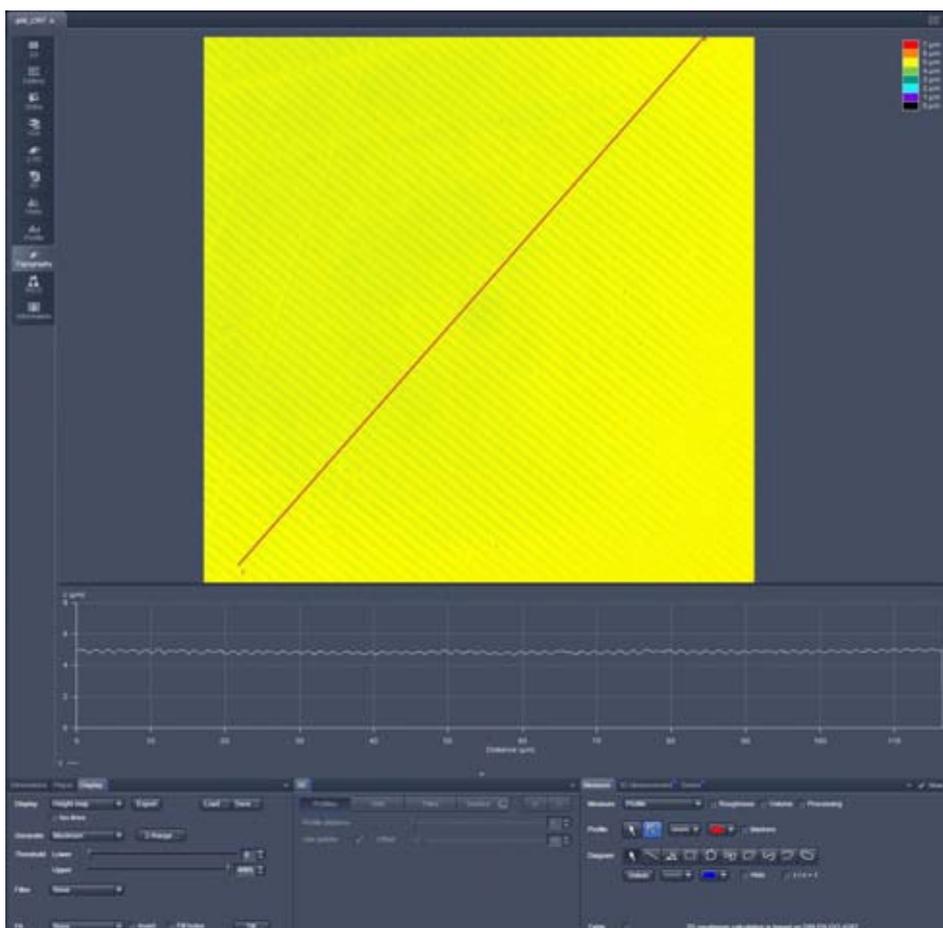


Fig. 6-65 Center Screen Area, Topography view

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.

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 check box 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.



Fig. 6-66 Image view control block - Display

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. 6-67 Z-Range pop up to define height thresholds

- 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.

- 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 check box **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 check box **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.

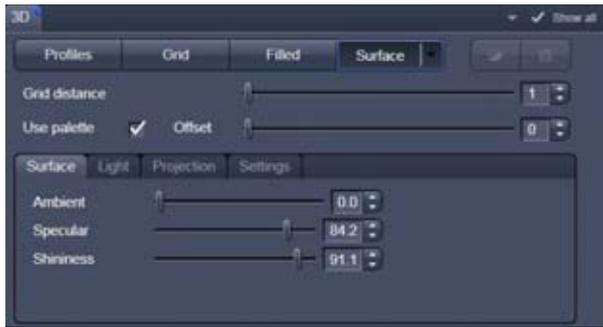


Fig. 6-68 Image view control block - 3D

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. 6-68).

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)

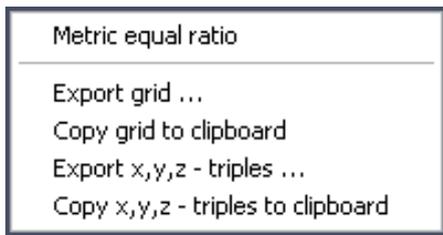


Fig. 6-69 Context menu using 3D Grid

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. 6-69).



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. 6-70 Surface renderer items

In case of **Surface** the renderer item can be set, clicking into the right part of the **Surface** button (Fig. 6-70).

OpenGL - software

The graphics calculation is performed using the installed software.

OpenGL - hardware

The graphics calculation is accelerated by using the installed graphics processor.

Additionally the shading model can be adjusted using the **Surface**, **Light** and **Projection** tabs:

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. 6-71 Surface renderer: Surface 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** check box shows you the "sun" within the image tab.

View angle: Determines the perspective, 0.0 degree: parallel projection, else: central projection.



Fig. 6-72 Surface renderer: Light tab

Scale Z: Determines the zoom factor for the Z / height dimension.

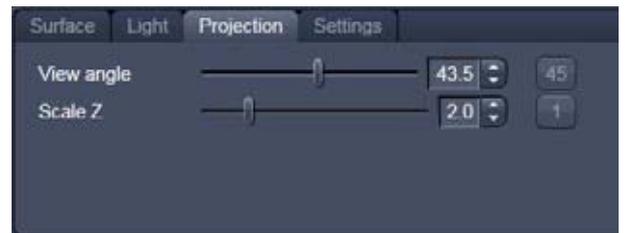


Fig. 6-73 Surface renderer: Projection tab

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.

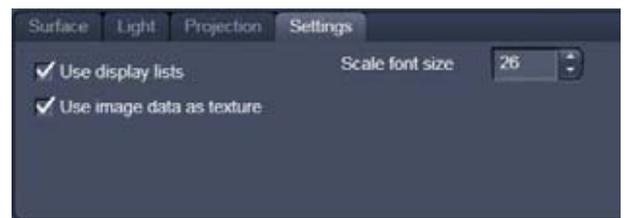


Fig. 6-74 Surface renderer: Settings tab

Load / Save (see Fig. 6-68) 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 **3D View (Image VisArtplus)**.

-  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.

6.12.3 Control Block: Measure

The topography measurement functions are available in the **Measure** control block (Fig. 6-75). The measurement functions can be performed in Height map, Maximum intensity and 3D display modes.

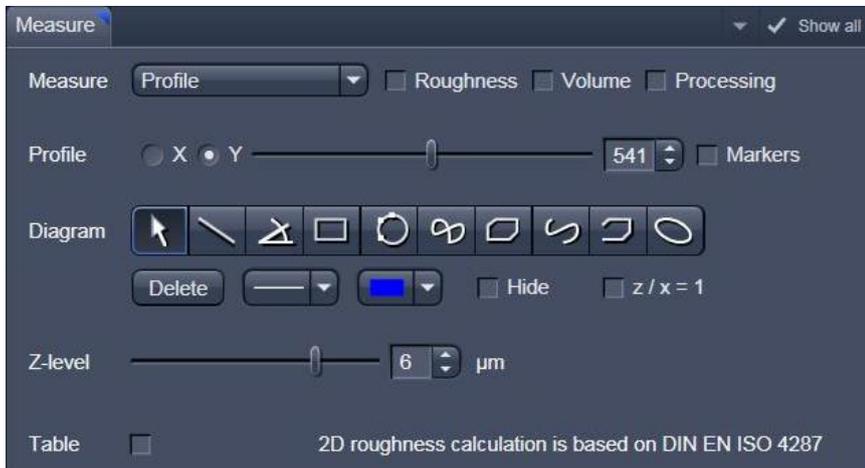


Fig. 6-75 Image view control block: Measure

Automated convention in height statistics analysis:

Topo Filters	None, median,	FFT High	FFT Low
Data formats	P primary profile	R roughness	W waviness
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 check boxes 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)

6.12.3.1 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:

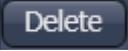


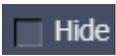
With the slider the profile line can be move on the 3D topography, in X and Y direction.

In order to use **Markers** to measure distances within the profile diagram, tick this check box:



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, dx/dy 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.
	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.
	Ticking this box will hide all drawing elements, but not the markers.
	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  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).

6.12.3.2 Z-histogram Measurement

Chose Z-histogram within the Measure dropdown menu. The Z-histogram will be shown as a diagram below the image.

6.12.3.3 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.

6.12.3.4 Roughness Measurement in 2D (Profile) and 3D

- Chose **Profile** in the **Measure** line to get 2D roughness values.
- Tick **Roughness** check box: 
 - 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.

(1) 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

(2) 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

$$- R_c = \frac{1}{N_y} \cdot \sum_{j=1}^{N_y} \cdot z(x, y_j) \quad N_x, N_y \dots \text{number of pixels in X- or Y-direction}$$

Arithmetic mean deviation of all profile height values R_a

$$- R_a = \frac{1}{N_y} \cdot \sum_{j=1}^{N_y} \cdot [z(x, y_j) - R_c]$$

- Quadratic mean deviation of all profile height values R_q

$$- R_q = \sqrt{\frac{1}{N_y} \cdot \sum_{j=1}^{N_y} \cdot [z(x, y_j) - R_c]^2}$$

- 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 :

$$- R_z = \frac{z_{\max 1} - z_{\min 1} + z_{\max 2} - z_{\min 2} + z_{\max 3} - z_{\min 3} + z_{\max 4} - z_{\min 4} + z_{\max 5} - z_{\min 5}}{5}$$

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} :

$$- R_{\max} = \text{Max} (z_{\max 1} - z_{\min 1}, z_{\max 2} - z_{\min 2}, z_{\max 3} - z_{\min 3}, z_{\max 4} - z_{\min 4}, z_{\max 5} - z_{\min 5})$$

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!

(3) 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

(4) Calculation of roughness parameters:

The following roughness parameters are calculated:

- Mean height of all surface height values S_c

$$-RS_c = \frac{1}{N_x \cdot N_y} \cdot \sum_{i=1}^{N_x} \cdot \sum_{j=1}^{N_y} \cdot z(x_i, y_j) \quad N_x, N_y \dots \text{number of pixels in X- or Y-direction}$$

- Arithmetic mean deviation of all surface height values RS_a

$$-RS_a = \frac{1}{N_x \cdot N_y} \cdot \sum_{i=1}^{N_x} \cdot \sum_{j=1}^{N_y} \cdot [z(x_i, y_j) - RS_c]$$

- Quadratic mean deviation of all surface height values RS_q

$$-RS_q = \sqrt{\frac{1}{N_x \cdot N_y} \cdot \sum_{i=1}^{N_x} \cdot \sum_{j=1}^{N_y} \cdot [z(x_i, y_j) - RS_c]^2}$$

- Skewness of the distribution of all surface height values RS_{SK}

$$RS_{SK} = \frac{1}{N_x \cdot N_y \cdot RS_q^3} \cdot \sum_{i=1}^{N_x} \cdot \sum_{j=1}^{N_y} \cdot z^3(x_i, y_j)$$

- Kurtosis of the distribution of all surface height values S_{KU}

$$RS_{KU} = \frac{1}{N_x \cdot N_y \cdot RS_q^4} \cdot \sum_{i=1}^{N_x} \cdot \sum_{j=1}^{N_y} \cdot z^4(x_i, y_j)$$

- 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 :

$$-RS_z = \frac{z_{\max 1} - z_{\min 1} + z_{\max 2} - z_{\min 2} + \dots + z_{\max 25} - z_{\min 25}}{25}$$

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} :

$$-RS_{\max} = \text{Max} (z_{\max 1} - z_{\min 1}, z_{\max 2} - z_{\min 2}, \dots, z_{\max 25} - z_{\min 25})$$

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!

6.12.3.5 Volume Measurement in 3D

- Tick the check box  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:

$$\frac{\Sigma (\text{surface area}_{ij}) - \Sigma (\text{projected area}_{ij})}{\Sigma (\text{projected area}_{ij})} * 100 \%$$

$$\text{projected area} = x * y$$

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)

6.12.4 Control Block: 3D Measurement

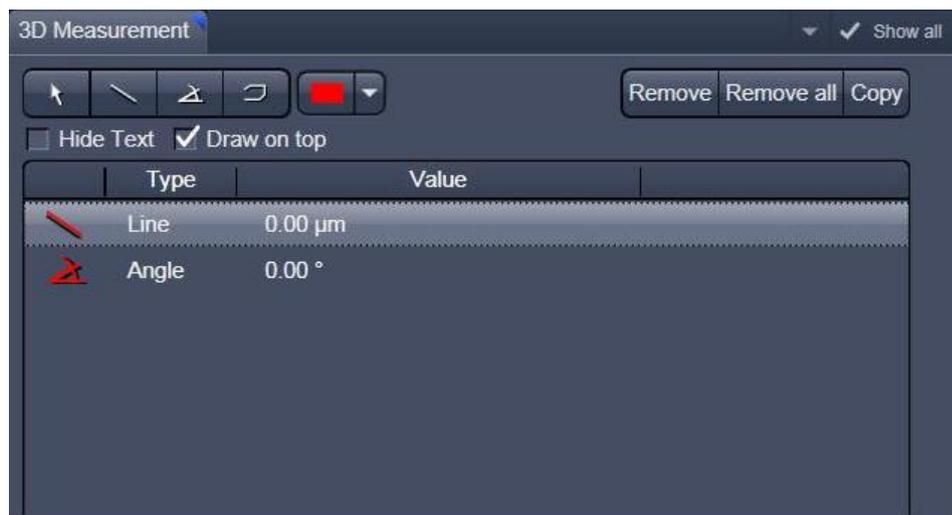


Fig. 6-76 Image view control block: 3D Measurement

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.

6.12.5 Control Block: Series

See section **Series** on 3D (VisArt plus).

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.

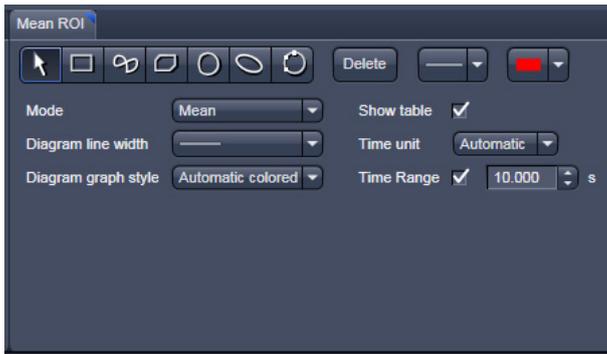


Fig. 6-77 Mean of ROI View Option control block

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 blocks apply. The additional view-specific **Mean of ROI View Option** control block is shown in Fig. 6-77.

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. 6-78 and Fig. 6-79).

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 or trigger events are marked with little symbols on the time-axis of the graph (see Fig. 6-78).

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).

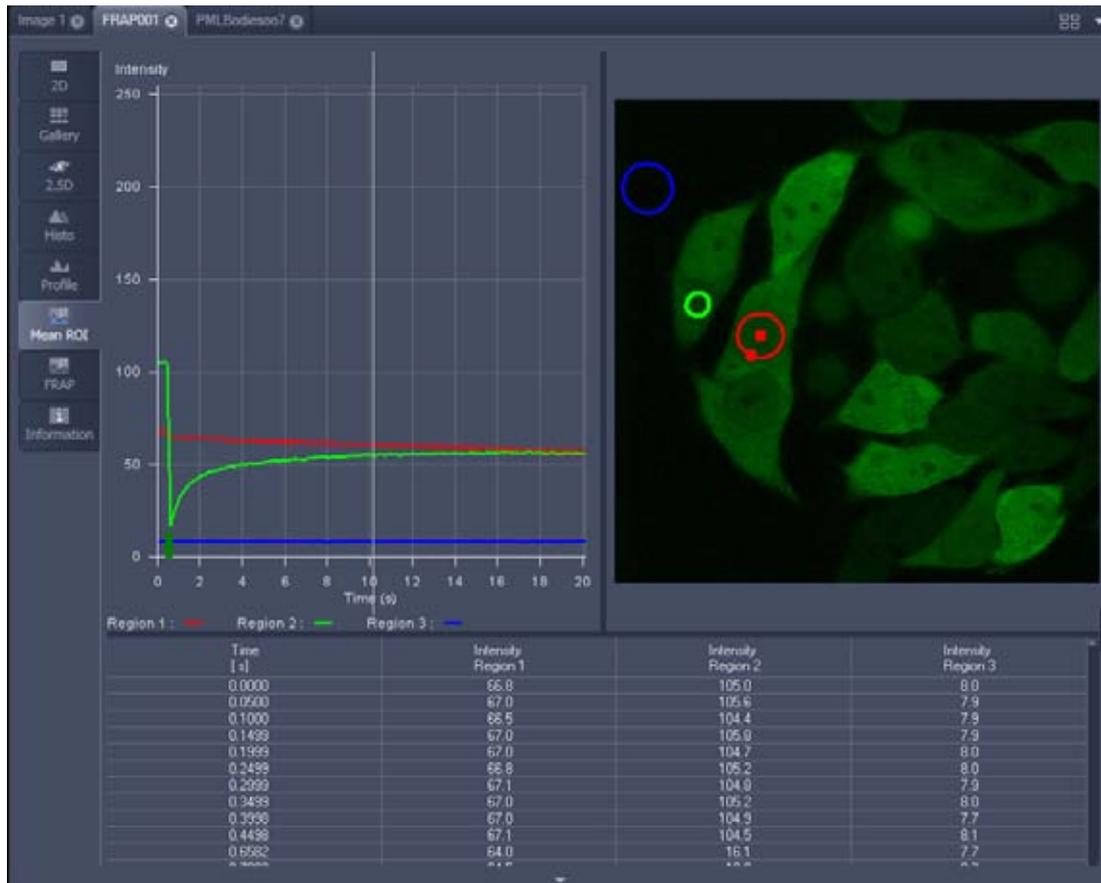


Fig. 6-78 Image Display window, Mean ROI display for time series in single plane

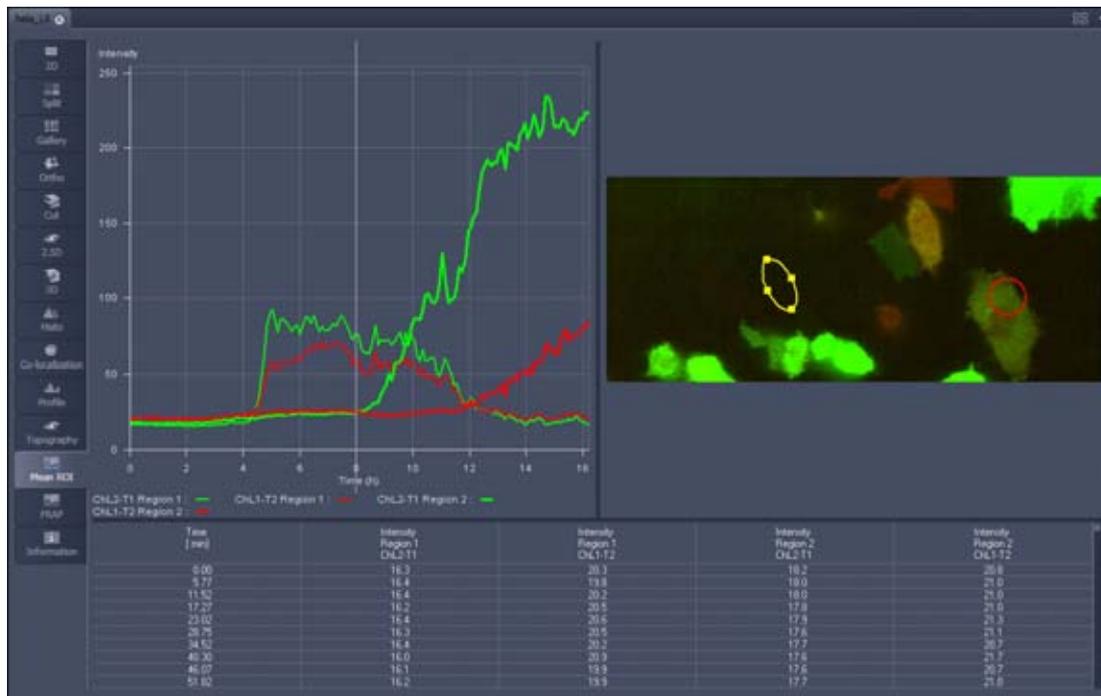


Fig. 6-79 Image Display window, Mean ROI display for time series of Z-Stack

The **Mean of ROI** view options control block contains the following function elements:

#	Type	X	Y	Width	Height	Lock
1		117	85	51	37	
2		239	176	40	40	
3		376	121	84	113	

ROIs can be managed in the Overlay View Option control block.



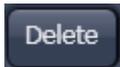
Arrow button: Activation of the mouse button for resizing or movement of the ROI in the **Image Display** window.



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 Ellipse 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.



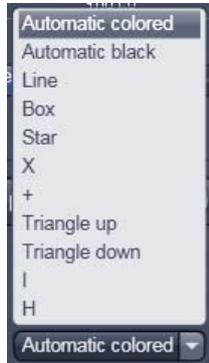
Polyline and 3D Polyline button: Activates polyline drawing mode. The first click sets the starting point, each additional click adds a further line, a double-click on the starting point closes the figure and ends the procedure.



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 width of the curves in the diagram.

Diagram graph style pull down: choose from a set of graph styles

Show Table tick 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 check 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.

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.

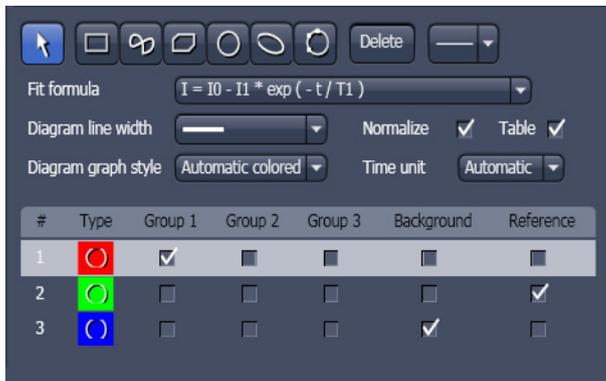


Fig. 6-80 FRAP View Option control block

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. 6-80.

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. 6-81, Fig. 6-83 and Fig. 6-84).

To access the Kinetic / FRAP analysis view type, load or acquire a time series data set and click on the **FRAP** View tab.

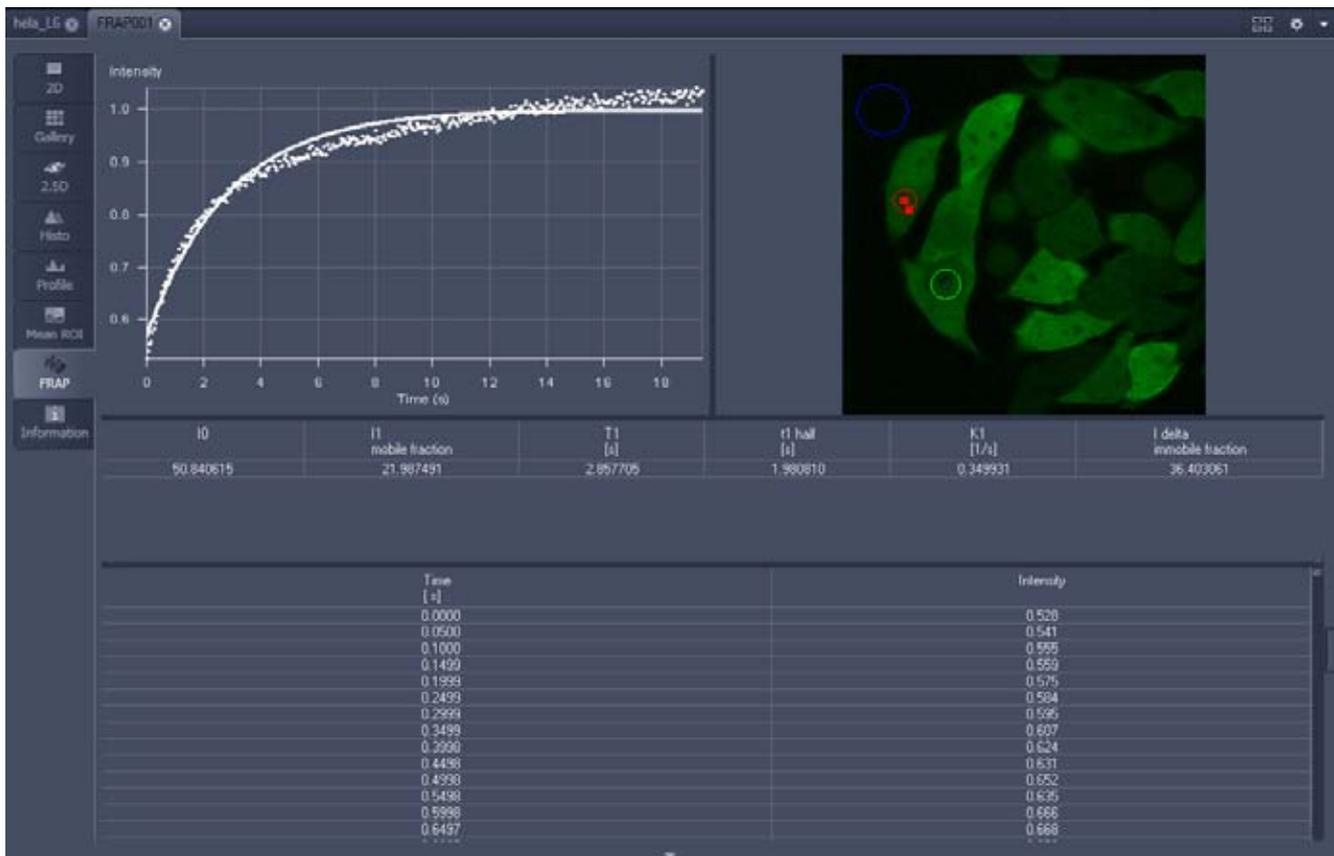


Fig. 6-81 Image display of a time series in the FRAP View tab

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.
- Analysis 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** Check box in the list of ROIs: Mark the region of interest which represents the mean background intensity to be used for data correction.
- Reference Region** Check box 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** Check boxes 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.
Group 1, 2, 3

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. 6-82). 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.

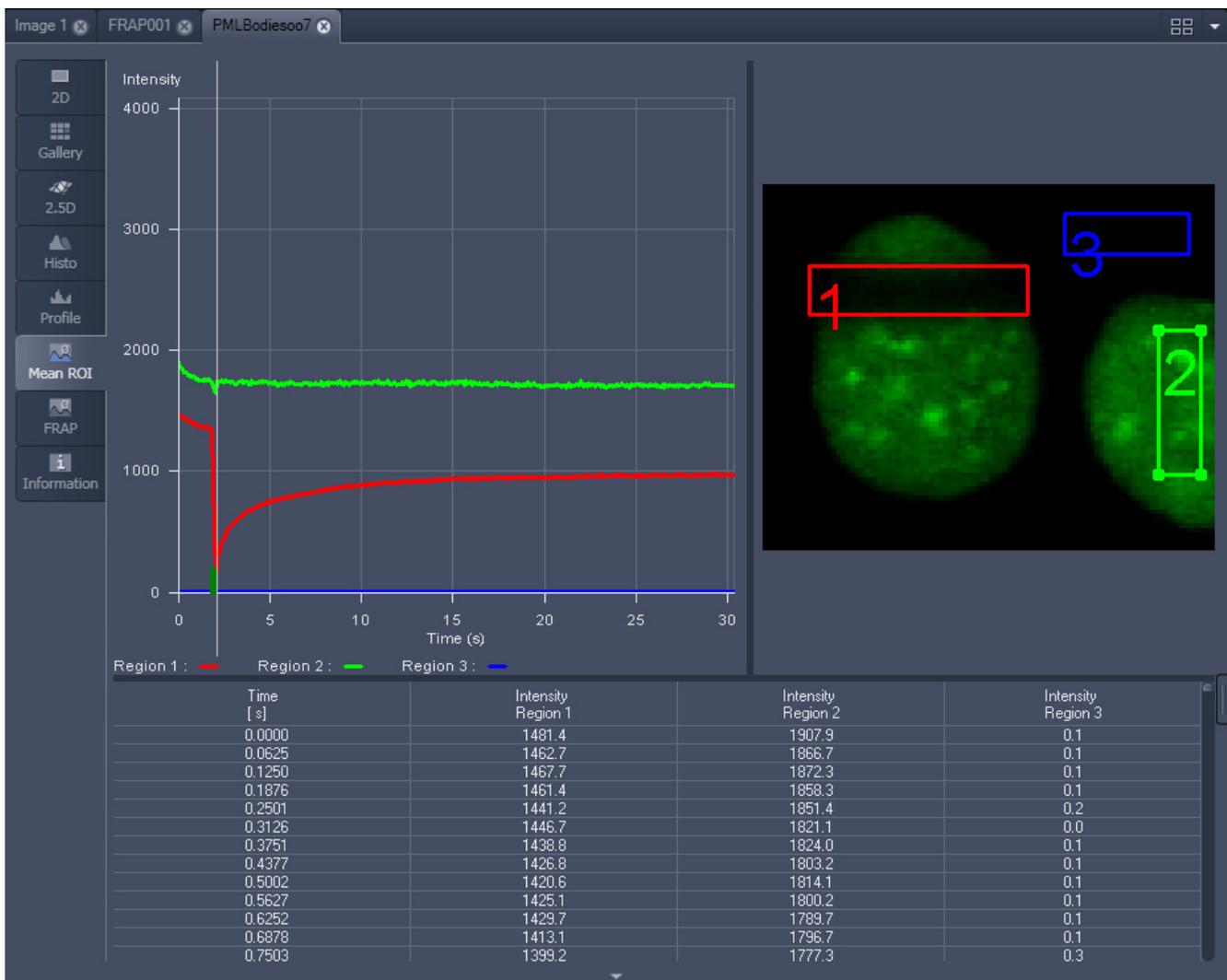


Fig. 6-82 FRAP data displaying the first bleached image of an images series in the Mean of ROI View Type

- Switch to the **FRAP** View tab.
 - Mark the check boxes 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 check box **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:
$$t_{\text{half}} = \frac{\ln 0.5}{-T1}$$

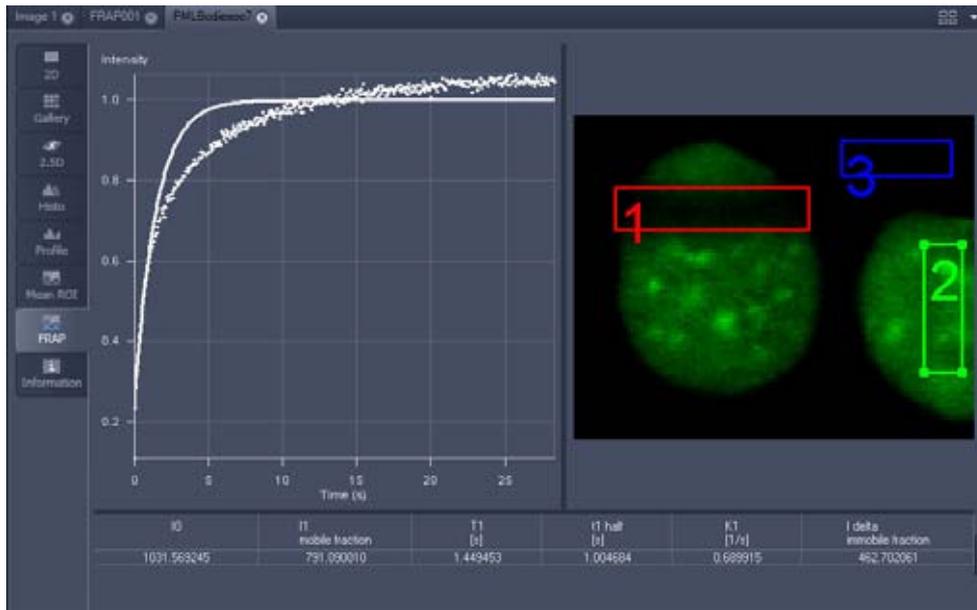


Fig. 6-83 Image window displaying the analysis of FRAP data using a mono exponential fit

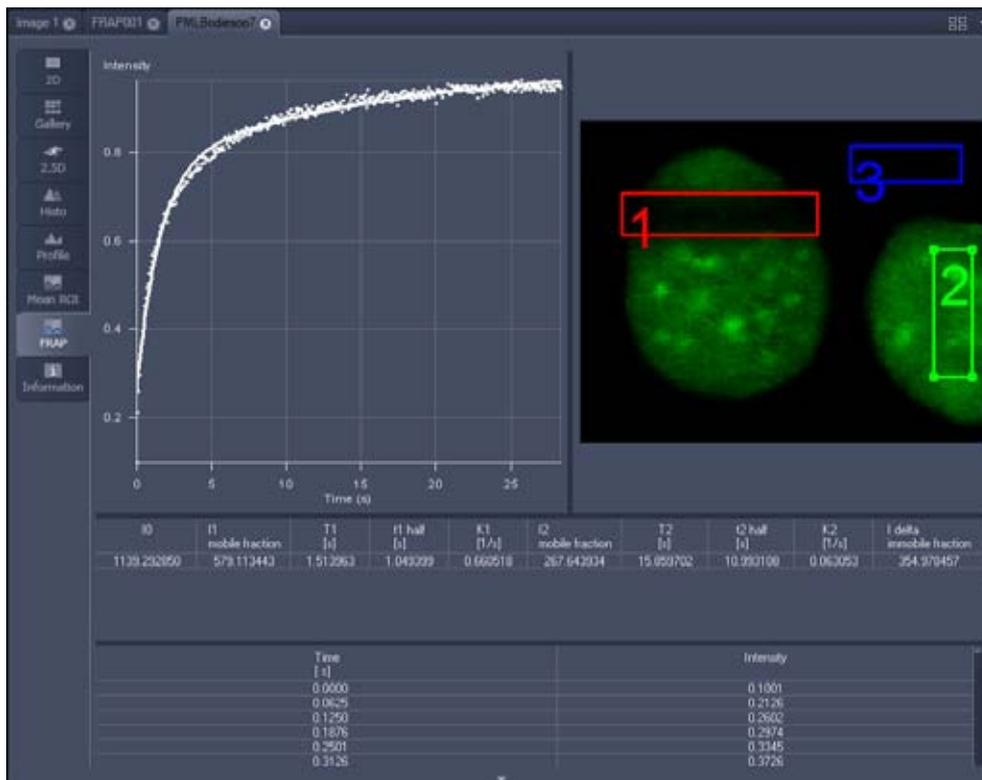


Fig. 6-84 Image window displaying the analysis of FRAP data using a double exponential fit

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. 6-85. 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.

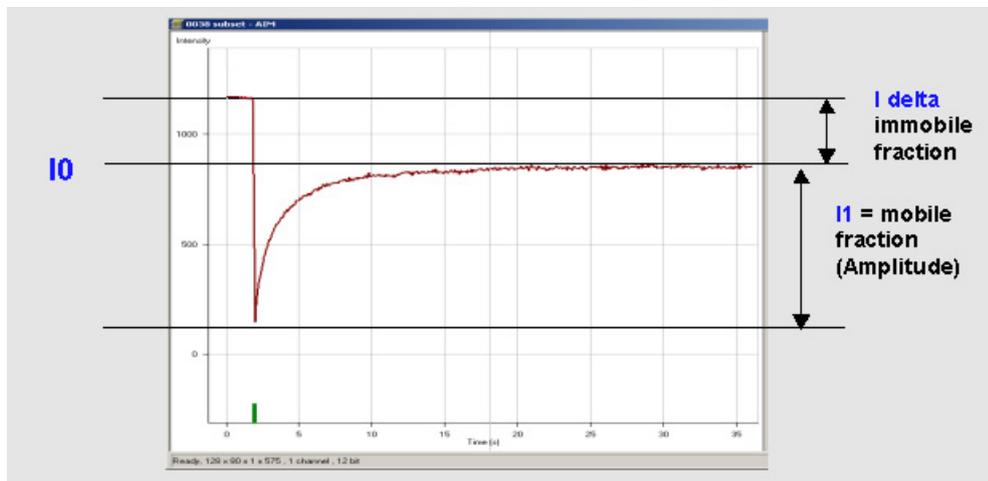


Fig. 6-85 Schematic FRAP curve with marks at the relevant data points. **I0**, **I1** and **I delta** which are calculated performing the Kinetic Analysis



Fig. 6-86 Lambda Coded - specific Dimensions View Option control block

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. 6-87).

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. 6-86.

Any changes done with this View Option control block are effective immediately.

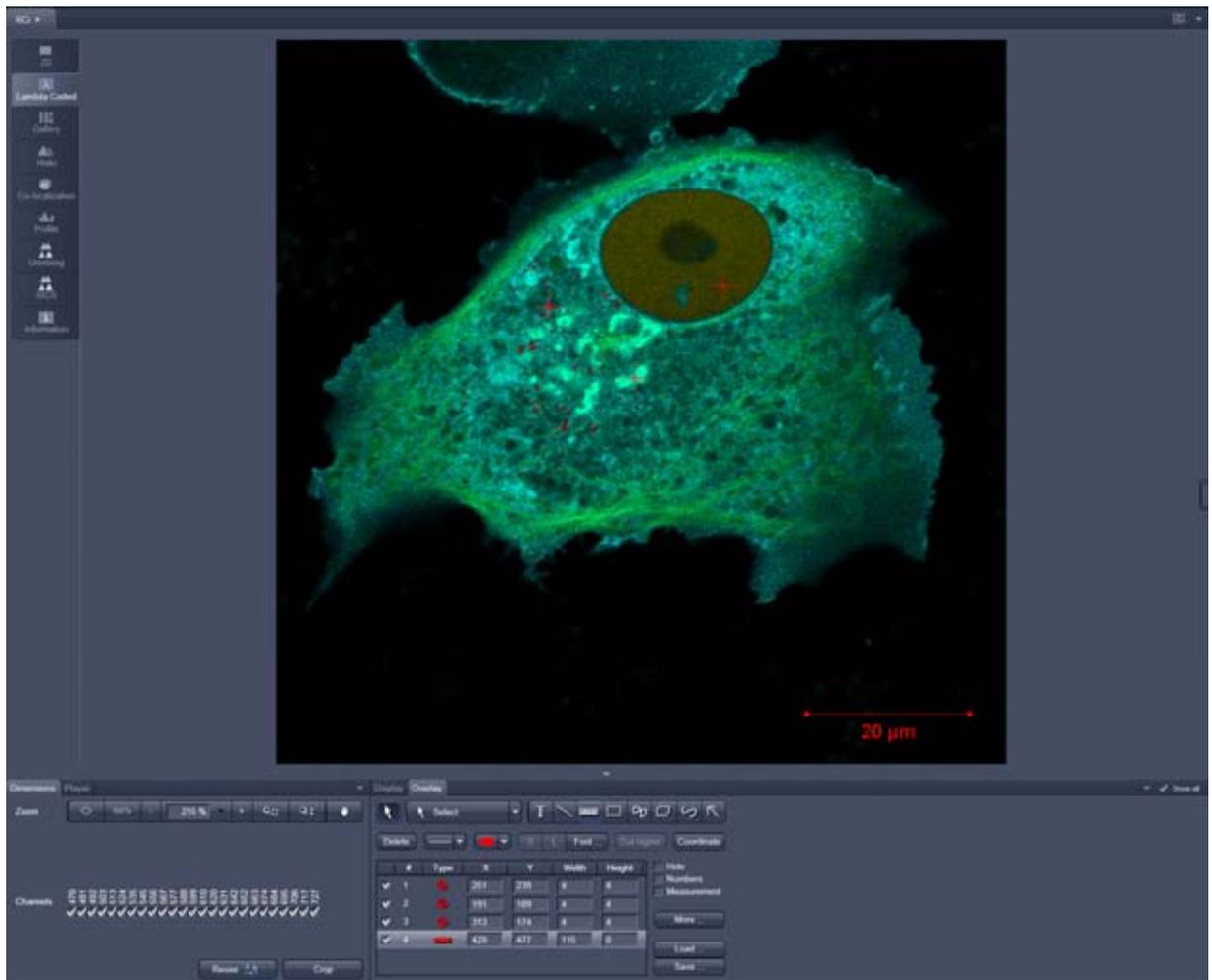


Fig. 6-87 Dimensions view option control block for Lambda Coded View

6.16 FRET View

In the **FRET** View type (Fig. 6-88), 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!

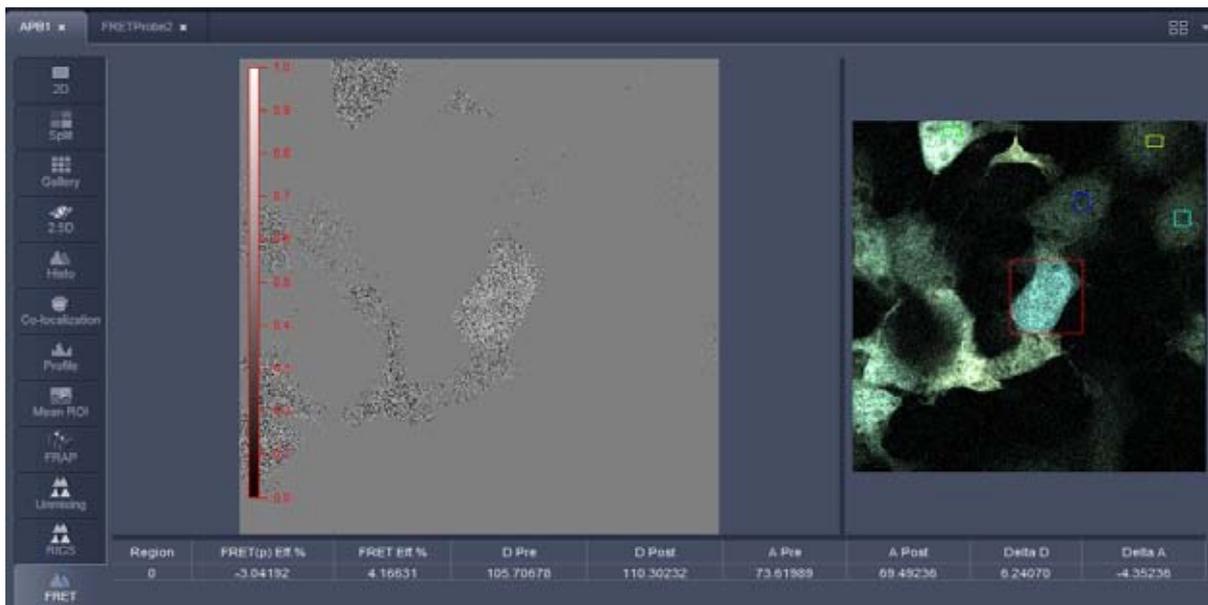
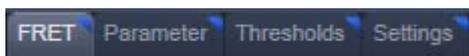


Fig. 6-88 FRET Display of Time series image having performed Acceptor Photobleaching

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.

6.16.1 Tools in the FRET View Options Control Block for Acceptor Photobleaching

FRET tab (Fig. 6-89):

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 **Overlay**).

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**.

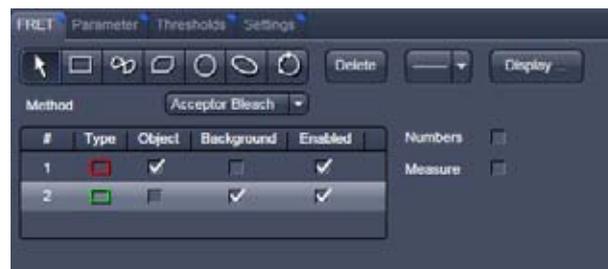


Fig. 6-89 FRET View Options Control Block, FRET tab

Parameter tab (Fig. 6-90):

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.:**

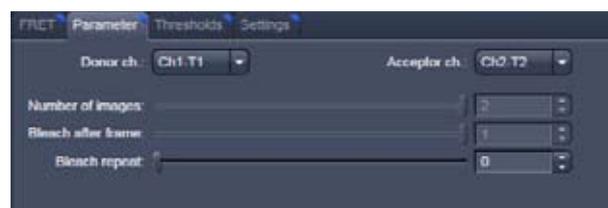


Fig. 6-90 FRET View Options Control Block, Parameter tab

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.

Thresholds tab (Fig. 6-91):

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.



Fig. 6-91 FRET View Options Control Block, Thresholds tab

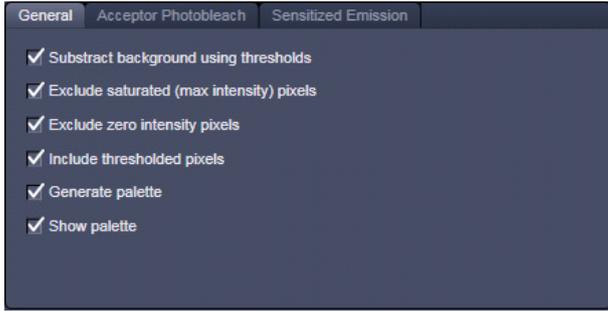


Fig. 6-92 FRET View Options Control Block, Settings tab, General

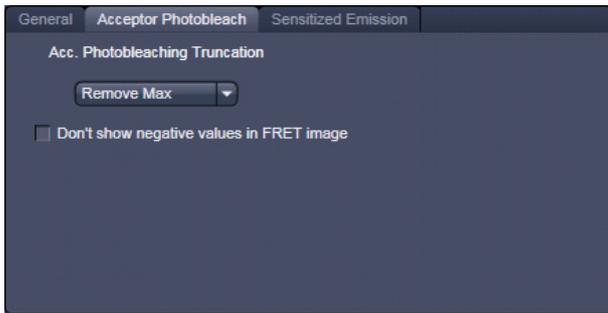


Fig. 6-93 FRET View Options Control Block, Settings tab, Acceptor Photobleach



Fig. 6-94 FRET View Options Control Block, FRET tab

Settings tab (Fig. 6-92):

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. 6-93) 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**).

6.16.2 Tools in the FRET View Options Control Block for Sensitized Emission

FRET tab (Fig. 6-94):

Within this tab overlay regions can be defined. (The drawing tools correspond to the drawing tools described in section **Overlay**).

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 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.

Parameter tab (Fig. 6-95):

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.

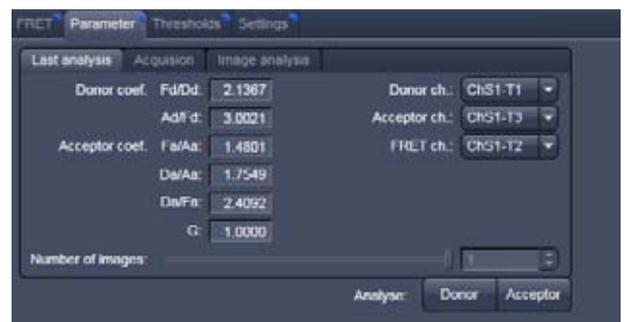


Fig. 6-95 FRET View Options Control Block, Parameter tab, Last Analysis

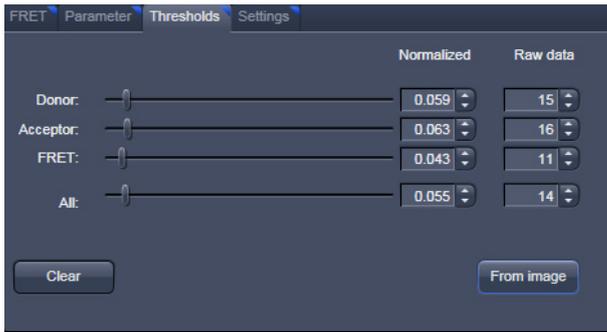


Fig. 6-96 FRET View Options Control Block, Thresholds tab

Thresholds tab (Fig. 6-96):

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.

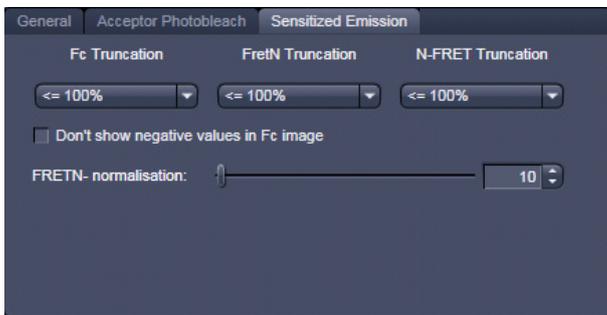


Fig. 6-97 FRET View Options Control Block, Settings tab, Sensitized Emission

Settings tab (Fig. 6-92 and Fig. 6-97):

This tab provides access to the definition of the parameters used for the image analysis.

The tab **General** (Fig. 6-92) 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. 6-97) 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 FRET-method.

6.17 Unmixing View

In the **Unmixing View** type, Lambda or channel 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 and all types of multi-channel images as input (only if the PMTs of the LSM system are calibrated),
- 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. 6-98 and Fig. 6-99.

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. 6-100). 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 or a multichannel image and click on the **Unmixing** tab.

 The Unmixing View is available (→ displayed) for all Lambda stacks and for all multi-channel images acquired on hardware with calibrated detectors. This includes all LSM 710, LSM 710 NLO, LSM 7 MP (if NDD detectors calibrated) and LSM 700 systems.

 Detector calibration is a service task. This calibration allows that reference spectra can be acquired at different detector gain settings than the multi-labeled image acquired for unmixing!



Fig. 6-98 Unmixing View Option control block



Fig. 6-99 Unmixing - Extract - View Option control block

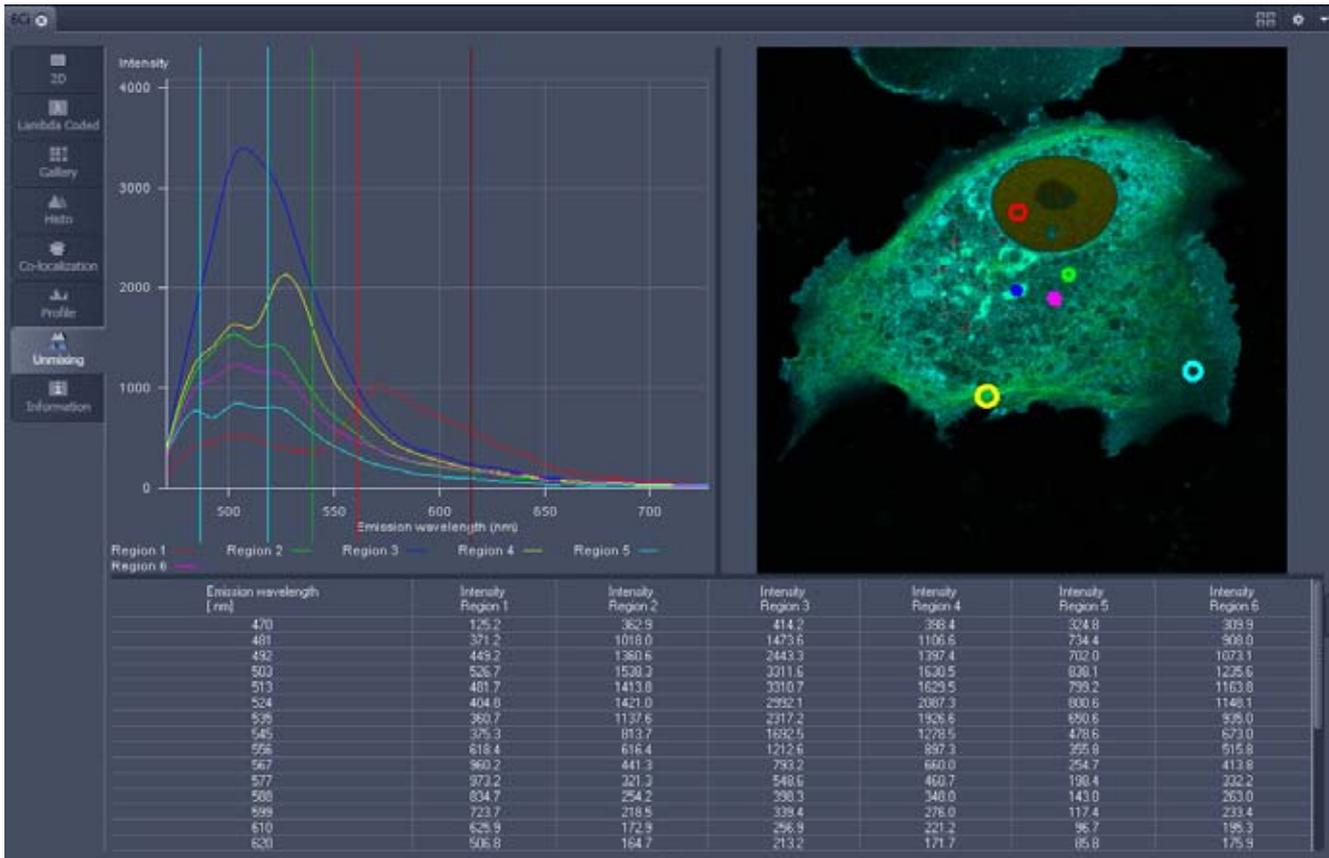


Fig. 6-100 Unmixing Display of a Lambda stack acquired on an LSM 710

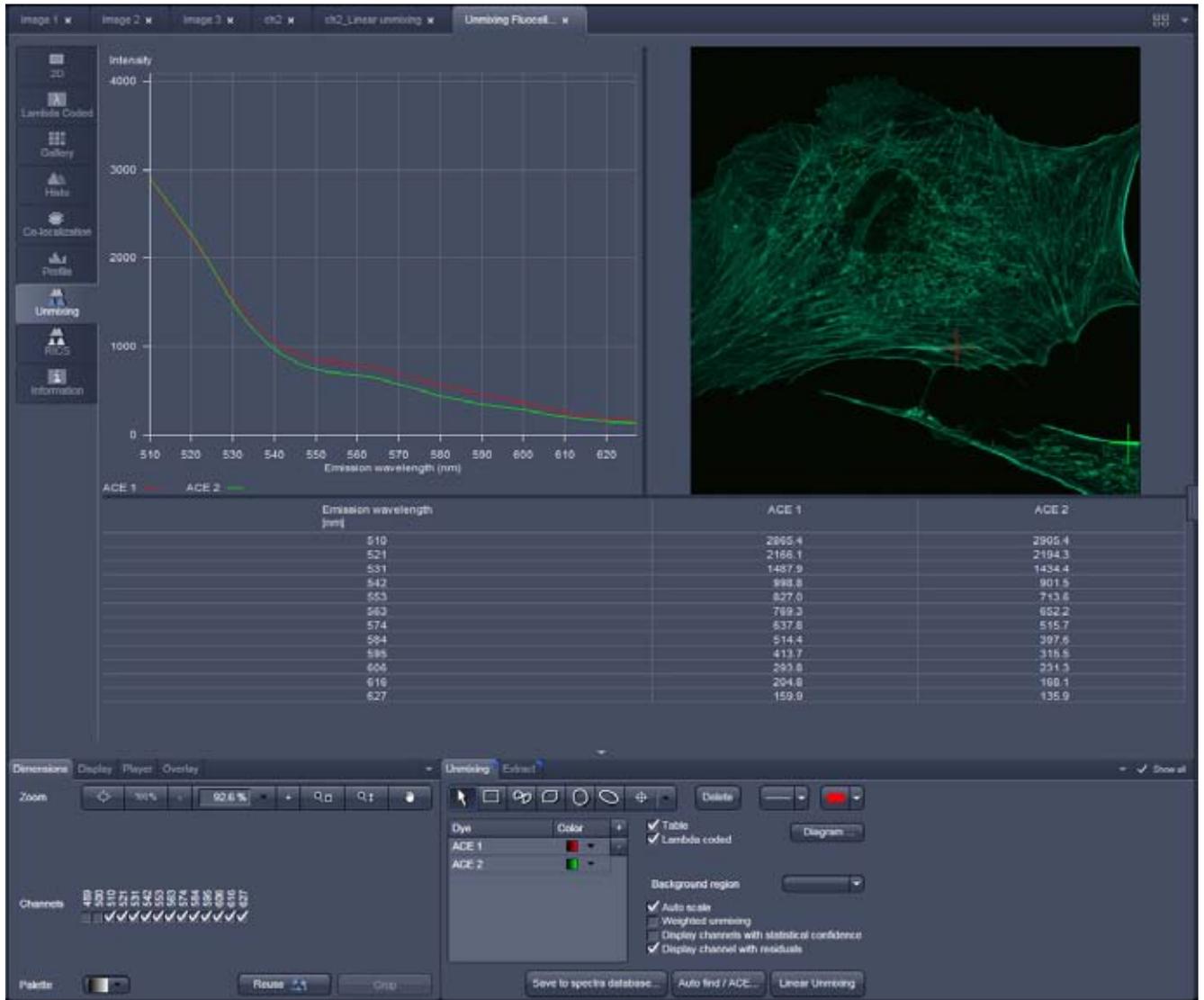


Fig. 6-101 Unmixing Display of a Lambda stack with a spectral dataset acquired on an LSM 700 with a variable secondary dichroic

Tools in the Unmixing View Options Control Block

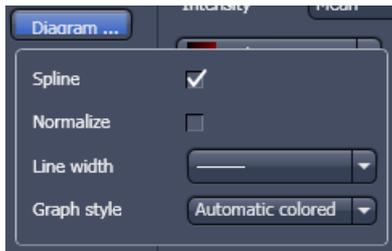
– Tools for display customization and drawing:

Drawing tools

Drawing tools work the same as in the Overlay View Option control block



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



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!

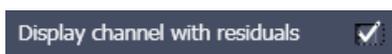
– Tools for unmixing:



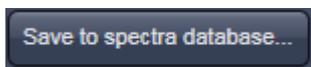
Select ROI for background subtraction



Automatically balances the intensity of unmixed channels to equal levels.



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 **Linear Unmixing**).



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. 6-102).



Auto Find / ACE ("Automatic Component Extraction")

Automatically searches the image for regions with pure signals (Fluorescence originating from one single dye).



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.

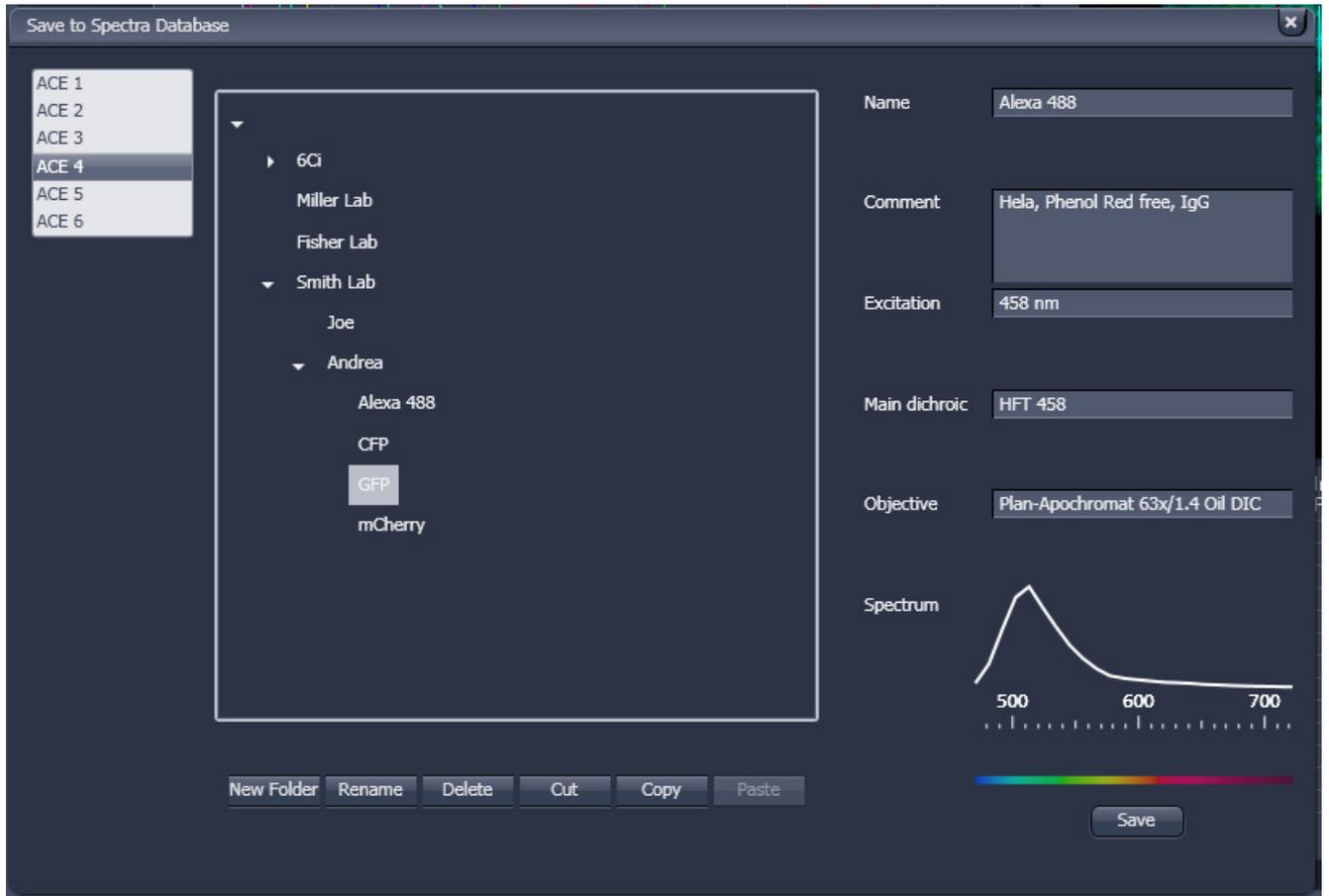


Fig. 6-102 Save to Spectra Database: database interface with subfolder structure

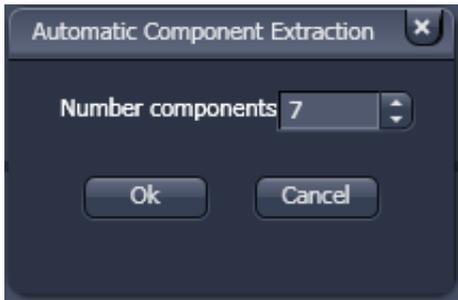


Fig. 6-103 ACE, number of components selection window

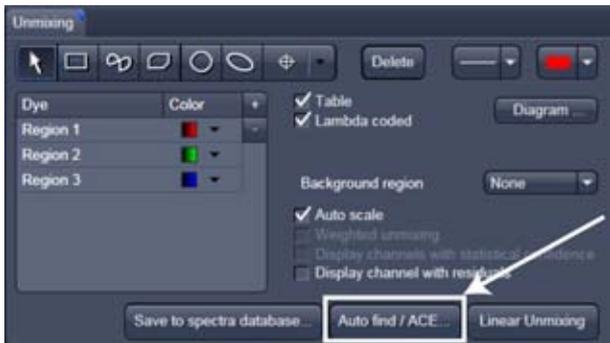


Fig. 6-104 Automatic Component Extraction function

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. 6-104).
- Select the number of components in the sample and click **Ok** (Fig. 6-103).
- Review the results in graph.
- When obtained spectra appear reasonable, unmix the data set by clicking the **Linear Unmixing** button (Fig. 6-104).

- 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.

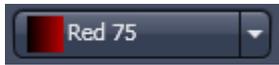
However, it provides a solution for Emission Fingerprinting in those cases where reference spectra are not accessible via single-labeled controls.

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:

These tools (Unmixing – Extract) are only available for Lambda stacks on an LSM 710 with 34 channel Quasar detector. Hence, they are not available for LSM 700 or LSM 7 LIVE Hardware (tool tab grayed).

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.

6.18 Raster Scanning Image Correlation Spectroscopy (RICS)

RICS is an image analysis tool that allows retrieving dynamic information from intensity images or image stacks.

RICS analyses the two dimensional spatial correlation in x and y by shifting the image by spatial lag increments and calculating the self similarity of the image.

In this way the correlation image is of the same dimensions as the original intensity image, but it allows the retrieval of diffusion coefficients and numbers of molecules by comparing the correlation function to model diffusion functions.

In order that RICS algorithms work, the pixel size should be at least 5 fold smaller than the $1/e^2$ beam waist of the excitation light.

For example, the beam waist of a C-Apochromat 40x W NA 1.2 objective is 300 nm, so the pixel size should be at least as small as 60 nm. Best results are obtained, if pixel sizes are 10 fold smaller than the beam waist.

Another pre-requisite is the correct scanning speed. This should match the diffusion coefficient of your molecule. As rule of thumbs for molecules diffusing with $D > 100 \mu\text{m}^2/\text{s}$ a speed of 4 $\mu\text{s}/\text{pixel}$ is adequate.

For molecules diffusing with a $D = 10 - 100 \mu\text{m}^2/\text{s}$ a speed approximating 8-32 $\mu\text{s}/\text{pixel}$ dwell time will be appropriate.

Especially too high and slow speeds should be avoided. All molecules with extreme low diffusion coefficients ($D = 0.1 - 10 \mu\text{m}^2/\text{s}$) should be sampled at 32-100 $\mu\text{s}/\text{pixel}$ dwelling time.

Deviations from these settings will result in intensity images not appropriate for RICS analysis.

Last not least molecular concentrations should stay between 1 nm to 1 μm . RICS works best with time series of 20-100 images. More than one image is needed to first improve on the statistics, and second to have the possibility to remove immobile and slowly moving structures.

The RICS procedure can therefore be divided by acquisition of a time series, removing slow moving and immobile structures, computing the correlation and then fitting the data to model functions.

6.18.1 Access to RICS

If you have taken an image or an image series or have opened an old image or image stack, a click on the **RICS** tab of the **View** tab list will make the RICS functionality available.

The right column of the **Image** window will display the Fit table, the middle image the original intensity image and the left one the computed correlation image using a default LUT (look up table) and a scale bar for the correlation (Fig. 6-105). Below, the result table is displayed.

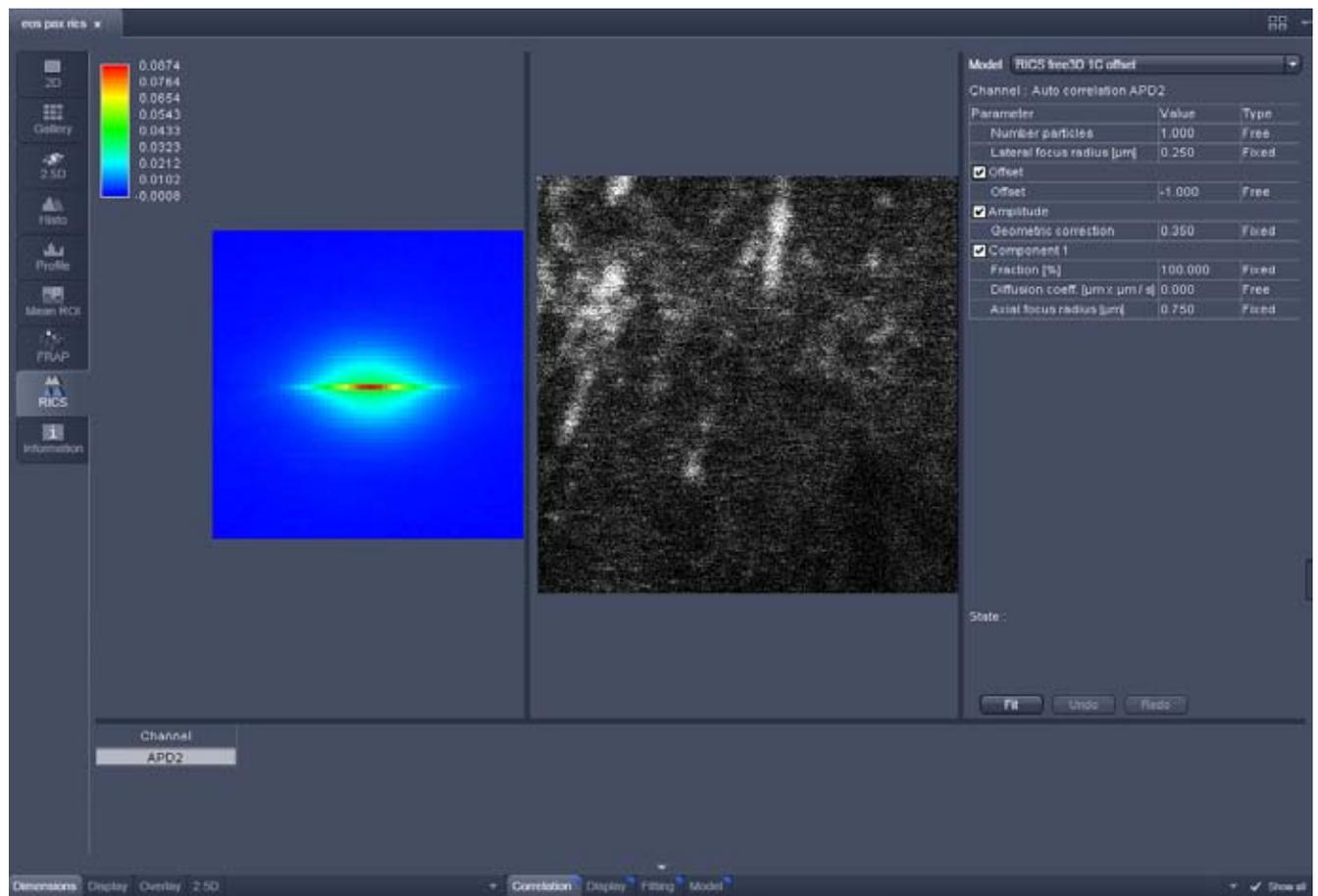


Fig. 6-105 RICS window with RICS register active

6.18.2 Control of View Functionalities

All required settings and manipulation for the RICS analysis can be called up with the View tabs. They divide into **View** for image manipulation, **RICS** for correlation analysis and **2.5D** for display functionalities. The **View** and **2.5D** options are described at respective sites of the manual dealing with image display. Here RICS functionalities are detailed.

6.18.3 Correlation Functionalities

Activate **Correlation** under the **RICS** view. The Correlation options will be displayed in the View control window (Fig. 6-106).

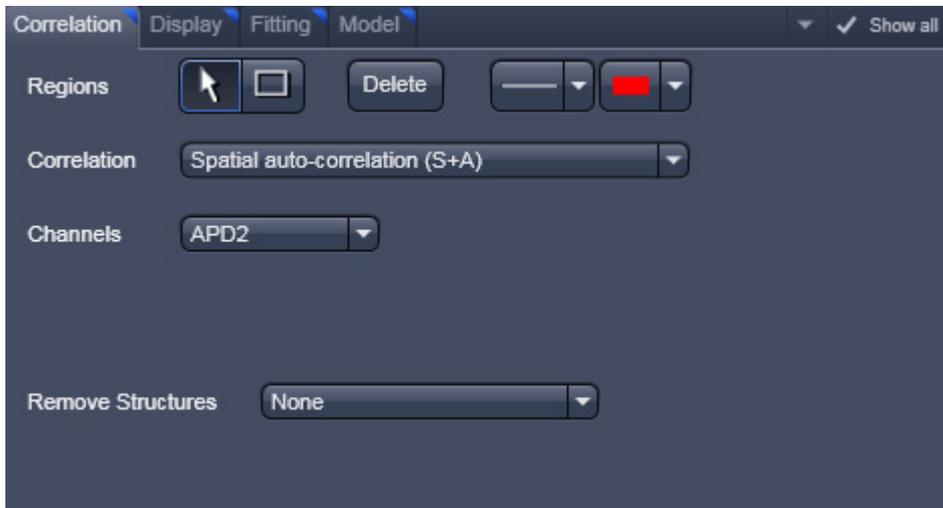


Fig. 6-106 Correlation control window

Regions allow you to draw regions of interest (ROI) within the intensity image. Activate the **Rectangle** button and draw a region within the intensity image by holding down the left mouse button. Any rectangular shaped region can be drawn. The correlation will be newly computed taken into account now only the ROI.

If the **Arrow** button is active, the ROI can be moved around the image by drag and drop. If the **Arrow** button is deselected, a new ROI will be drawn.

For each ROI a correlation image will be computed (Fig. 6-107). You can view each correlation by selecting the respective row in the **Result** table at the bottom. It is not possible to view two correlation images at the same time.

If a ROI is selected with the Arrow button active, then pressing the **Delete** button will remove the ROI from the intensity image.

With the **Line** button you can select the line width and with the Color rectangle button the color of a selected ROI via drop down menus.

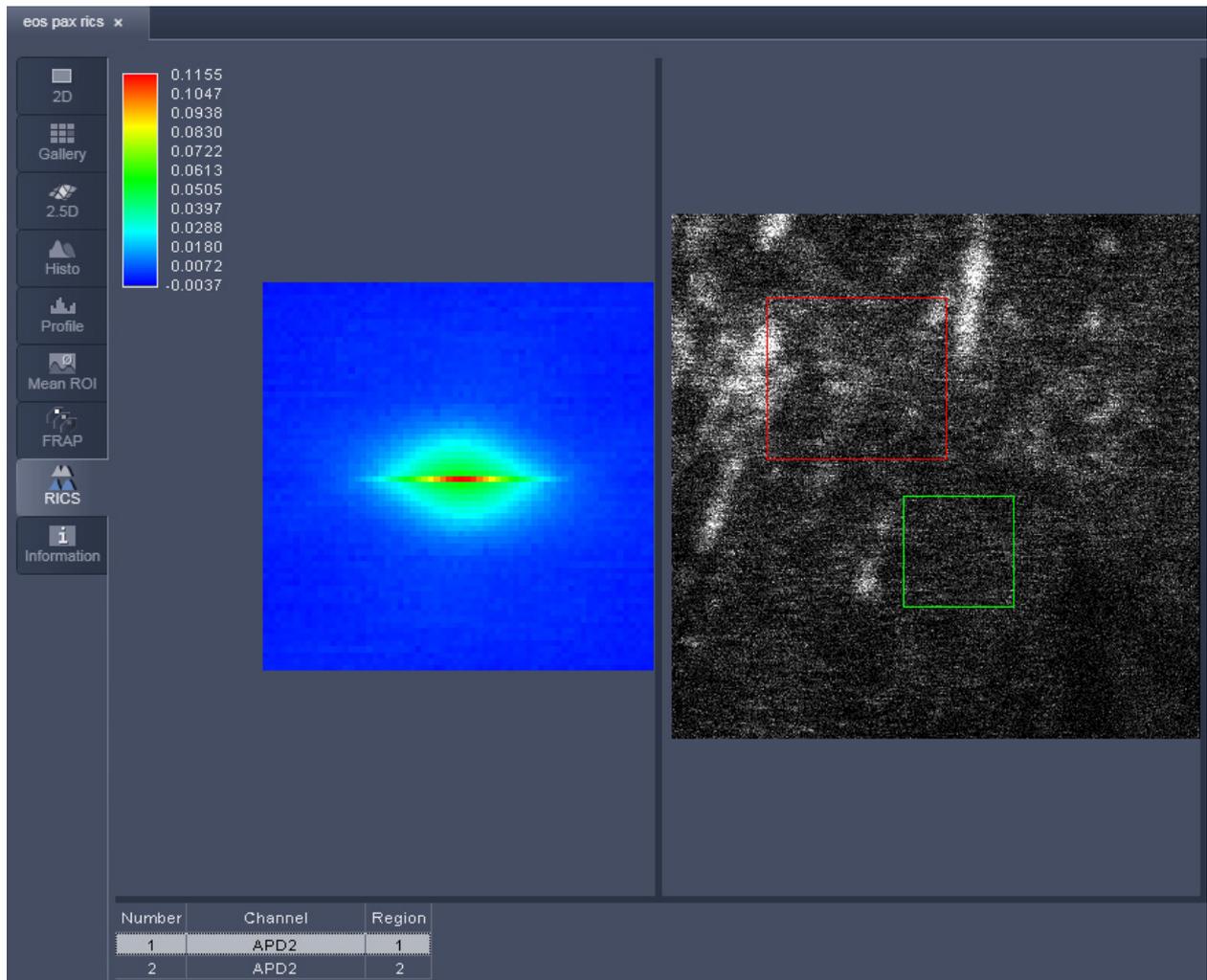


Fig. 6-107 Image window with two selected regions of interest in the intensity image

Correlation allows you to select the type of correlation analysis you want to perform. For a one channel recording the only option is **Spatial auto-correlation (S+A)**. For a two channel recording you can select from the pull down menu between **Spatial auto-correlation (S+A)** and **Spatial cross-correlation (S+C)** (Fig. 6-108).

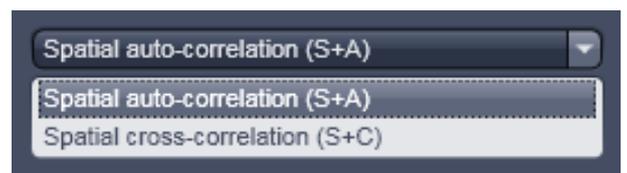


Fig. 6-108 Correlation pull down menu

Channels allow you to select the channel for which a correlation should be performed. For a one channel record auto-correlation setup, only the active channel is available. For a two channel auto-correlation setup you can select the channel by the pull down menu (Fig. 6-109). The correlation of the active channel will be computed and displayed. Both channels will be displayed in the **Result** table and the correlation of the selected row will be displayed.

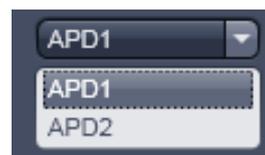


Fig. 6-109 Channels pull down menu for auto-correlation



Fig. 6-110 Channels pull down menu for cross-correlation



Fig. 6-111 Remove structures pull down menu



Fig. 6-112 Averaging frames enter box

For a two channel cross-correlation setup, two drop-down menus will be displayed (Fig. 6-110). The upper channel will be the one that will be cross-correlated against the lower channel. In addition, two auto-correlations for each channel will be displayed. All correlations will be displayed in the **Result** table and the correlation image of the selected row will be presented. Note, if both channels are identically selected, the cross-correlation all three correlations will be identical auto-correlations for the double selected channel.

Remove structures is needed to remove the contribution of immobile or slowly moving structures from the correlation. You can select what kind of average subtraction you want to perform from the pull down menu (Fig. 6-111). If **None** is selected, no subtraction will be done.

If **Slowly moving structures** is the choice the **Average frames** enter box will become available (Fig. 6-112). Choose a moving average frame number that would clean up the correlation from slow moving structures by entering a number in the box, by using the arrows or the slider.

Using 5 frames is a good compromise for nearly all situations. This creates a new stack of $n-m+1$ frames, where n = number of frames from original stack and m = number of moving average frames.

Subtraction of the moving average frames from the original frames starts at $m/2$ and ends at $m/2+(n-m+1)$ frames of the original stack. Thus the first and last frames of the original stacks are not used. To avoid negative pixels by this procedure a scalar is added to each subtracted frame that is computed from all pixels of the moving average that was used for subtraction. The result is a correlation image, that is not any more afflicted by slow moving and immobile structures and from which fast dynamic processes can be calculated (Fig. 6-113).

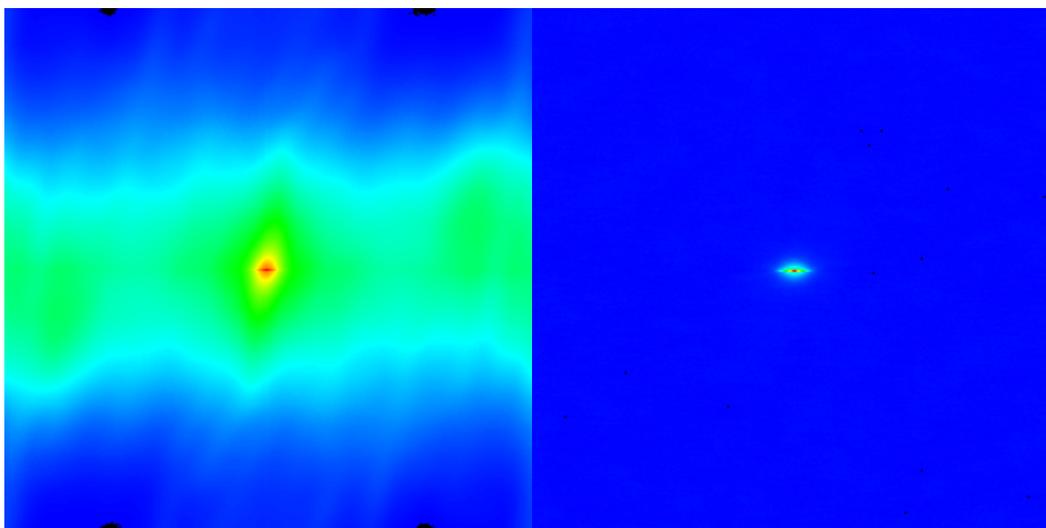


Fig. 6-113 Correlation image before (left) and after (right) removal of slow moving structures

When **Immobile structures (+Stack)** is selected, the average image from the stack (pixel by pixel) is subtracted from every image and a scalar is added to avoid negative pixels. The scalar is computed from the average of all pixels from all frames of the stack. When **Immobile structures (+Plane)** is selected, the average image from the stack (pixel by pixel) is subtracted from every image and a scalar is added to avoid negative pixels. The scalar is computed from the frame to which it is added. Both procedures are equally valid in most of applications.

6.18.4 Display Functionalities

Activate **Display** under the **RICS** view. The Display options will be displayed in the View control window (Fig. 6-114).

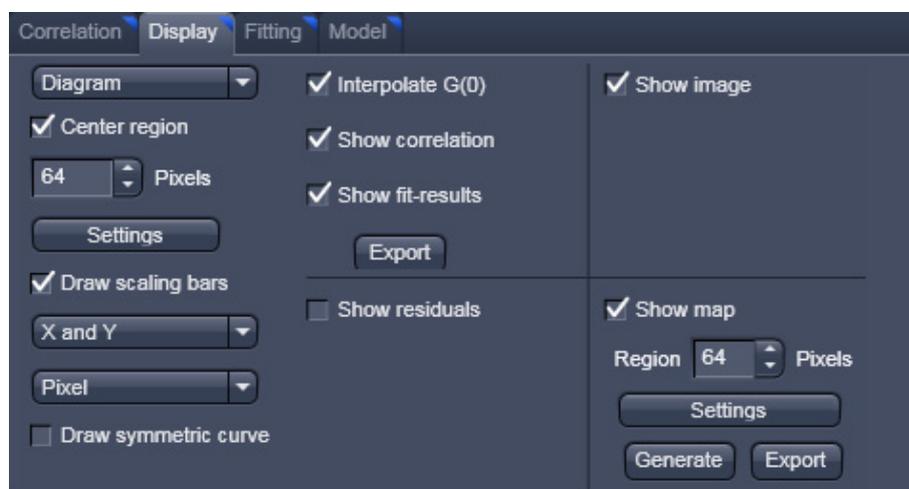


Fig. 6-114 Display control window

In the **Representation** pull down menu you can choose how the correlation function should be displayed.

Four options are available (Fig. 6-115):

Diagram will display the horizontal and vertical profiles of the correlation function and the fit / residuals if activated;

Image will display the correlation and the fit / residuals if activated as two dimensional images;

Table will display the coordinates of the horizontal and vertical profiles of the correlation and the fit / residuals if activated that can be copied or saved via a right mouse click from the **Table Export** menu (Fig. 6-116);

2.5 D will display the correlation function and the fit / residuals if activated as pseudo 3D images (Fig. 6-117).

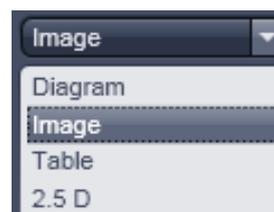


Fig. 6-115 Representation pull down menu

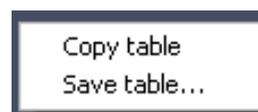
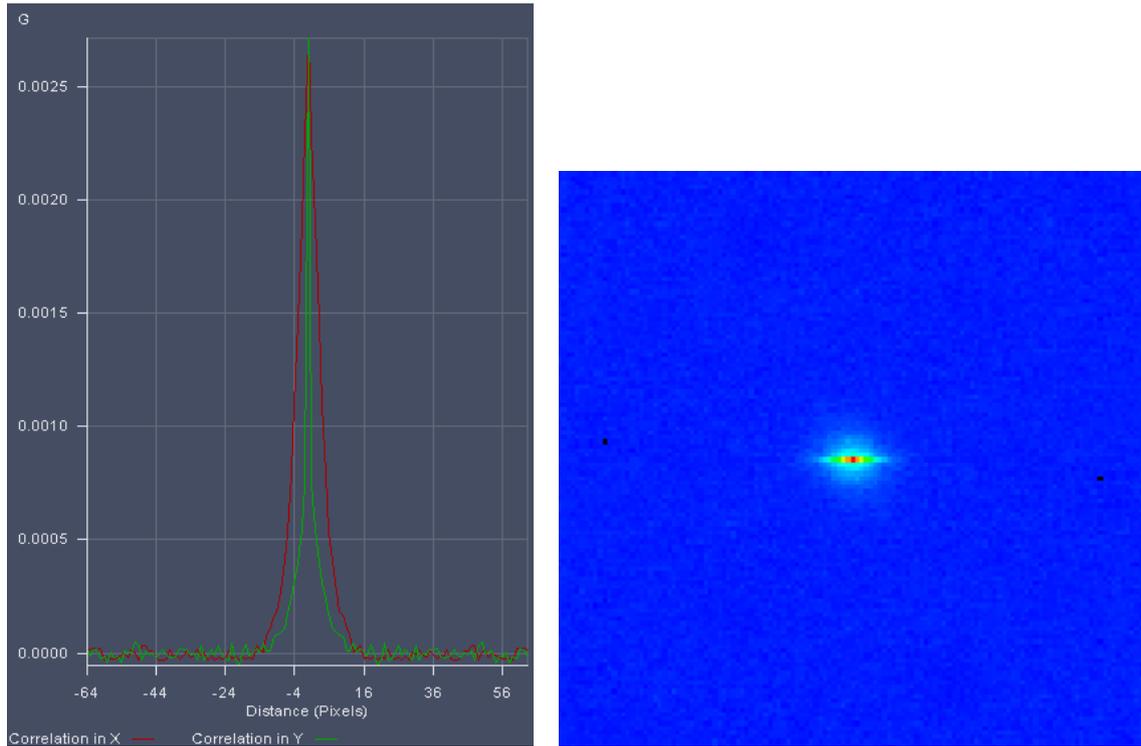


Fig. 6-116 Table export menu



Distance	Correlation X	Correlation Y
-9	0.0001845	0.0000794
-8	0.0002719	0.0000889
-7	0.0004087	0.0001104
-6	0.0005320	0.0001700
-5	0.0007900	0.0002392
-4	0.0010917	0.0003138
-3	0.0014632	0.0003965
-2	0.0018991	0.0005512
-1	0.0022843	0.0007451
0	0.0027150	0.0027150
1	0.0022843	0.0007451
2	0.0018991	0.0005512
3	0.0014632	0.0003965
4	0.0010917	0.0003138
5	0.0007900	0.0002392
6	0.0005320	0.0001700
7	0.0004087	0.0001104
8	0.0002719	0.0000889
9	0.0001845	0.0000794

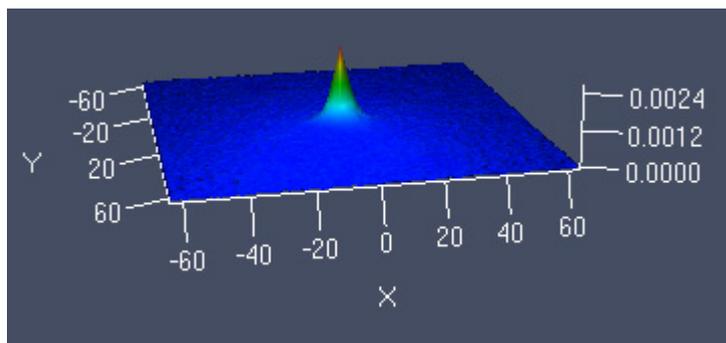


Fig. 6-117 Correlation displayed clockwise as Diagram, Image, Table and 2.5 D



Note, that for different representations different options will become available.

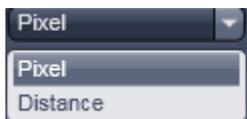


Fig. 6-118 Pixel pull down menu

For the **Diagram**, **Table** and **2.5D display**, the **Pixel** pull down menu becomes available (see Fig. 6-118). Here you can choose to display distances as integer pixels or metrically in μm .

For the Diagram and Table, the **X and Y** pull down menu and the **Draw symmetric curve** check box will be additionally available. In the **X and Y** pull down menu you can choose if you want to display correlations in both (x and y) or only one (x or y) direction (see Fig. 6-119 and Fig. 6-120).



Fig. 6-119 X and Y pull down menu

If the **Draw symmetric curve** check box is checked (Fig. 6-120), the correlation will be displayed symmetrically around the zero spatial lag with negative and positive lag values displayed. If the box is unchecked, only the positive lag values will be displayed.



Fig. 6-120 Draw symmetric curve check box

For the Diagram only the **Settings** popup menu is available (see (Fig. 6-121),. Here you can assign for the **X and Y Dimensions** individual colors to the **Correlation**, **Fit results** and **Residuals** diagrams via the corresponding color palette drop down menus. You can also define for each dimension the line width by entering a number in the **Line width display boxes** or using the arrows. By checking the left and right **Auto-Scale check boxes**, the diagrams for Correlation & Fit results as well as the diagram for the Residuals, respectively, will be auto scaled to the lowest and highest value. By de-checking you can enter your own minimum and maximum values into the corresponding **Min.** and **Max. display boxes** by typing or by using the arrows. All values between the minimum and maximum will be scaled accordingly. Values lying outside will not be displayed.



Fig. 6-121 Settings popup menu

If the **Center region** box is checked the correlation will be centered on the pixel numbers that can be put into the displayed **Pixels** input box. This allows you to display only the relevant part of the correlation and avoid areas of no content.

If the **Draw scaling bar** box is checked, a Scaling will appear for any correlation diagram and deviated diagram (Fit, Residuals, Map). For the intensity image, no scaling bar is available.

If the **Use image palette** box is checked, the look up table (LUT) selected for the intensity image will be taken for the correlation diagram and Maps; the LUT will not apply for Fit and Residuals. If the **Interpolate G(0)** box is checked the zero spatial correlation value will be interpolated.

Checking and unchecking the **Show fit-results** box will toggle between the correlation and fit images. Note, if this box is activated, the **Interpolate G(0)** box will disappear. Pressing the export button below the **Show fit-results** box will export the correlation image or fit image, whatever is active as an image in .lsm format.

If **Table**, **Image** or **Diagram** was selected from the **Representation** pull down menu, the 2D image will be exported. If 2.5 D was selected, a pseudo 3D image will be stored. If the Show residuals box is checked, the Residuals will be displayed in a format as selected in the **Representation** pull down menu (Fig. 6-115). By activating this box the **Hide G(0)** error check box becomes available as well. Check the box in order to omit the zero spatial correlation value to be included in the fit.

Pressing the export button below the **Hide G(0)** box will export the residuals image. If **Table**, **Image** or **Diagram** was selected from the **Representation** pull down menu, the 2D image will be exported. If 2.5 D was selected, a pseudo 3D image will be stored. Activating / Deactivation the **Show image** check box will display or remove the intensity image from the **Center image** window.

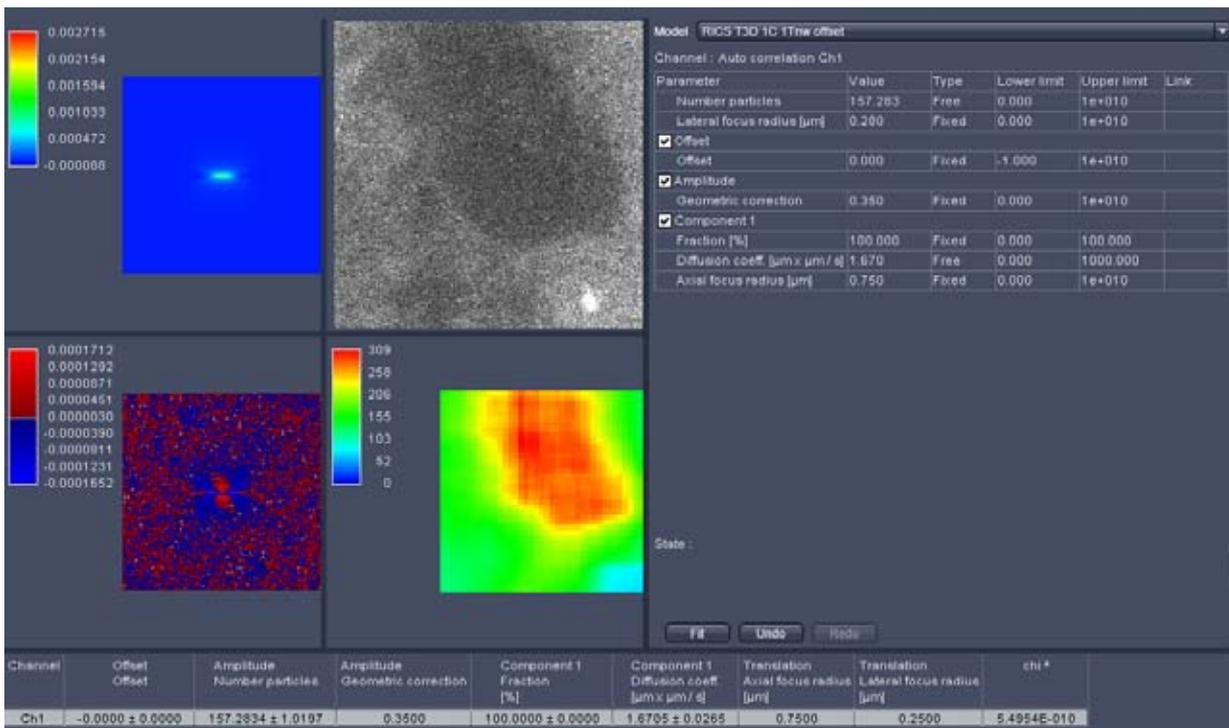


Fig. 6-122 Center Image window displaying Fit image, Intensity image, Fit table, Residuals Image, Map and Result table (from top left to bottom right)

Note that the **Draw scaling bar**, **Use image palette** and **Hide G(0)** check boxes will only be available in the RICS **Show all** mode. To activate the **Show all** mode activate the RICS **Show all** button on the **View Control** bar.

If the **Show map** box is checked, a map, if being created before, will be displayed and the map options will be visible. In the **Region in Pixels** input box you determine the size of the region in an m x m format to be used for mapping the intensity image. This size should naturally be smaller than the image size, but it is recommended not to fall below 64 x 64 pixels to obtain enough signal-to-noise ratio (SNR).

If you press the **Settings** button, the settings menu will pop up (Fig. 6-123).

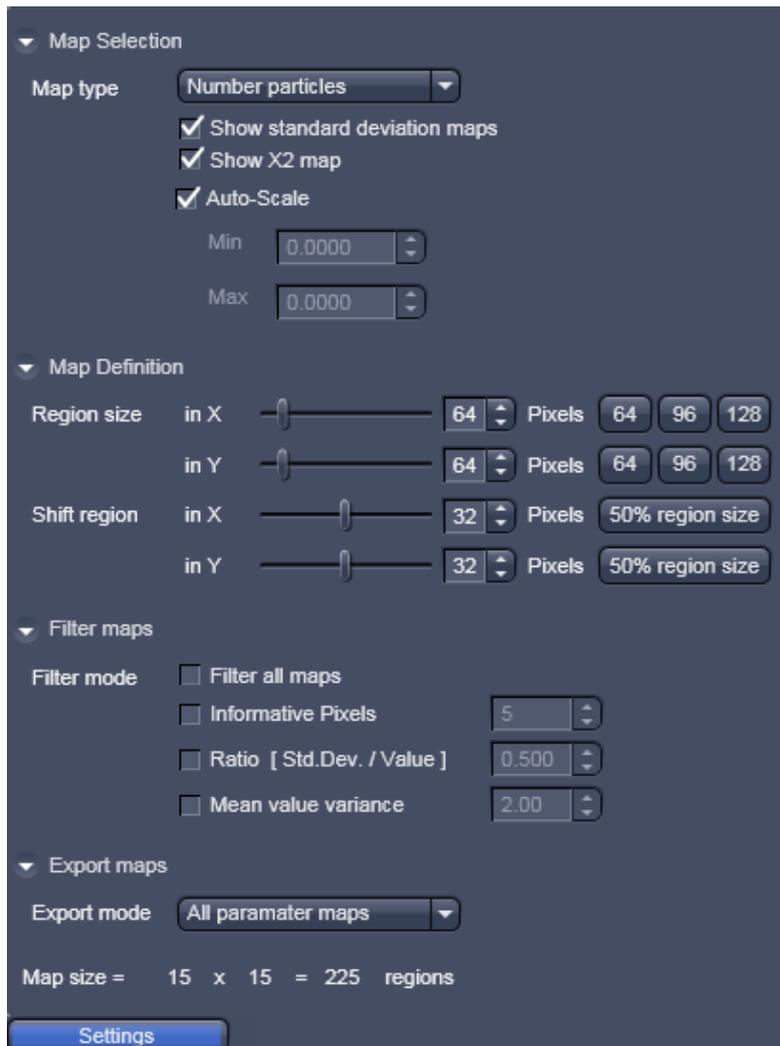


Fig. 6-123 The Map settings menu

In **Map Selection** you can determine the map type with the **Map type** pull down menu (Fig. 6-124). You can choose between the **Number particles** and the **Diffusion coeff. ($\mu\text{m} \times \mu\text{m} / \text{s}$)** parameter maps, that will display the relative molecule numbers –please note that if average subtraction is performed the information on absolute numbers is lost - and diffusion coefficients of the mapped areas.

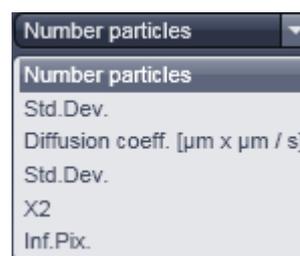


Fig. 6-124 Map type pull down menu

You can also display the informative pixels by choosing **Inf.Pix**. This will display a map that indicates, how many pixels contributed meaningfully to the correlation before dropping to zero.

If the **Auto Scale** check box is activated, the maps are scaled automatically between the highest and lowest value. If unchecked you can enter in the **Min** and **Max input boxes** own minimum and maximum values. All values lying between will be accordingly scaled. Note that all values lying outside the

minimum and maximum will be displayed as black in the maps. This option is helpful in eliminating extreme outliers.

In the **Show all** mode you have further the option to display the **Std.Dev.** (standard deviation) maps of both parameter maps as well as the X^2 (Chi square) map by checking the **Show standard deviation maps** and **Show X2 map** boxes.

In **Map definition** you can define the region size in an m x n format. Please note, if you don't choose a size as an integer part of the intensity image, some rest areas to the right and bottom of the image will not be taken into account. Region sizes can be put into the **Region sizes in X / Y in Pixels** input boxes by using the slider, arrows or typing. Default values in an m x m format can be obtained by pressing the **64**, **96** or **128** buttons. The step size with which the region will be shifted from data point to data point can be put in the **Shift region in X / Y in Pixels** input boxes by using the slider, arrows or typing. The default value of a shift size half the region size can be obtained by pressing the **50% region size** button.

In **Filter maps** different **Filter modes** can be selected to sort out outlier values. Extreme outliers will affect the scaling of the map so that interesting values can be hidden. By checking the **Filter all maps** box, chosen filters will be applied for all maps. If not selected only the map chosen in the **Map type** pull down menu will be filtered. You can choose between Informative Pixels, ratio and mean / variance filter by checking the respective **Informative Pixels**, **Ratio (Std.Dev./Value)** and **Mean value variance** boxes. Thresholds can be set in each case via the input boxes by typing or using the arrows. Note that filters can be combined.



Fig. 6-125 Export mode pull down menu

In Export maps one can select which maps should be exported as an .lsm image by selecting between the **Current map**, that is only the map selected in the Map-type pull down menu, **All parameter maps**, that are the **Number particles** and **Diffusion coeffic. ($\mu\text{m} \times \mu\text{m} / \text{s}$)** maps, and **All maps**, that are the parameter maps, **Std.Dev.** maps and X^2 maps (Fig. 6-125).

The map size in terms of region numbers is displayed in the info area of the **Settings** menu.

By pressing **Generate** a map is created with region and shift sizes as determined in the settings. During creation of the new map, an old map will be overwritten. The progress in calculation is displayed in the intensity image by an overlay region and the black map is filled with white regions (Fig. 6-126).

The **Generate** button will only be active, if the correlation was fit to a model beforehand to ensure the correct one was selected.

Only at the end of the map, the scaling is calculated and the map displayed in the chosen palette. By pointing the pointer to a region in the map, the corresponding site from which this data point was derived will be displayed in the intensity image by the overlay region. By pressing **Export** maps will be saved according to the settings in an .lsm format or the type you choose in the Windows Explorer popup menu.

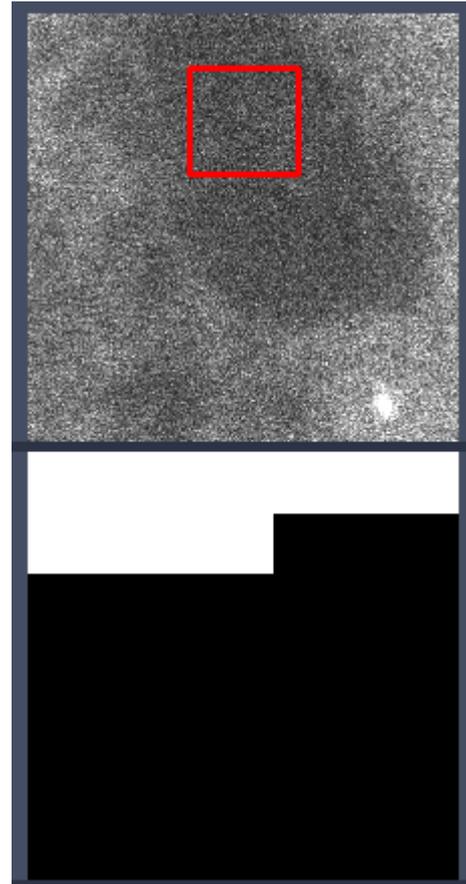


Fig. 6-126 Progress indicator during creating a map in the intensity (upper panel) and map (lower panel) images

6.18.5 Fitting Functionalities

Activate **Fitting** under the **RICS** view. The Fit options will be displayed in the View control window (Fig. 6-127).

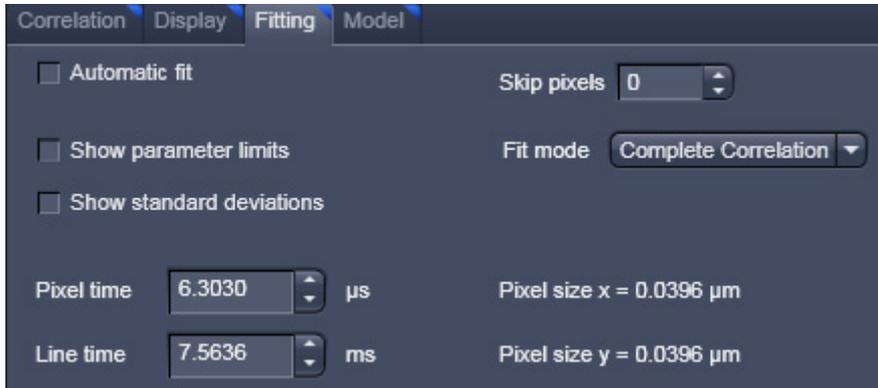


Fig. 6-127 Fit control window

If the **Automatic fit** check box is selected, a fit will be performed automatically, whenever the correlation input intensity image is changed. For example, if a region of interest is drawn or moved within an intensity image, the fit will automatically be performed according to the default or loaded model. If the check box is deselected, a fit will only be performed when pressing the **Fit** button in the **Fit** table (Fig. 6-128).



Fig. 6-128 Fit table

You can select a model from the **Model** pull down menu in the **Fit** table. Every stored model will be listed (Fig. 6-129). Below the **Model** pull down menu the active channel is displayed in the **Channel** display area. The table below lists the terms and parameters of the selected model in the **Parameter** column, its current value in the **Value** column, the type of the parameter in **Type** column that can be set as **Free** (freely floating with initial guess), **Fixed** (no fit) and **Start** (freely floating with start value) from the **Type** pull down menu between (Fig. 6-130), the lower and upper limits assigned by typing in the desired number to the parameter in the **Lower limit** and **Upper limit** columns and the type of global linkage in the **Link** column, which presently is not activated. Terms in the parameter columns can be activated or deactivated by ticking or un-ticking the respective check boxes.



Fig. 6-129 The model pull down menu of the Fit table

In the **State** display area any abnormal settings will be displayed. For example, if Number particles and Geometric factor are both set to free, the system will comment that one has to be fixed (Fig. 6-131).



Fig. 6-130 Type pull down menu



Fig. 6-131 The State display area with message

To perform a fit press the **Fit** button. The active correlation function will then be fitted according to the settings in the **Fit** table. Pressing the **Undo** button will cancel the commands in the reverse order of their placement. The **Redo** button will become active once the **Undo** button has been pressed once. It allows recalling cancelled commands in the order of their cancellation.

Fit results and statistics will be listed in the **Result** table (Fig. 6-132). It lists the channel and all parameters (with or without standard deviation according to the settings) that are present in the **Fit** table as well as the χ^2 (chi²) value of the fit.

Channel	Offset Offset	Amplitude Number particles	Amplitude Geometric correction	Component 1 Fraction [%]	Component 1 Diffusion coeff. [µm ² µm / s]	Translation Axial focus radius [µm]	Translation Lateral focus radius [µm]	chi ²
Ch1	-0.0000 ± 0.0000	157.2834 ± 1.0197	0.3500	100.0000 ± 0.0000	1.6705 ± 0.0265	0.7500	0.2500	5.4954E-010

Fig. 6-132 The Result table



Fig. 6-133 Result table menu

By clicking with the right mouse button into the **Result** table the table's menu box will pop up (Fig. 6-133). You have the option to copy the table to the clipboard by selecting Copy, or by choosing save to save the data as a text file. Please note that the options **Select all**, **Select all channels** and **Select all regions** is currently not available.

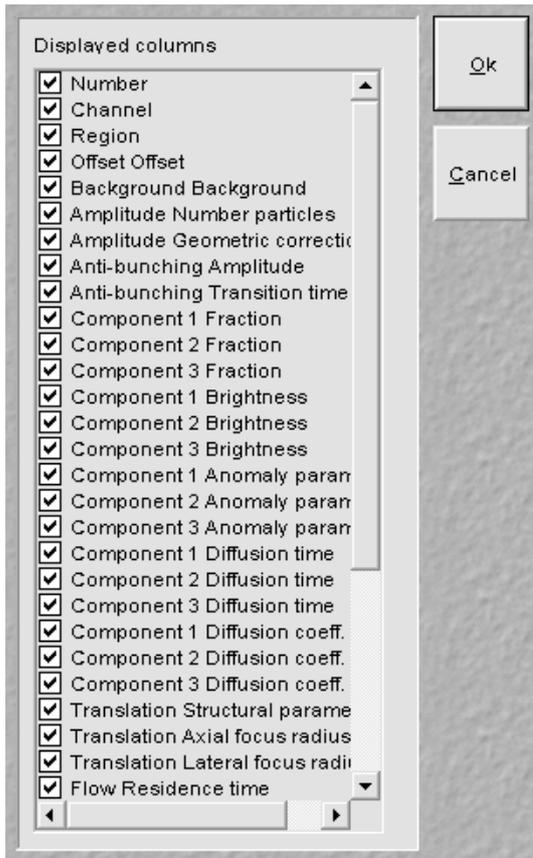


Fig. 6-134 Properties sub menu

By selecting **Properties** the **Properties** submenu will appear (Fig. 6-134). The table lists all available parameters. You can select the parameters you want to have displayed in the result table by checking the corresponding boxes.

If the **Show parameter limits** check boxed is ticked, the **Upper limits** and **Lower limits** columns are displayed in the Fit table, otherwise they are not visible. If the **Show standard deviations** check box is ticked, standard deviation for floating parameter values are displayed, otherwise they are suppressed.

Pixel dwelling times in μs and line times in ms of the scan are displayed in the **Pixel times in μs** and **Line times in ms** input boxes in the **Show all** mode. They are read from the Meta data of the image and are fixed values for the Fit. If you want to simulate with other numbers, they can be entered in the input boxes via the arrows or typing. Note, if numbers are changed and the image saved under the same name, the real values will be lost. Pixel sizes in μm are only accessible from the **Information** register.

The **Skip pixels** input box in the **Show all** mode allows setting the numbers of spatial lag pixels that should be omitted for the Fit. The number can be entered by typing or using the arrows.

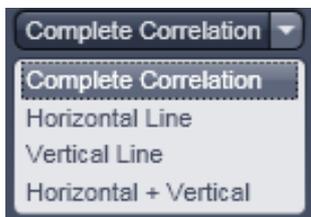


Fig. 6-135 Fit mode pull down menu

With the **Fit mode** input box, only available in the **Show all** mode, the data set for fitting can be defined (Fig. 6-135). You can select between fitting the **Complete Correlation**, the **Horizontal Line** only, the **Vertical Line** only, or the combined **Horizontal + Vertical** lines.

In the **Show all** mode, the size of the Pixels in x and y dimensions are shown in the **Pixel size display area**. Note that the sizes cannot be edited.

6.18.6 Model Functionalities

Activate **Model** under the **RICS** view. The **Model** options will be displayed in the View control window (Fig. 6-136).

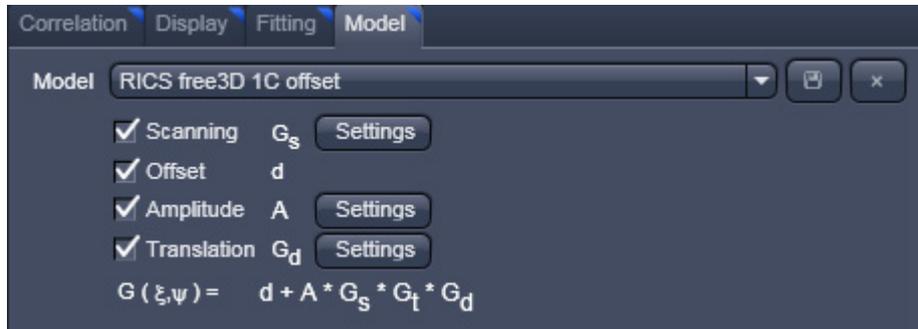


Fig. 6-136 Fit control window

With the **Model** pull down menu you can select a saved pre-defined model from the list. You can rename a created or changed model by typing in a new name in the display box. If the **Ok** button is pressed, the model will be saved under the new name (Fig. 6-137). If the model name was not changed, you will be prompted to acknowledge to overwrite the old existing one in the **Save Model Warning** menu (Fig. 6-138). Press **Yes** to overwrite, **No** to keep the old model.

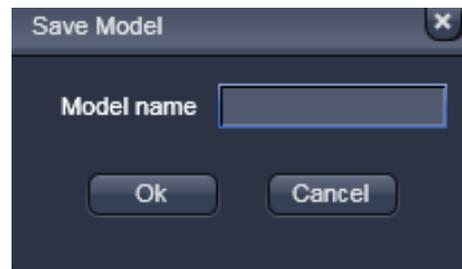


Fig. 6-137 Model Save menu

Type in a name in the **Model name** display box and press **Ok**, if you want to save the model, or press **Cancel** without saving. You can delete a model by pressing the **Cross** button. You will be prompted to acknowledge the action within the popped up **Delete Model** menu (Fig. 6-139). Press **Yes** to delete, **No** if the model should be kept.

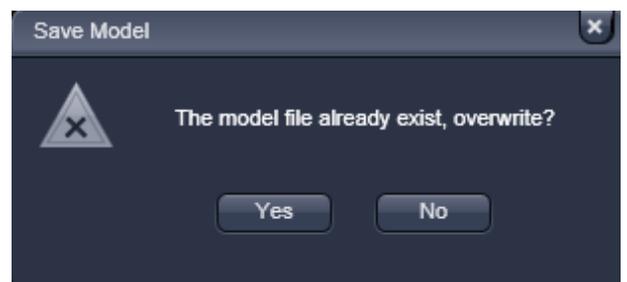


Fig. 6-138 Save Model Warning menu

Below the Model pull down box the available terms to assemble a model are listed. If you want to take a term into account, check the corresponding box. Four terms are available: **Scanning** G_s , **Offset** d , **Amplitude** A and **Translation** G_d . The assembled model will be displayed in the spatial correlation $G(\xi, \psi)$ display area.

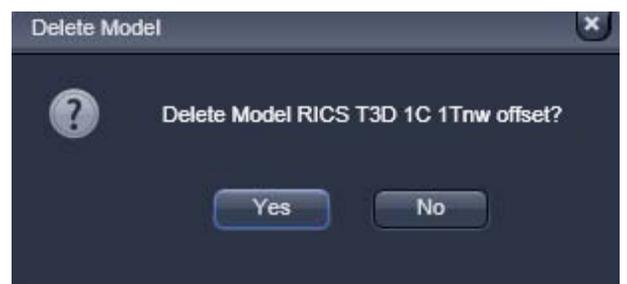


Fig. 6-139 Delete Model menu

For three of the terms (G_s , A and G_d) settings menus are available. To open a settings menu, press the corresponding **Settings** button.

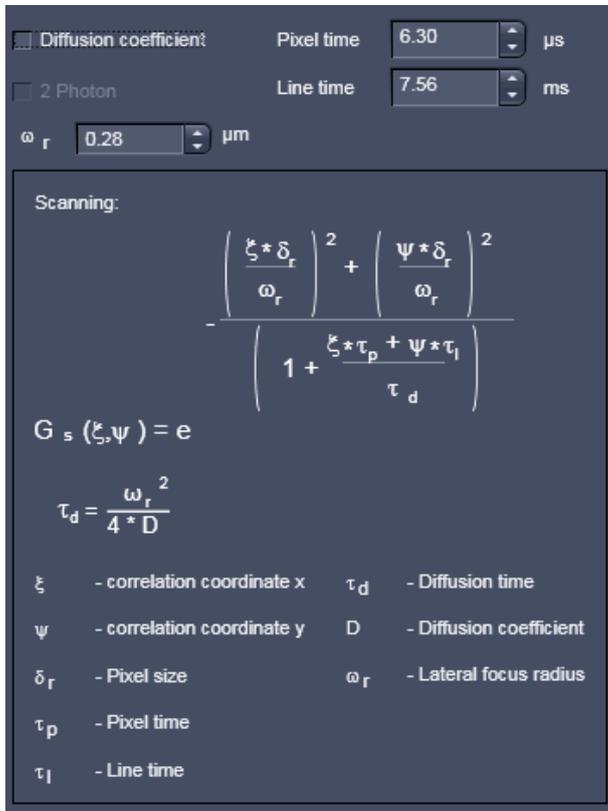


Fig. 6-140 Settings for the Scanning term

Scanning $G_s(\xi, \psi)$ describes the scanning term of the model function. Scanning is only active in the **Show all** mode. To open the **Scanning** settings press the **Settings** button (Fig. 6-140).

If the **Diffusion coefficient** check box is ticked, the diffusion coefficient will be fitted, otherwise the diffusion time. In case the box is checked, the **2 Photon** check box will be accessible. If ticked diffusion time scales with 1/8th of the diffusion coefficient, if un-ticked with 1/4th. The 1/e² radius of the laser beam can be entered within the **ω_r in μm** input box by typing or using the arrows. Pixel dwelling times and line times can be entered in the **Pixel time in μs** and **Line time in μs** input boxes. These values are useful for simulations when fixed. They will be overwritten with the correct Meta data from the image, whenever an image is loaded. If these Meta data are not available, values have to be provided. In the **Scanning information** box the formulas for the Scanning term $G_s(\xi, \psi)$ and the conversion formula between diffusion time τ_d and diffusion coefficient D are displayed as well as a description of the parameter variables.

Offset d describes any scalar added to the correlation function. It will be necessary, if the correlation function does not decay to zero.

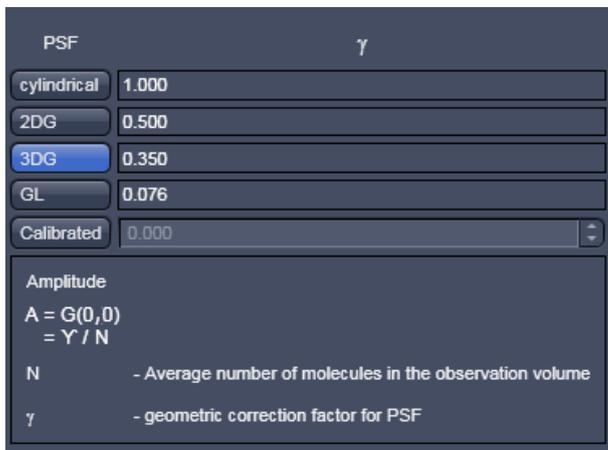


Fig. 6-141 Settings for the Amplitude term

Amplitude A describes number of particles taking into account the form of the point spread function in relation to the studied structure expressed in the geometric correction factor γ . To open the **Amplitude** settings press the **Settings** button (Fig. 6-141). You can select a **cylindrical** PSF with $\gamma=1$, a two dimensional Gaussian (**2DG**) PSF with $\gamma=0.5$, a three dimensional Gaussian (**3DG**) PSF with $\gamma=0.35$ or a Gaussian-Lorentzian (**GL**) PSF with $\gamma=0.076$ by pressing the respective button. Alternatively, if **Calibrated** is active, you can enter a calibrated value via the **Calibrated** input box.

In the **Amplitude information** box the formulas for the Amplitude term A is displayed as well as a description of the parameter variables.

Translation $G_d(\xi, \psi)$ describes the translational diffusion term of the model function. To open the **Translation** settings press the **Settings** button (Fig. 6-142).

Fractional intensities

Diffusion coefficient ω_r 0.280 μm

2 Photon

Components: 1, 2, 3

Free / anomalous: free, free, free

Dimension: 3D, 3D, 3D

Molecular brightness: 0, 0, 0

$$G_d(\tau) = \sum_{i=1}^3 \frac{\Phi_i}{\left(1 + \left(\frac{\tau}{\tau_{d,i}}\right)^{\alpha_i}\right)^{e_{d1}} * \left(1 + \left(\frac{\tau}{\tau_{d,i}}\right)^{\alpha_i} \frac{1}{S}\right)^{e_{d2}}}$$

$\Phi_i = \frac{f_i * \eta_i^2}{\sum_{i=1}^3 (f_i * \eta_i)^2}$
 $C = \frac{N}{L_A * \pi^{3/2} * \omega_r^2 * \omega_z}$
 $S = \frac{\omega_z}{\omega_r}$
 $\tau_{d,i} = \frac{\omega_r^2}{8 * D_i}$

	e_{d1}	e_{d2}
1D	0.5	0
2D	1	0
3D	1	1

- i - Index of component (1, 2, 3)
- Φ_i - Fractional intensity
- f_i - Fraction of molecules
- η_i - Molecular brightness
- $\tau_{d,i}$ - Diffusional correlation time
- S - Structural parameter
- ω_z - Axial focus radius
- ω_r - Lateral focus radius
- α_i - Anomaly parameter
- N - Total number molecules
- D_i - Diffusion coefficient
- c - Concentration

Fig. 6-142 Settings for the Translation term

If the **Fractional intensities** box is checked, the **Molecular brightness** input boxes will become available. Enter the absolute or relative molecular brightness of the molecules, which have to be determined beforehand. If unchecked, molecular brightness will not be taken into account and the **Molecular brightness** input box will not be displayed.

If the **Diffusion coefficient** check box is ticked, the diffusion coefficient will be fitted, otherwise the diffusion time. In case the box is checked, the **2 Photon** check box will be accessible. If ticked diffusion time scales with $1/8^{\text{th}}$ of the diffusion coefficient, if un-ticked with $1/4^{\text{th}}$. The $1/e^2$ radius of the laser beam must be entered within the ω_r in μm input box, which is also displayed only in case the Diffusion coefficient box by typing or using the arrows.

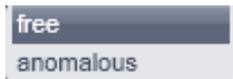


Fig. 6-143 Free anomalous pull down menu

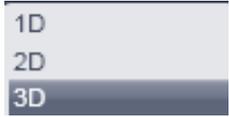


Fig. 6-144 Dimension pull down menu

Components allow selecting **1**, **2** or **3** components by pressing the respective button. **Free / anomalous** allow via pull- down menus to select between free translational or anomalous translational diffusion for each component (Fig. 6-143).

Dimension offers selection via pull down menus of one-, two- or three-dimensional diffusion (Fig. 6-144).

In the **Translation information** box the formulas for the general translational term $G_s(\xi, \psi)$ and useful conversion formulas are displayed as well as a description of the parameter variables.

6.19 Polarization Imaging

Linear (plane) polarized light, which is light whose wave goes only one direction, exciting a fluorescent molecule with a preferred dipole orientation results in polarized emitted light. It provides a contrast-enhancing method that is especially useful in the study of molecules that are fixed in their orientation or are greatly restricted in their rotational diffusion. Anisotropy is directly related to polarization and is defined as the ratio of the polarized light component intensity to the total light intensity.

In polarization microscopy using the LSM 710 the sample is irradiated with vertical polarized light (in respect to the optical table) from a laser source. The emitted fluorescence is passed sequentially through emission polarizers (analyzers) that are housed in the **Notch filter cascade** and that transmit either the vertical (I_{VV}) or horizontal (I_{VH}) polarized emitted light onto the Quasar detector (L format fluorescence polarization). Since the vertical component of the emission light is parallel polarized to the vertical polarized excitation light, it is often also referred to as the parallel component ($I_p, I_{||}$). Likewise, the horizontal polarized emission light is also designated as the perpendicular ("senkrecht" in German) component (I_s, I_{\perp}). In the Software the "p" and "s" designations are used.

Polarisation **P** and anisotropy **r** are defined as:

$$P = \frac{I_p - I_s}{I_p + I_s} \text{ and } r = \frac{I_p - I_s}{I_p + 2 \cdot I_s} \text{ with } 0 \leq r \leq 1.$$

Hence they can be interconverted to each other in the following way:

$$P = \frac{3 \cdot r}{2 + r} \text{ and } r = \frac{2 \cdot P}{3 + P}.$$

In a completely polarized sample ($I_s=0$) the anisotropy $r=1$. In a completely non-polarized sample ($I_s=I_p$) anisotropy $r=0$.

The formulas for polarization **P** and anisotropy **r** as given above are strictly true only, if the optical transmission for both emission polarizers are identical. Any differences must be corrected by introducing a correction factor **G** that is multiplied with I_p . Hence the anisotropy **r** in such a case would be calculated according to:

$$r = \frac{I_p - G \cdot I_s}{I_p + 2 \cdot G \cdot I_s}$$

G can be measured using horizontally polarized excitation light and is defined as

$$G = \frac{I_{HV}}{I_{HH}}.$$

However, since in the LSM 710 the polarization of the excitation light can not be changed easily from vertical to horizontal, G has to be determined with an isotropic fluorescent dye solution as the ratio between the intensities I_p and I_s .

As the formulas implies:

Anisotropy **r** is the preferential display as anisotropy of single species will be simply additive. Note that the ZEN Software provides for a formula to display the anisotropy directly in ratio imaging. Images could also be computed in the **Processing** tool's **Calculator**.



Fig. 6-145 Light path with Notch filter control button

6.19.1 Light Path for Polarization Imaging

In case emission polarizers (analyzers) are available, you have a **Notch filter cascade** selection button available situated on top of the MBS button (see Fig. 6-145).

Set up a multitrack configuration using exactly the same settings with the exceptions of the analyzers. To this end press the **Notch filter cascade** button. This opens up the **Polarizer selection box**. Check the polarizer which you want to use with track 1. The options are **P** (for the parallel polarized light transmission) and **S** (for vertical polarized light transmission) (see Fig. 6-146).

If you want to ratio image, then it is best for convenience to choose **P** for track 1. Check the other polarizer for track 2.

For ratio imaging this would be **S**. For ratio imaging check the **R1 channel box** and assign **Source 1** to the selected channel-T1 and **Source 2** to the selected channel-T2. You can use the single PMTs **Ch1** and **Ch2** or the spectral detector **ChS**.



Fig. 6-146 Light path with Notch filter cascade option

6.19.2 Determination of G-factor

In order to determine the G-factor for the LSM 710 system, you need an isotropic medium like a fluorescent dye at a 1 mM concentration, for example fluorescein. Use the multi track configuration with P and S analyzers selected and with a beam path and channel settings appropriate to your dye. Take a single image and activate in the **Channel** tab the **Ratio** track. In the **Ratio** panel select **Ratio Type 1**. Set all editable summands to 0 and all factors to 1 (see Fig. 6-147).

For an inverted microscope, use for convenience a glass bottom dish and pipette enough solution in to cover the glass well. For an upright stand, squeeze a drop of solution between the glass bottom dish and a cover slip.

If you have chosen **P** in the Notch filter cascade for Track 1 and assigned it to Ch1-T1 and **S** for track 2 assigned to Ch1-T2 then the G factor is the mean intensity value of the R1 channel. For display activate the **Histo** register of the Image and select the R1 channel (see Fig. 6-148).



Fig. 6-147 Channels panel with and Ratio Type 1 formula in the Ratio1 channel selected

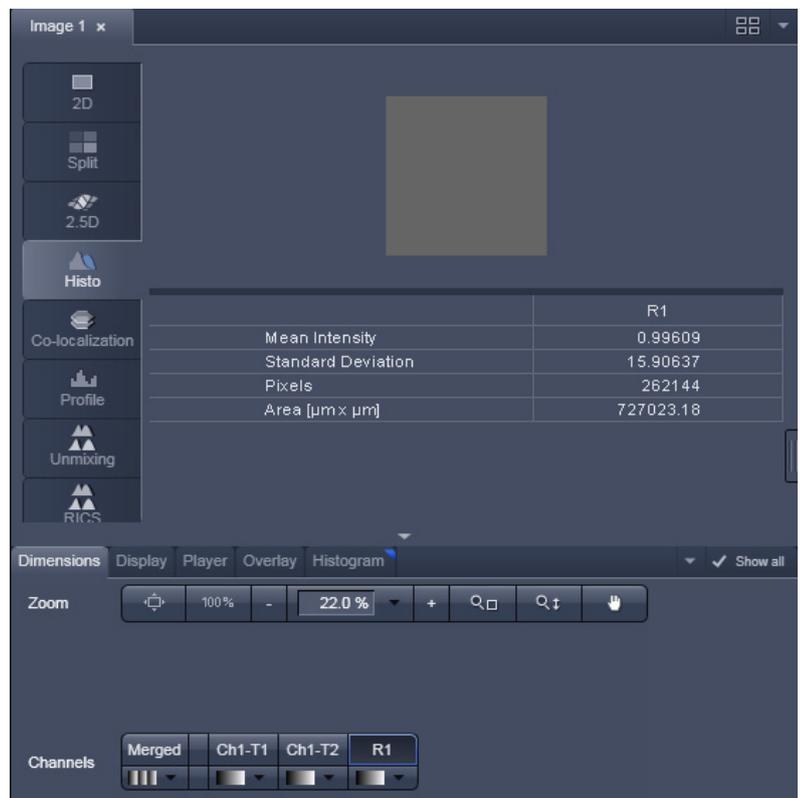


Fig. 6-148 Histogram display with Ratio1 channel activated

The G-factor is therefore defined as:

$$G = \frac{Ch1 - T1}{Ch1 - T2} = \frac{I_P}{I_S}$$

6.19.3 Obtaining an Anisotropy Image

Set up a multitrack configuration with alternating analyzers: **P** in track 1, **S** in track 2, leaving all other settings equal (see Fig. 6-149). Assign track 1 to **Ch1-T1**, track 2 to **Ch1-T2**. Alternatively assign to Ch2-T1 and Ch2-T2 or ChS1-T1 and ChS1-T2, whatever physical channel you want to use.



Fig. 6-149 Channels control with Ratio Type 3 formula selected in the Ratio1 channel using a G factor of 1.18

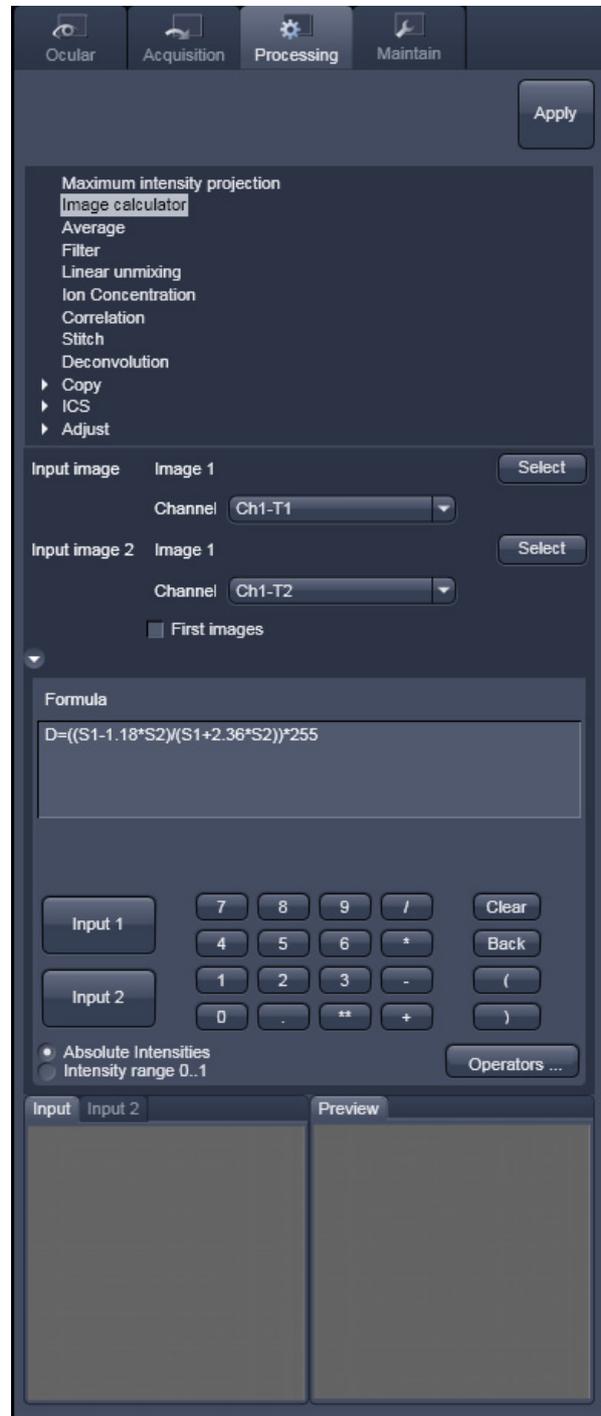


Fig. 6-150 Image calculator with anisotropy formula and a chosen G-factor of 1.18 with absolute intensities selected

In the **Ratio1** channel choose the **Ratio Type 3** formula. With Ch1-T1 and Ch1-T2 corresponding to I_p and I_s this formula corresponds to the formula for anisotropy r . For the factors put in the value for the G-factor in the numerator and $2 \times G$ in the denominator. Scale by Min/Max by checking the **Set by min/max** box and entering **0** in the **Min input box** and **1** in the **Max input box**. With a corresponding look up table like rainbow this will produce a false colored anisotropy image in the nRatio1 channel.

Alternatively you can use the image calculator with source 1 (S1) being the **P** track image (Ch1-T1) and source 2 (S2) the **S** track image (Ch1-T2) with consideration of the G-factor (see Fig. 6-150).

Proceed as follows. In the **Processing** tab select the **Image calculator**. Press **Select** and click on the desired input image. In the **Channel** pull down menu of **Input Image Image 1** select **ChX-T1**, where X stand for 1, 2 or S. Accordingly, in the **Channel** pull down menu of **Input Image Image 2** select **ChX-T2**.

Type in the anisotropy formula considering the G-factor of the system. Choose either Absolute Intensities in case you have to multiply the formula with $2^n - 1$ to rescale to an n bit image. For example, if the color depth was 8 bit, multiply with $2^8 - 1 = 256 - 1 = 255$. Alternatively, choose **Intensity range 0..1**, in which case you do not multiply with an extra factor. Press **Apply** and choose a respective look up table to obtain a false colored anisotropy image.

6.20 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

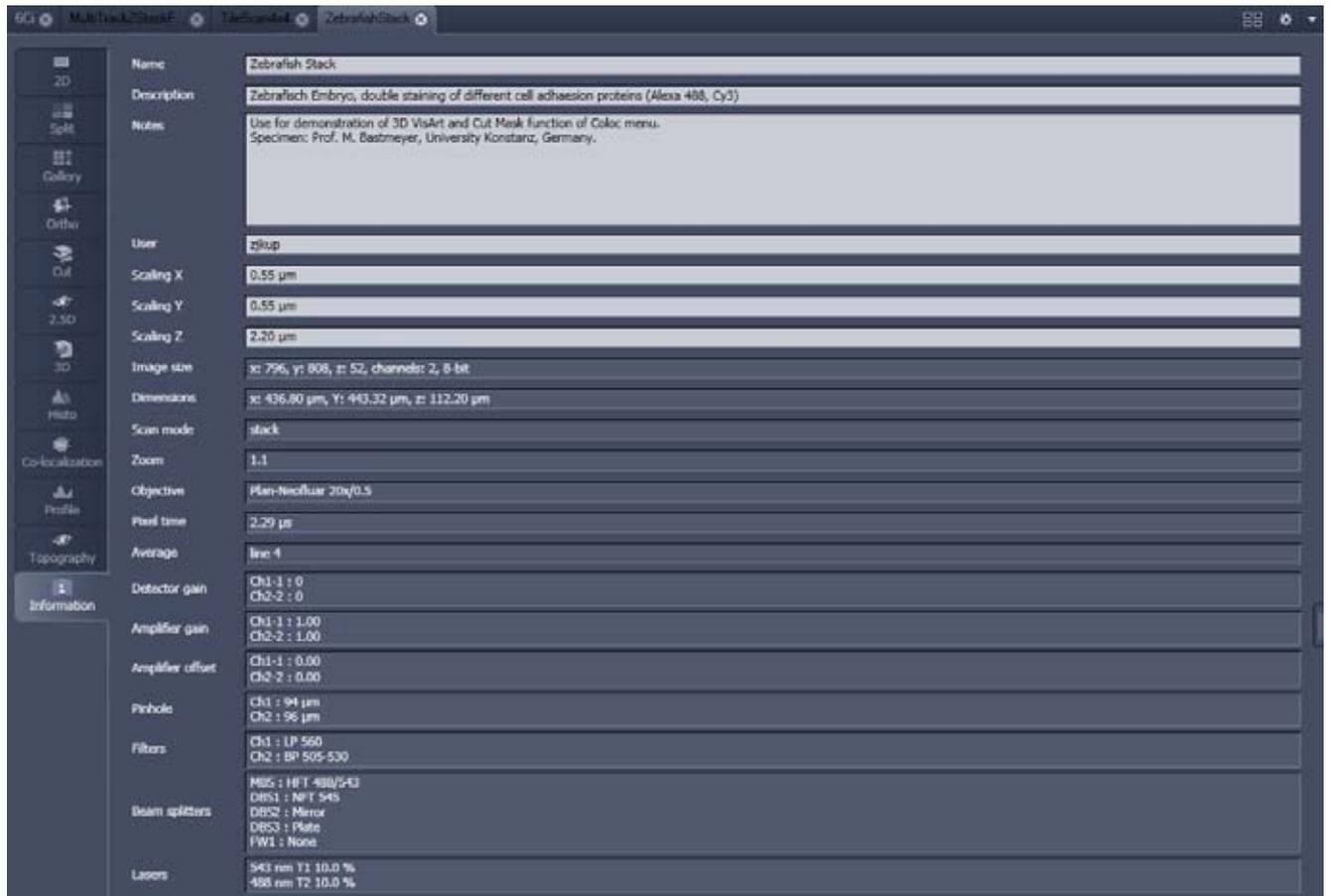


Fig. 6-151 Information View of a 2 channel Z-Stack

7 Right Tool Area: Data Management and Storage

7.1 General

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 **Information View**) in the image.

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 **Dimensions**) function is available in the ZEN Image Browser: Hardware parameters can be loaded without opening the files by clicking the **Re-use** button.

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.

Export

With the **Export** dialog in the **File** menu, the acquired data can be directly exported to various file types.

 In the **Save As** dialogue in the **File Menu** these formats can also be chosen as file type.

7.2 File Menu

- In the **Main** menu bar, click on **File**.
 - This displays the menu shown in Fig. 7-1.

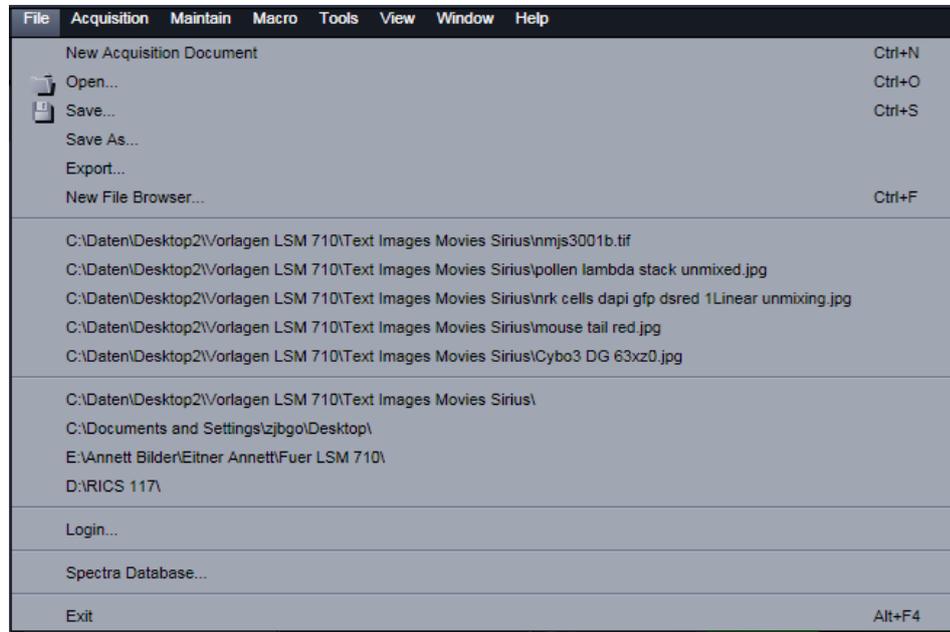


Fig. 7-1 File menu

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 **ZEN File Browser**).
- 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.

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.

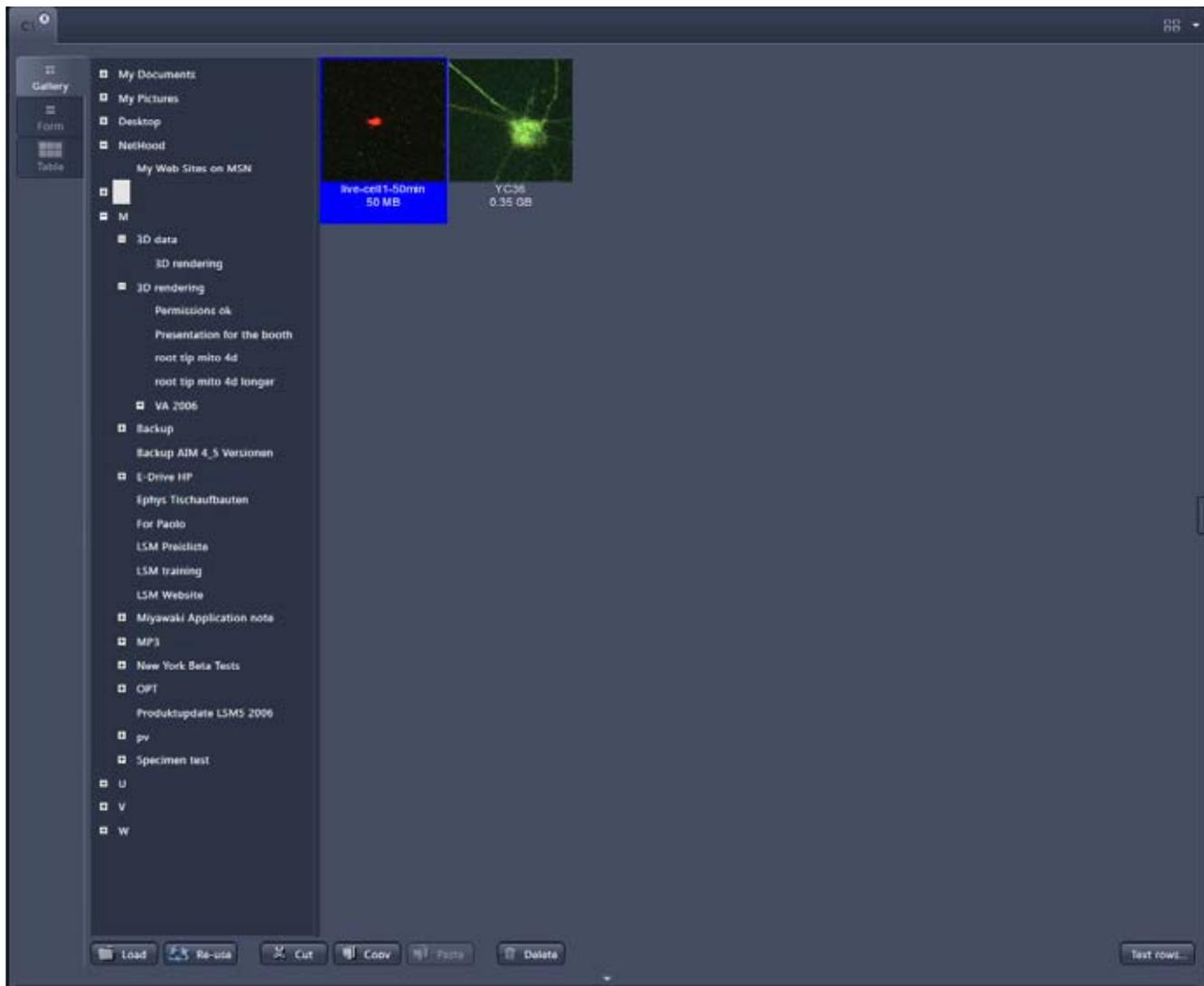


Fig. 7-2 ZEN File Browser

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. 7-3) fading in the right hand corner inform about missing hardware if applicable. The message box disappears after ~ 10 seconds.

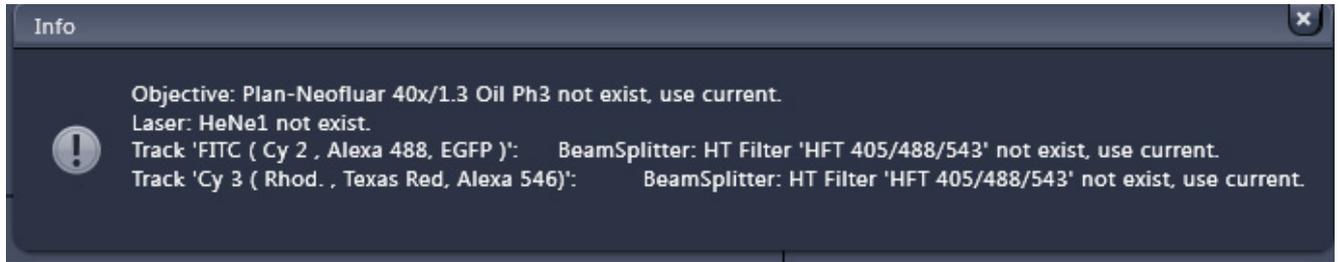


Fig. 7-3 Error message

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.

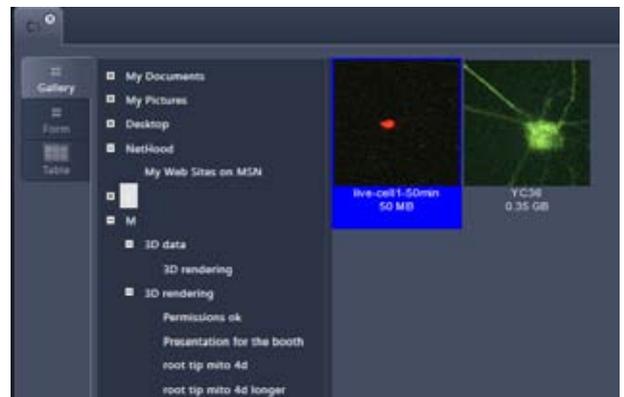


Fig. 7-4 Gallery view

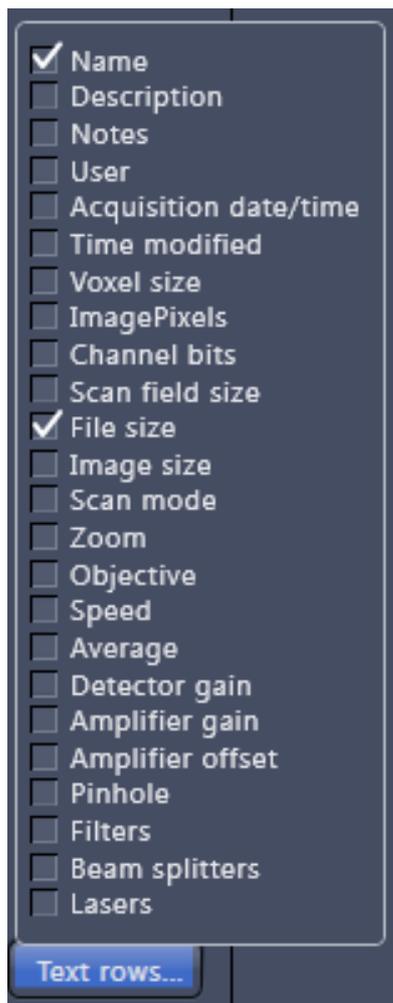
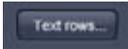


Fig. 7-5 Image information selection

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  button a choice of information is available (see Fig. 7-5).

The example below (Fig. 7-6) shows the use of the display of:

- file name,
- acquisition date,
- file size and
- scan mode.

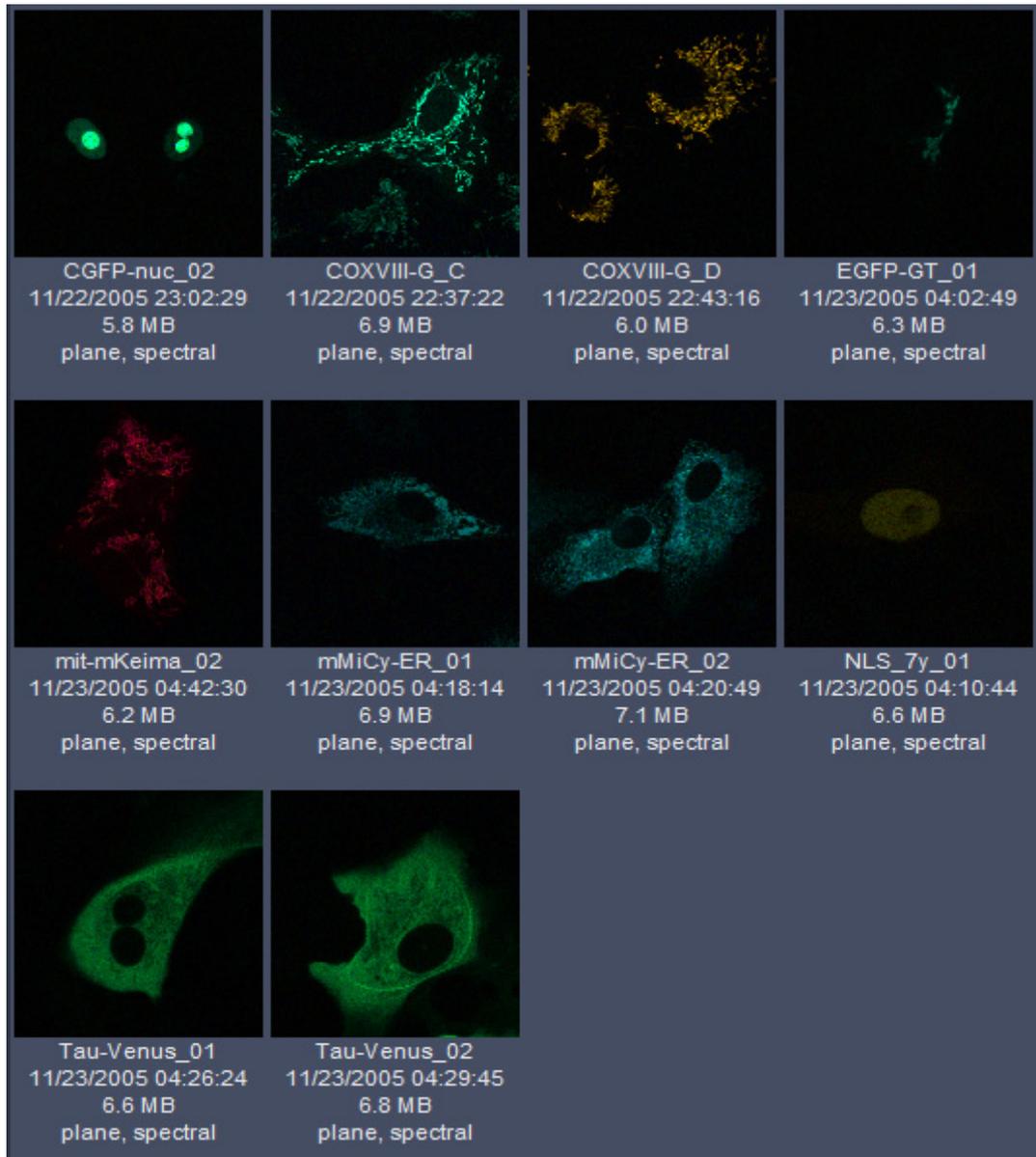


Fig. 7-6 Images with information

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

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.

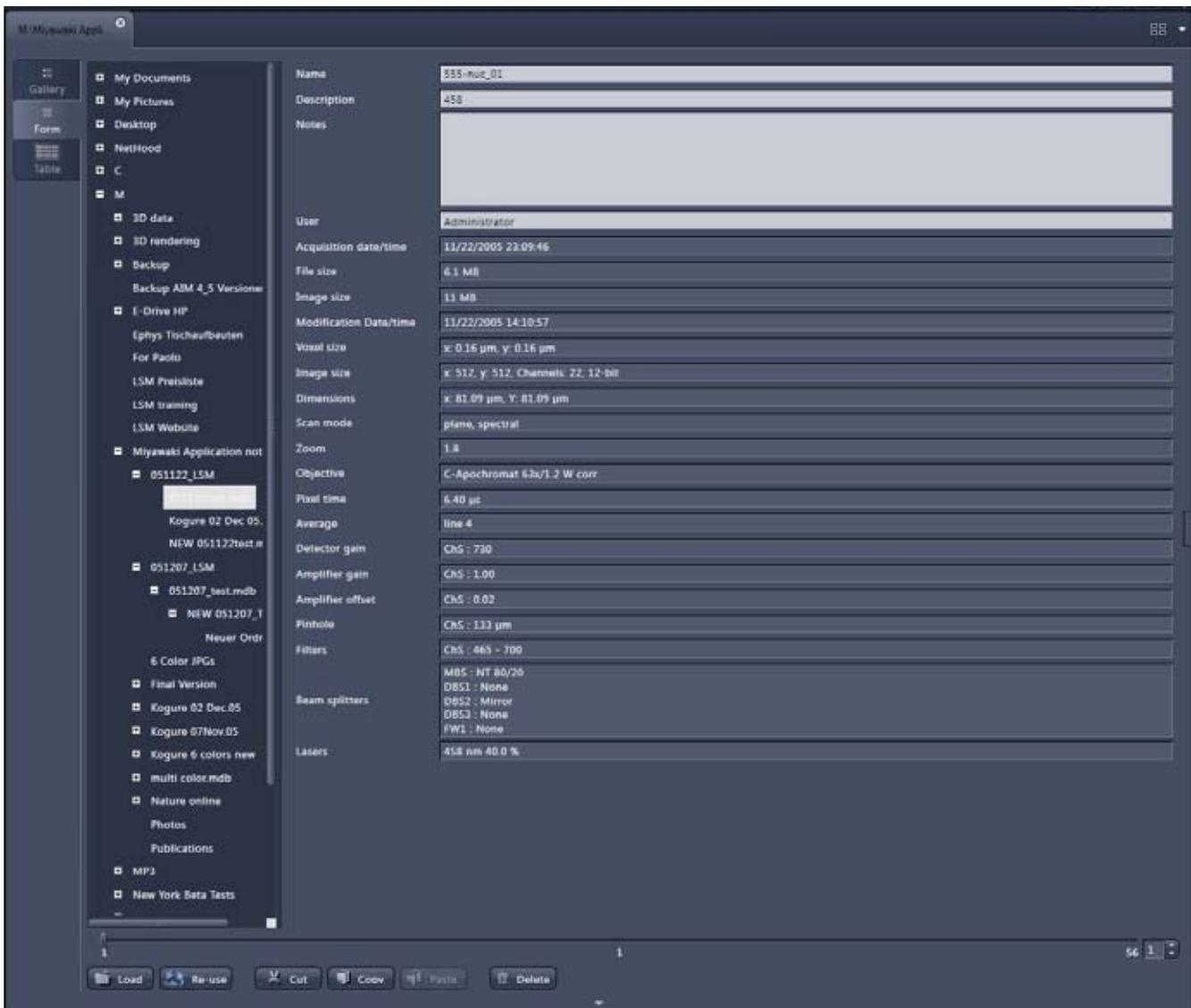


Fig. 7-7 Form view

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.

Name	Description	User	Acquisition date/time	Time modified	Voxel size	Image pixels	Channel bits	Scan #
555-nuc_01	458	Administrator	11/22/2005 23:09:46	11/22/2005 14:16:53	x: 0.16 µm, y: 0.16 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	81.09 µm x 81
555-nuc_02	458	Administrator	11/22/2005 23:15:04	11/22/2005 14:16:17	x: 0.16 µm, y: 0.16 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	81.09 µm x 81
7_595_01	458	Administrator	11/23/2005 07:56:11	11/22/2005 22:57:59	x: 0.19 µm, y: 0.19 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	97.31 µm x 97
7_595_01_urnia	458	Administrator	11/23/2005 07:56:25	11/22/2005 22:58:21		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_595_01_urnia_BK	458	Administrator	11/23/2005 07:58:56	11/22/2005 23:02:56		512 x 512 x 1 x 1	9 Channels, 16-bit	
7_595_02	458	Administrator	11/23/2005 08:06:41	11/22/2005 23:08:30	x: 0.11 µm, y: 0.11 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	58.30 µm x 58
7_595_02_urnia_BK	458	Administrator	11/23/2005 08:10:23	11/22/2005 23:11:07		512 x 512 x 1 x 1	9 Channels, 16-bit	
7_595_02_urnia	458	Administrator	11/23/2005 08:08:39	11/22/2005 23:09:17		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_595_03	458	Administrator	11/23/2005 08:14:41	11/22/2005 23:16:24	x: 0.14 µm, y: 0.14 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	72.99 µm x 72
7_595_03_urnia_BK	458	Administrator	11/23/2005 08:17:43	11/22/2005 23:18:19		512 x 512 x 1 x 1	9 Channels, 16-bit	
7_595_03_urnia	458	Administrator	11/23/2005 08:16:30	11/22/2005 23:17:55		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_595_04	458	Administrator	11/23/2005 09:21:30	11/22/2005 22:22:56	x: 0.12 µm, y: 0.12 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	63.46 µm x 63
7_595_04_urnia_BK	458	Administrator	11/23/2005 09:26:01	11/22/2005 22:25:16		512 x 512 x 1 x 1	9 Channels, 16-bit	
7_595_04_urnia	458	Administrator	11/23/2005 09:23:19	11/22/2005 22:24:05		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_595_05	458	Administrator	11/23/2005 09:31:14	11/22/2005 23:34:00	x: 0.12 µm, y: 0.12 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	60.62 µm x 60
7_595_05_urnia_BK	458	Administrator	11/23/2005 09:34:51	11/22/2005 23:35:33		512 x 512 x 1 x 1	9 Channels, 16-bit	
7_595_05_urnia	458	Administrator	11/23/2005 09:33:18	11/22/2005 23:34:07		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_595_06	458	Administrator	11/23/2005 09:40:11	11/22/2005 23:41:54	x: 0.14 µm, y: 0.14 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	72.99 µm x 72
7_595_06_urnia_BK	458	Administrator	11/23/2005 09:42:48	11/22/2005 23:43:17		512 x 512 x 1 x 1	9 Channels, 16-bit	
7_595_06_urnia	458	Administrator	11/23/2005 09:41:58	11/22/2005 23:43:06		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_595_07	458	Administrator	11/23/2005 09:46:29	11/22/2005 23:48:19	x: 0.16 µm, y: 0.16 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	81.09 µm x 81
7_595_07_urnia_BK	458	Administrator	11/23/2005 09:49:57	11/22/2005 23:50:10		512 x 512 x 1 x 1	9 Channels, 16-bit	
7_595_07_urnia	458	Administrator	11/23/2005 09:48:34	11/22/2005 23:49:40		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_CG_01	458	Administrator	11/23/2005 06:13:37	11/22/2005 21:15:30	x: 0.15 µm, y: 0.15 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	57.31 µm x 57
7_CG_01_urnia_BK	458	Administrator	11/23/2005 06:02:07	11/22/2005 22:04:32		512 x 512 x 1 x 1	9 Channels, 16-bit	
7_CG_01_urnia	458	Administrator	11/23/2005 06:17:04	11/22/2005 21:17:41		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_CG_01_urnia2	458	Administrator	11/23/2005 06:17:04	11/22/2005 21:19:54		512 x 512 x 1 x 1	9 Channels, 16-bit	
7_CG_02	458	Administrator	11/23/2005 06:20:40	11/22/2005 21:26:37	x: 0.16 µm, y: 0.16 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	76.82 µm x 76
7_CG_02_urnia	458	Administrator	11/23/2005 06:24:20	11/22/2005 21:28:33		512 x 512 x 1 x 1	9 Channels, 16-bit	
7_CG_02_urnia_BK	458	Administrator	11/23/2005 06:27:36	11/22/2005 21:29:44		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_CG_03	458	Administrator	11/23/2005 06:32:41	11/22/2005 21:36:06	x: 0.22 µm, y: 0.22 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	112.26 µm x 112
7_CG_03_urnia	458	Administrator	11/23/2005 06:36:24	11/22/2005 21:36:56		512 x 512 x 1 x 1	9 Channels, 16-bit	
7_CG_03_urnia_BK	458	Administrator	11/23/2005 06:38:09	11/22/2005 21:38:25		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_CG_04	458	Administrator	11/23/2005 06:42:29	11/22/2005 21:52:07	x: 0.24 µm, y: 0.24 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	121.64 µm x 121
7_CG_04_urnia	458	Administrator	11/23/2005 06:52:14	11/22/2005 21:52:41		512 x 512 x 1 x 1	9 Channels, 16-bit	
7_CG_04_urnia_BK	458	Administrator	11/23/2005 06:54:36	11/22/2005 21:55:08		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_CG_05	458	Administrator	11/23/2005 07:06:30	11/22/2005 22:09:27	x: 0.14 µm, y: 0.14 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	72.99 µm x 72
7_CG_05_urnia	458	Administrator	11/23/2005 07:08:22	11/22/2005 22:09:39		512 x 512 x 1 x 1	9 Channels, 16-bit	
7_CG_05_urnia_GT_BK	458	Administrator	11/23/2005 07:11:32	11/22/2005 22:12:29		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_CG_05_urnia_GT	458	Administrator	11/23/2005 07:11:00	11/22/2005 22:11:28		512 x 512 x 1 x 1	7 Channels, 16-bit	
7_CG_05_urnia_BK	458	Administrator	11/23/2005 07:10:21	11/22/2005 22:16:48		512 x 512 x 1 x 1	9 Channels, 16-bit	
EGFP-nuc_01	458	Administrator	11/22/2005 22:48:37	11/22/2005 14:00:54	x: 0.16 µm, y: 0.16 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	81.09 µm x 81
EGFP-nuc_02	458	Administrator	11/22/2005 22:02:29	11/22/2005 14:02:23	x: 0.16 µm, y: 0.16 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	81.09 µm x 81
DDMIII-G_C	458	Administrator	11/22/2005 22:37:22	11/22/2005 13:39:50	x: 0.17 µm, y: 0.17 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	95.06 µm x 95
DDMIII-G_D	458	Administrator	11/22/2005 22:43:16	11/22/2005 13:44:17	x: 0.16 µm, y: 0.16 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	81.09 µm x 81
EGFP-GT_01	458	Administrator	11/23/2005 04:02:49	11/22/2005 19:06:41	x: 0.11 µm, y: 0.11 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	58.30 µm x 58
EGFP-GT_02	458	Administrator	11/23/2005 04:06:24	11/22/2005 19:07:59	x: 0.11 µm, y: 0.11 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	58.30 µm x 58
ind-mKainic_01	458	Administrator	11/23/2005 04:36:40	11/22/2005 19:42:46	x: 0.14 µm, y: 0.14 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	72.99 µm x 72
ind-mKainic_02	458	Administrator	11/23/2005 04:42:30	11/22/2005 19:43:26	x: 0.16 µm, y: 0.16 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	81.09 µm x 81
mMCP-ER_01	458	Administrator	11/23/2005 04:18:14	11/22/2005 19:23:24	x: 0.19 µm, y: 0.19 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	97.31 µm x 97
mMCP-ER_02	458	Administrator	11/23/2005 04:20:49	11/22/2005 19:23:29	x: 0.19 µm, y: 0.19 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	97.31 µm x 97
NLS_7y_01	458	Administrator	11/23/2005 04:10:44	11/22/2005 19:15:01	x: 0.11 µm, y: 0.11 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	58.30 µm x 58
NLS_7y_02	458	Administrator	11/23/2005 04:14:12	11/22/2005 19:15:26	x: 0.11 µm, y: 0.11 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	58.30 µm x 58
SECFP-psn_01	458	Administrator	11/22/2005 23:24:56	11/22/2005 14:26:02	x: 0.12 µm, y: 0.12 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	63.46 µm x 63
Tau-Venus_01	458	Administrator	11/23/2005 04:26:24	11/22/2005 19:31:20	x: 0.14 µm, y: 0.14 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	72.99 µm x 72
Tau-Venus_02	458	Administrator	11/23/2005 04:29:45	11/22/2005 19:31:26	x: 0.14 µm, y: 0.14 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	72.99 µm x 72

Fig. 7-8 Table view



The columns displayed in the table are selectable from the **Table columns** button. The options shown in Fig. 7-9 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.

Fig. 7-9 Table view options

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 indicated 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 **Action Buttons**).

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. 7-10).

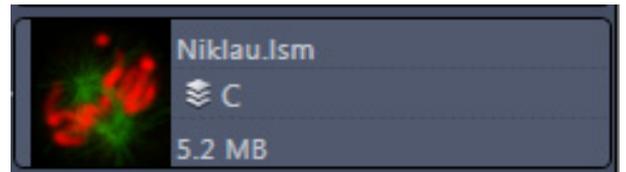
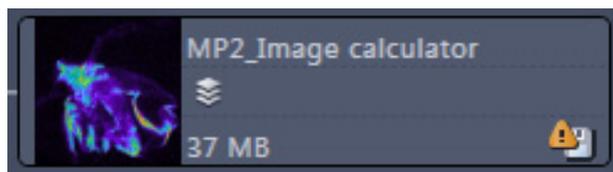
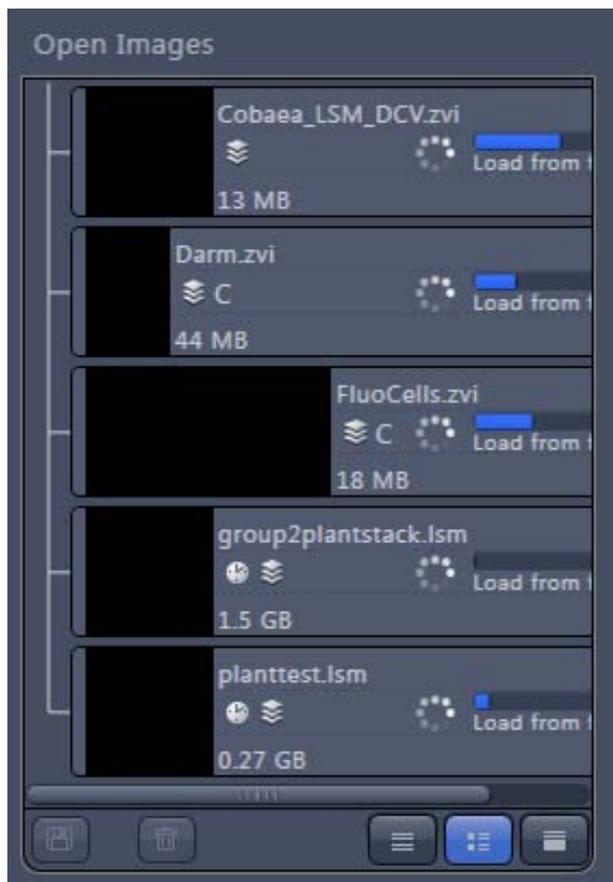


Fig. 7-10 2 Ch. Z-Stack image



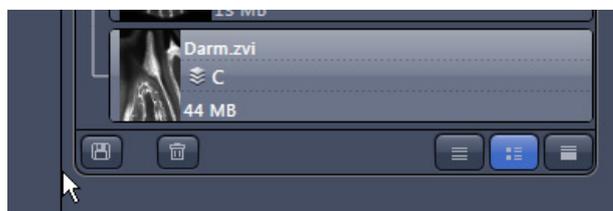
- In case the image is not saved or has been processed, a warning symbol is displayed on the right side (Fig. 7-11).

Fig. 7-11 Image with warning symbol



- A progress bar (Fig. 7-12) is shown for each image facilitating the overview if e.g. many processing function are running at the same time.

Fig. 7-12 Image progress bar



- In case long filenames are used the whole section can be dragged to the left and thereby enlarged (Fig. 7-13).

Fig. 7-13 Enlarging the image view

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**.

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.

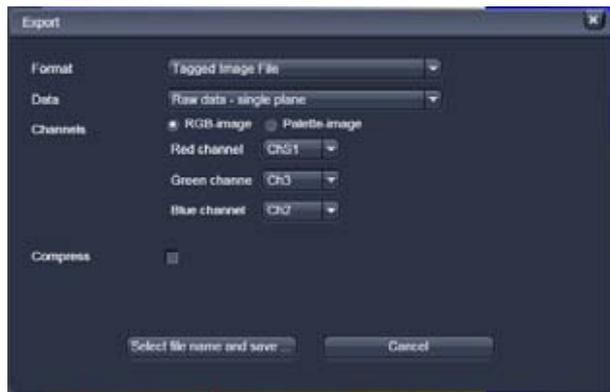


Fig. 7-14 Export window

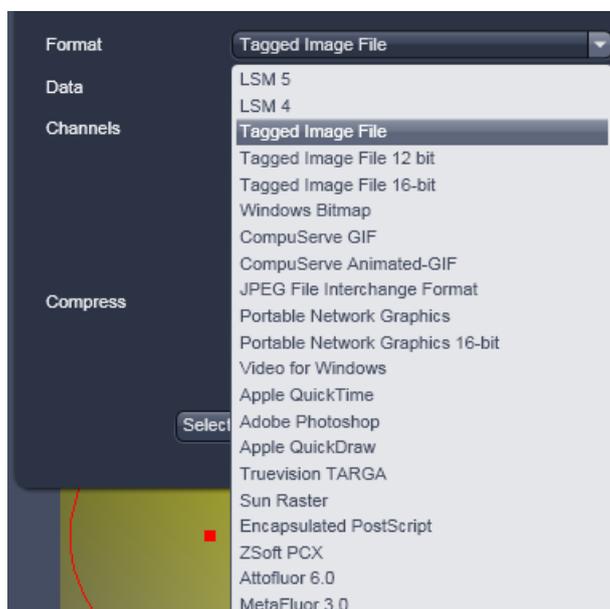


Fig. 7-15 Format selection



Fig. 7-16 Data type selection

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. 7-14).

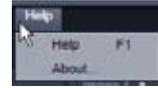
- Under **Format**, select the format to which the image is to be exported to (Fig. 7-15).

- Select the data type which the image is to be exported under **Data** (Fig. 7-16).
- 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.

7.8 Help – About



The **About** window can be accessed via the Help item in the menu bar clicking **About** opens the following dialog:



Fig. 7-17 About window

The panel hosts important information about the software version number, the license number and the available software modules.

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6 TOOLS, ADDITIONAL SOFTWARE

6.1 3D for LSM

6.1.1 Overview and Explanations

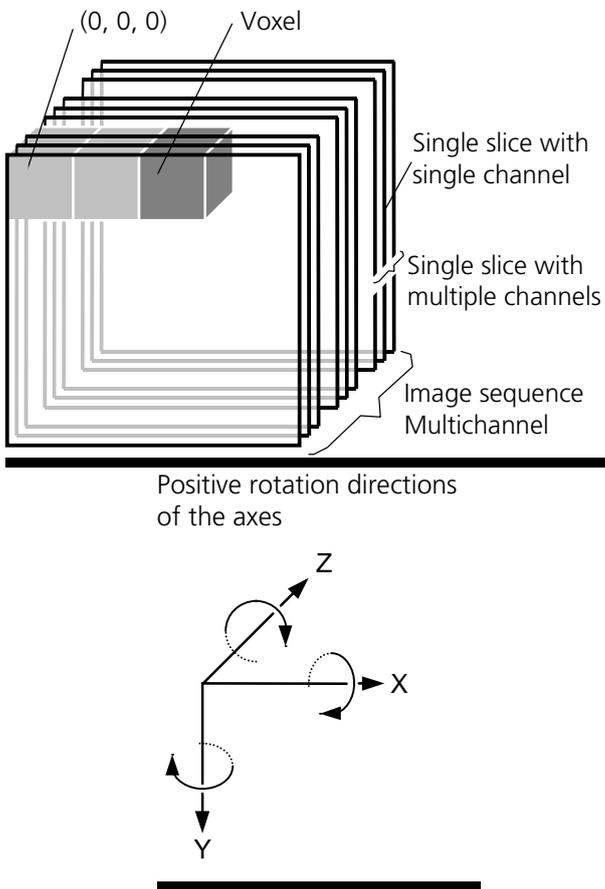


Fig. 6-1

- 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.

6.1.1.1 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.

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**.

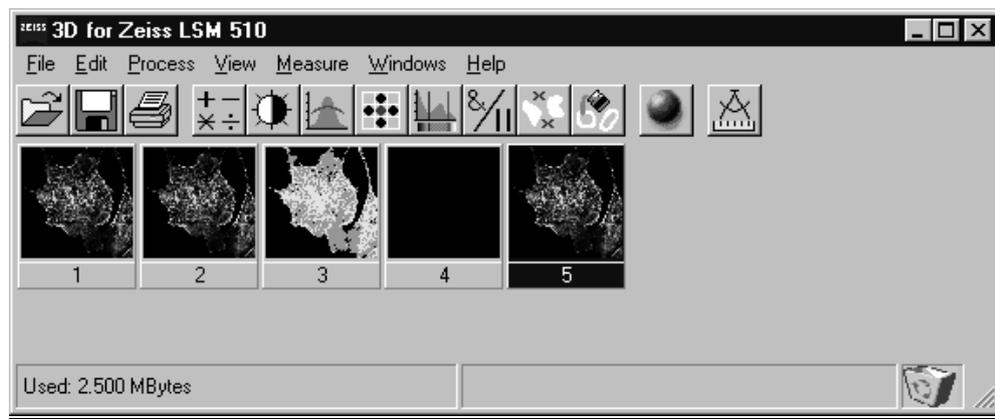


Fig. 6-2

Tool bar This menu shows all image processing functions.

Display window This window is used to display image sequences.

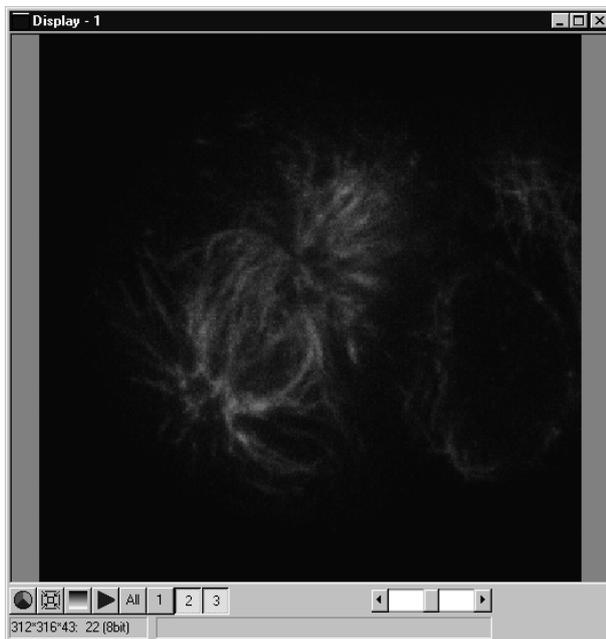


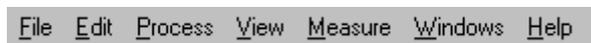
Fig. 6-3 Display window

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:

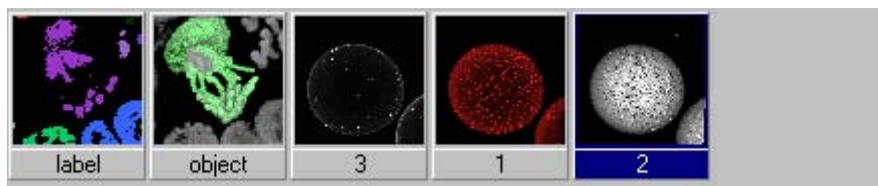
the **Menu**



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**

Smooths 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.

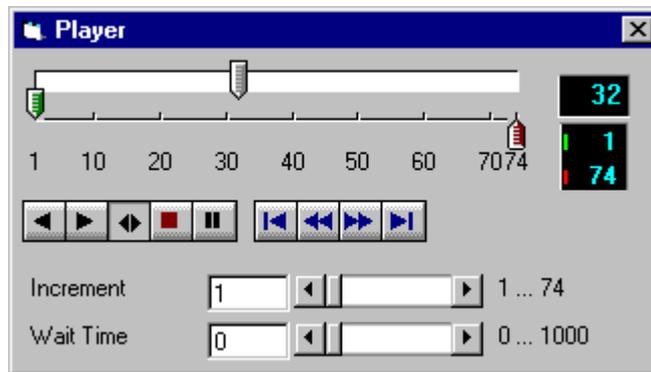


Fig. 6-4

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.

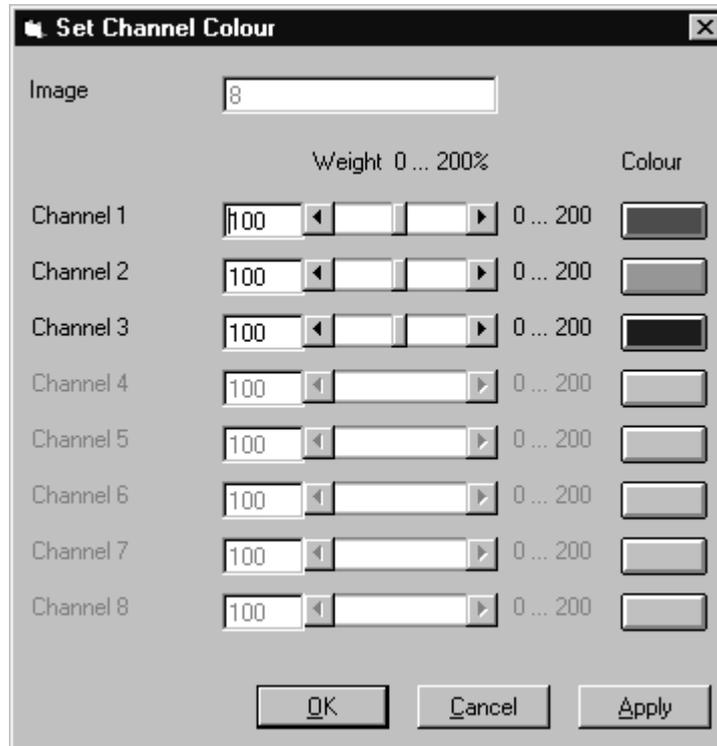


Fig. 6-5

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 (*.tif) or Carl Zeiss Vision (*.img) image sequence from a disk or network drive.

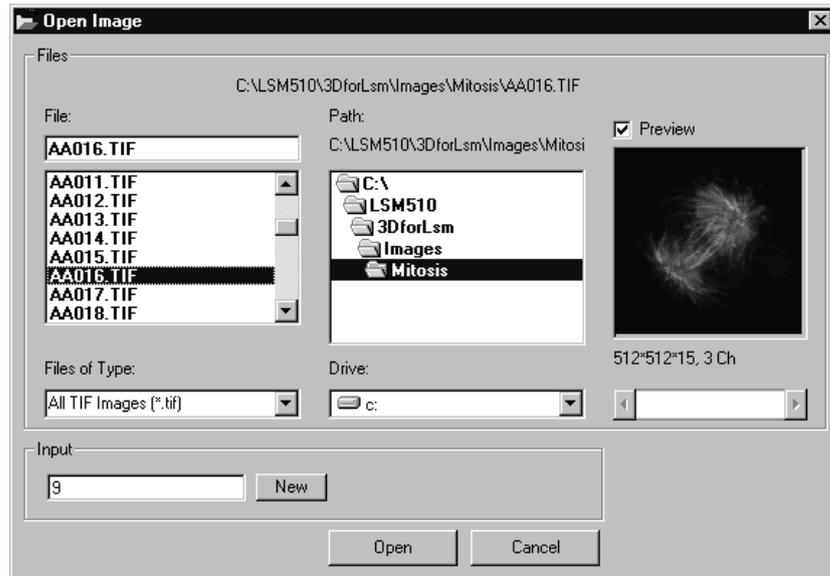


Fig. 6-6

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 (*.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 (*.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.

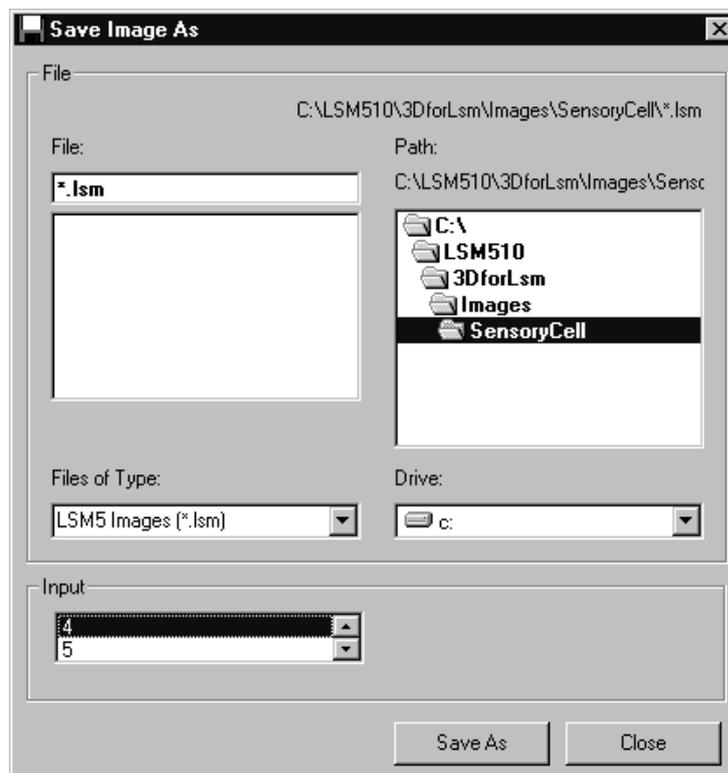


Fig. 6-7

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 (*.ism) and the Carl Zeiss Vision file format "KE Images (*0.img)" is supported.

By choosing the Carl Zeiss Vision file format "KE Images (*.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.

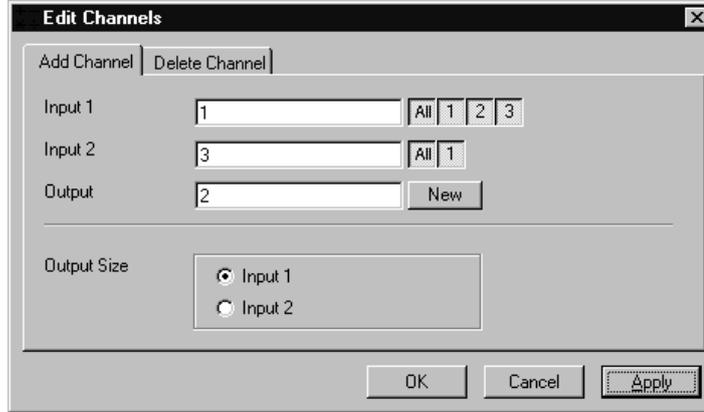


Fig. 6-9

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.

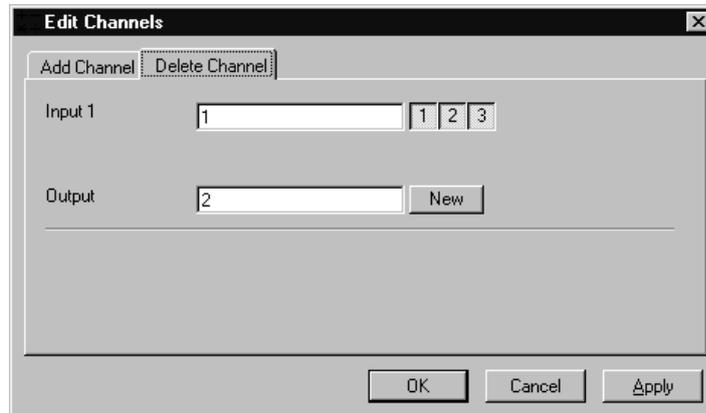


Fig. 6-10

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.

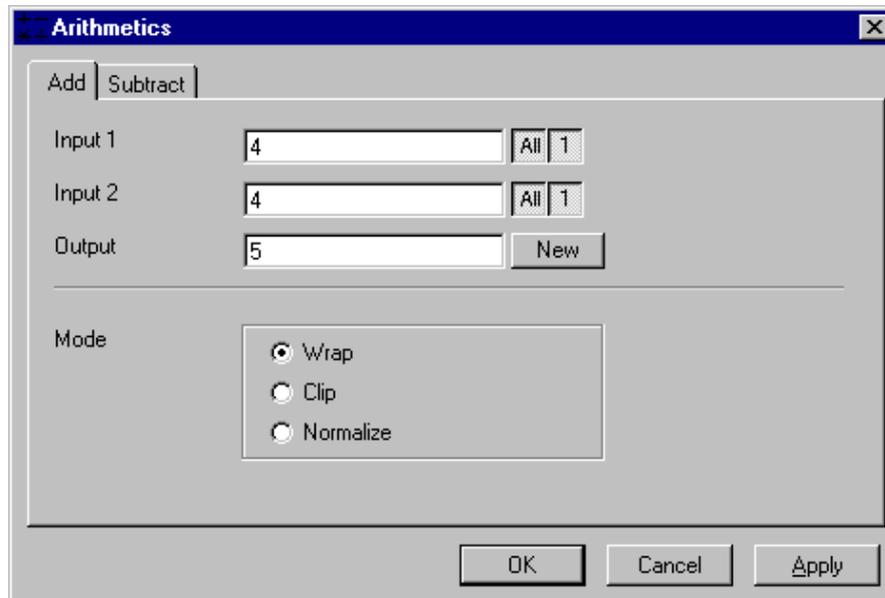


Fig. 6-11

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.

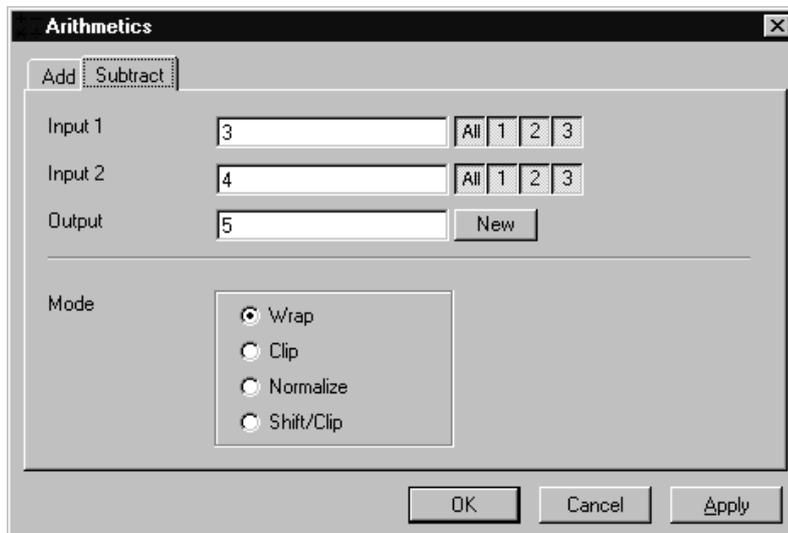


Fig. 6-12

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.

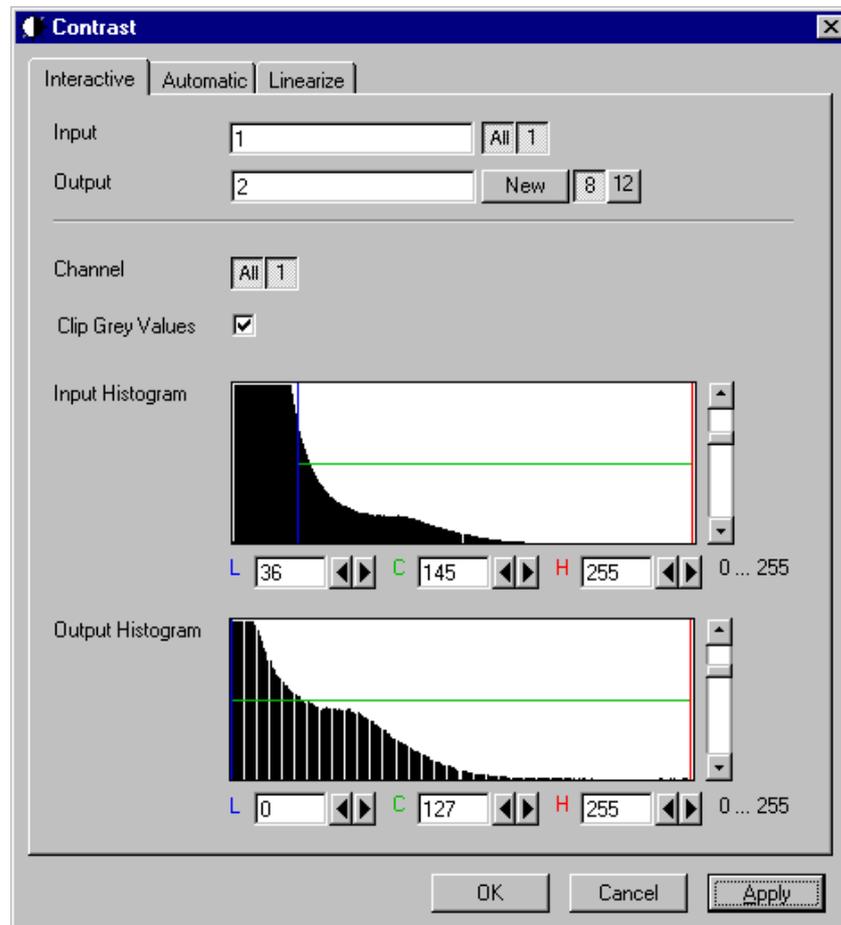


Fig. 6-13

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. Raising 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.

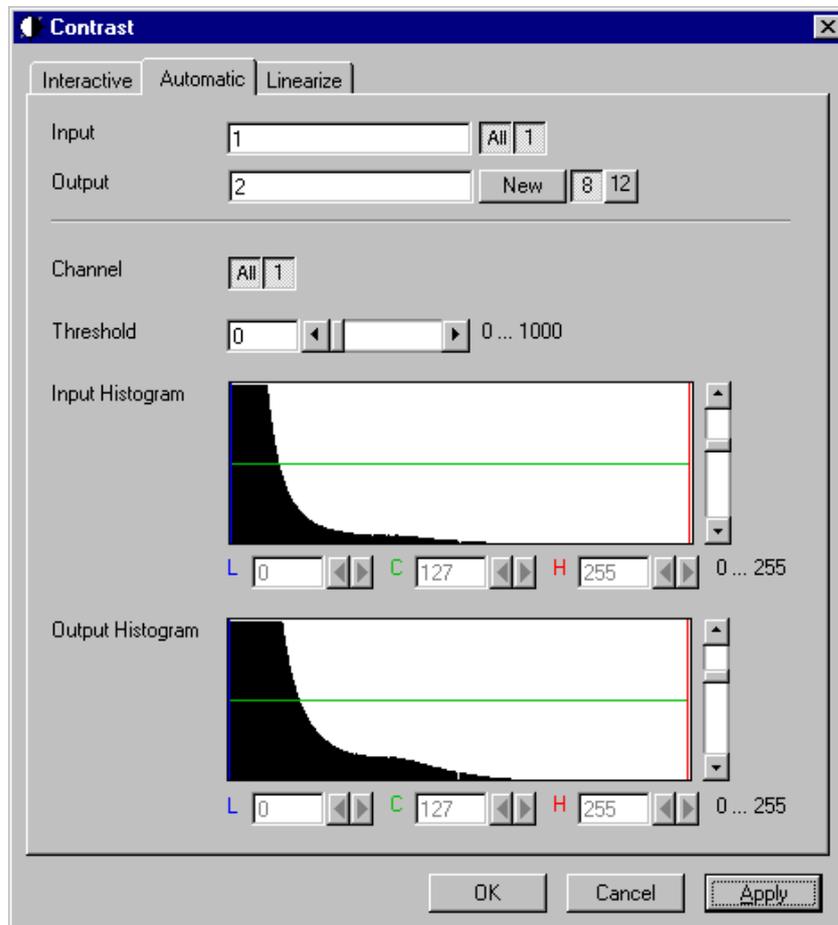


Fig. 6-14

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. Raising 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.

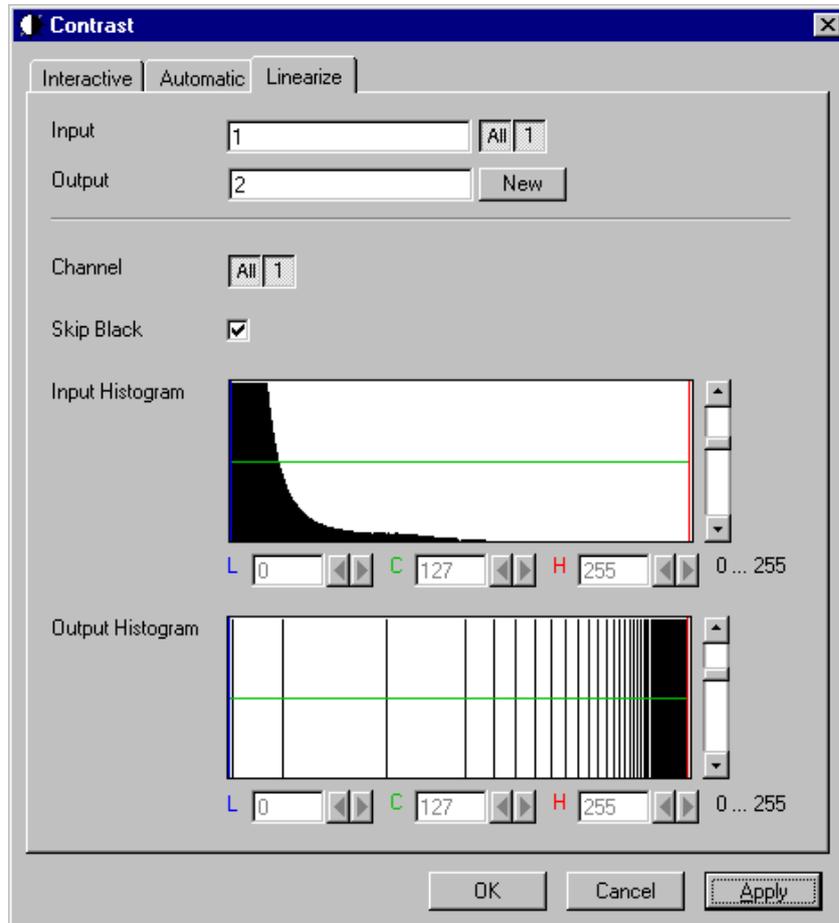


Fig. 6-15

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.

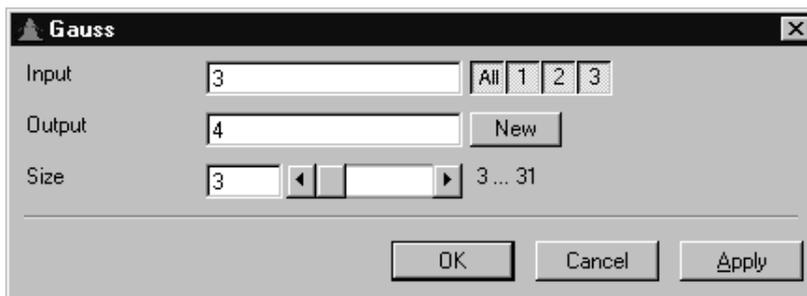


Fig. 6-16

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.

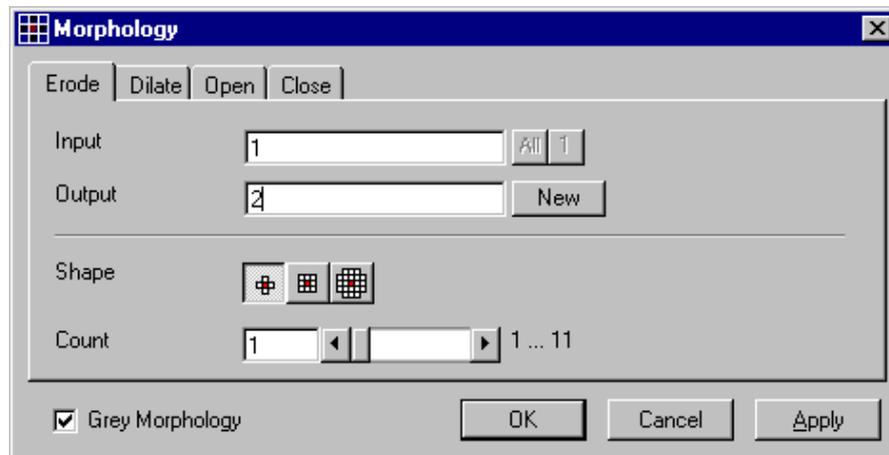


Fig. 6-17

As generalization of the morphology of two-dimensional images to three dimensions the structural elements are small volumina.

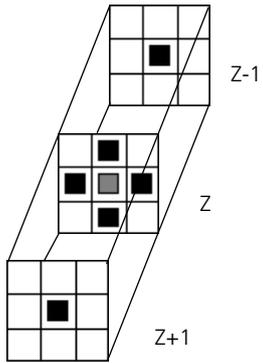
Literature

- 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

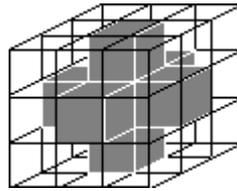
The input image sequence is analyzed voxel by voxel with a selected shape (**Shape**). The voxel to be analyzed is always the central voxel of the shape. The shape type determines which neighboring voxels are used to compute the resulting voxel.

The following structural elements are available for all morphological operations. They represent approximated spheres with an increasing radius.

Sequential image:

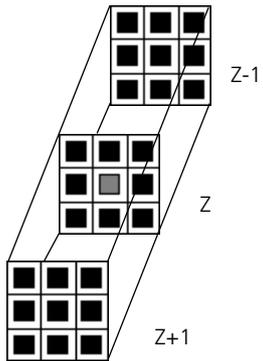


Volume view:

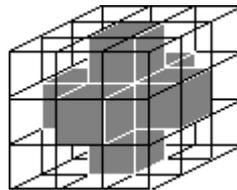


Cross shape

Sequential image:

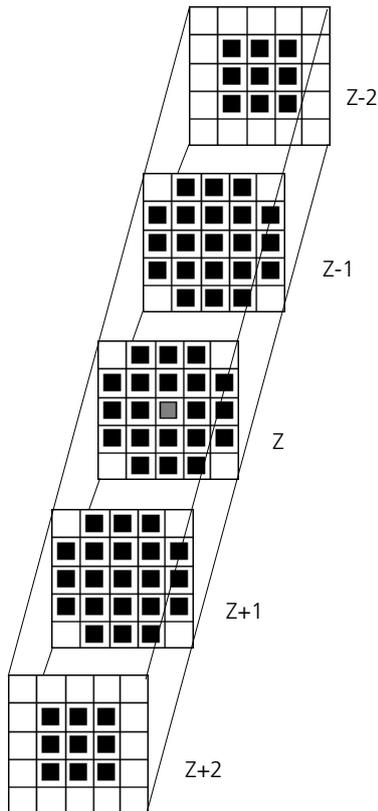


Volume view:

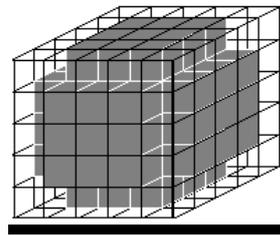


Cross shape

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 is 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** checkbox 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** checkbox 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

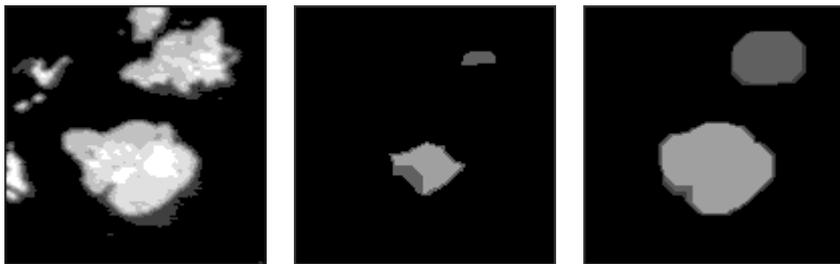


Fig. 6-18

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.

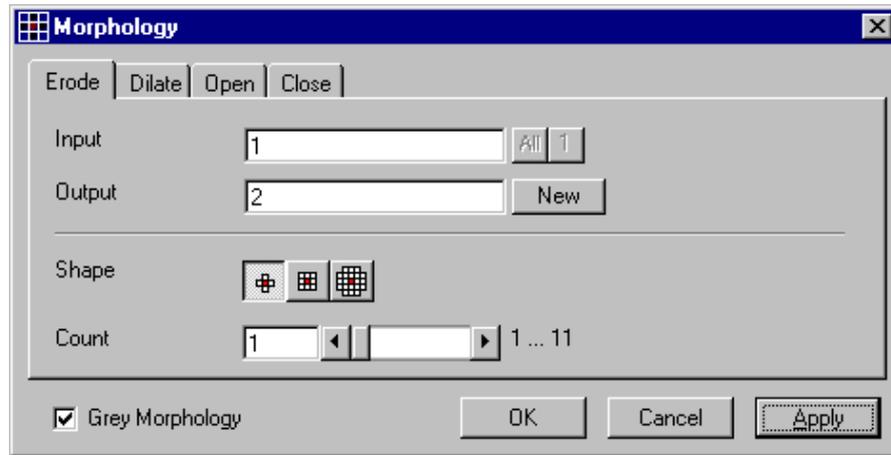


Fig. 6-20

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.

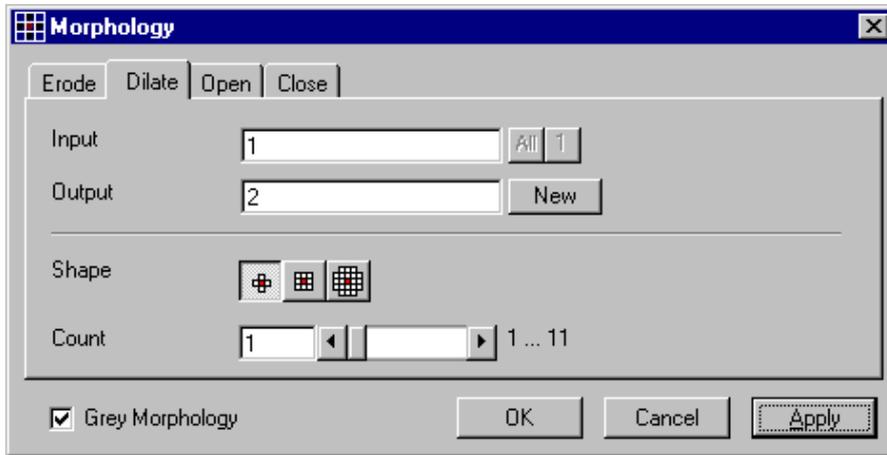


Fig. 6-21

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.

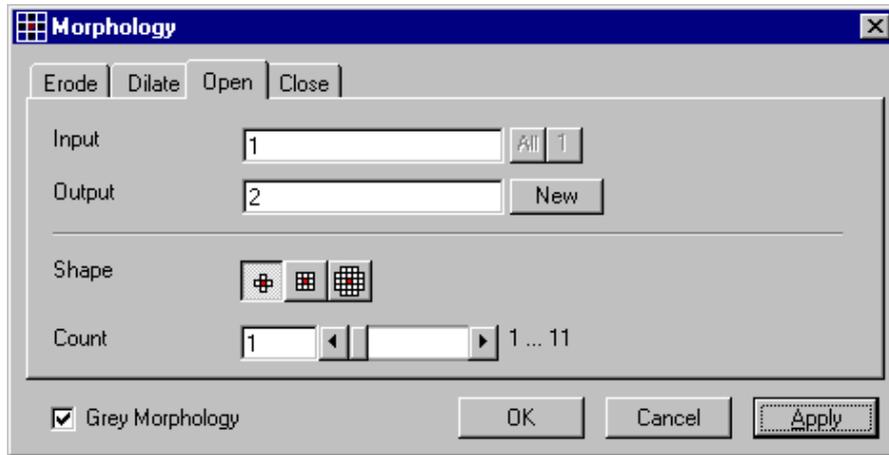


Fig. 6-22

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.

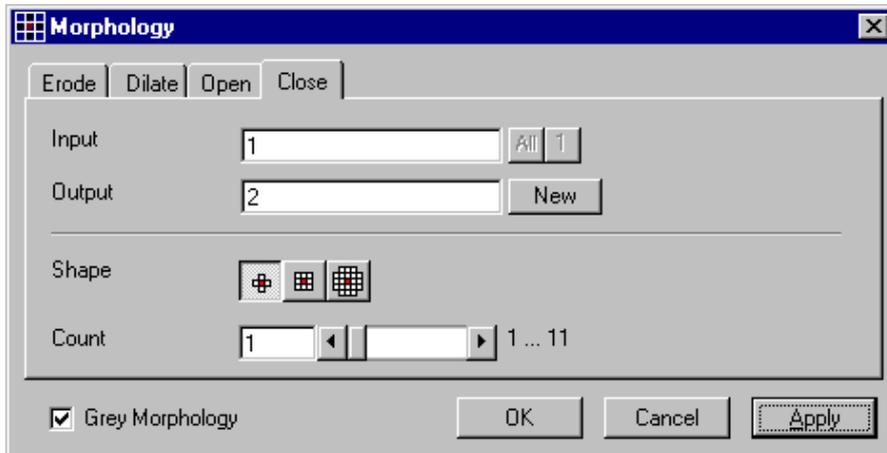


Fig. 6-23

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.

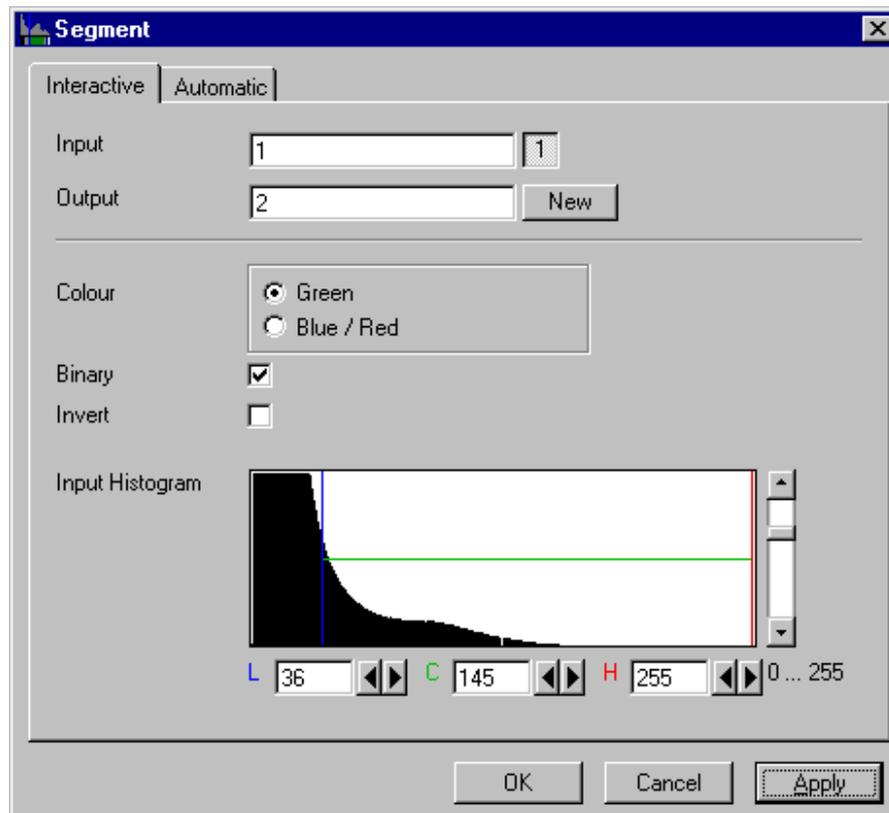


Fig. 6-24

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 **Low** and **High** are determined either by moving the borderlines in the grey value histogram or by the scroll bars underneath. Furthermore, the values for **Low**, **Center** and **High** 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 **Low** and **Low+1** are displayed in blue. The voxels with the grey values **High** and **High-1** are displayed in red.

If **Blue/Red** is selected, the voxels with grey values within the interval **Low**, **High** remain unchanged. Voxels with grey values less than **Low** are highlighted in blue; those with grey values higher than **High** 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 **Low** to **High** 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.

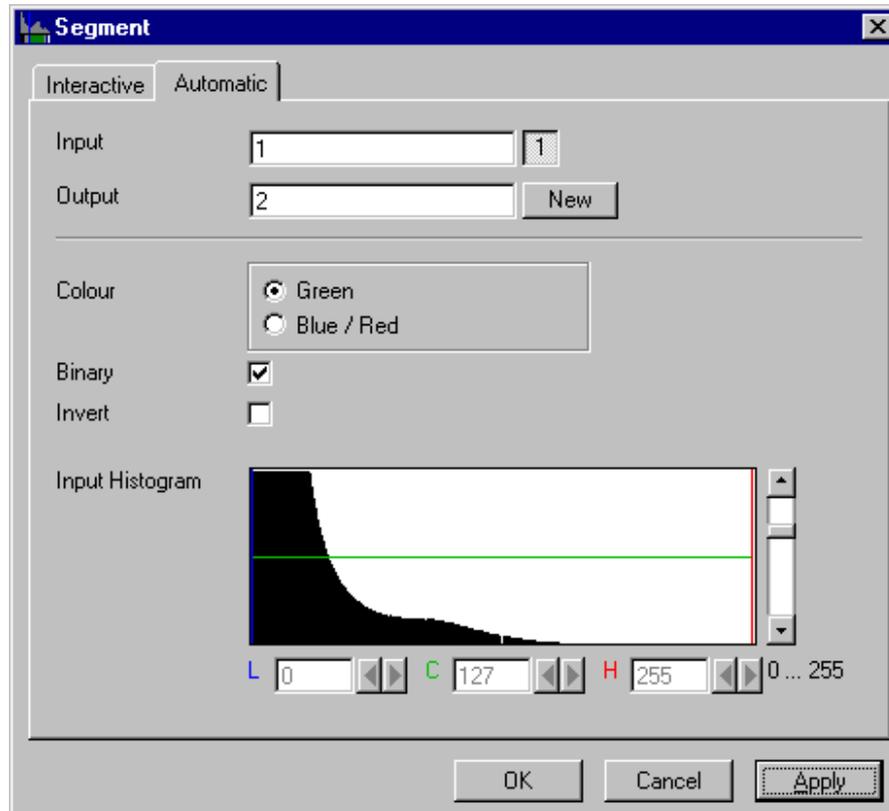


Fig. 6-25

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 **Low** and **Low+1** are displayed in blue. The voxels with the grey values **High** and **High-1** are displayed in red.

If **Blue/Red** is selected, the voxels with grey values within the interval **Low**, **High** remain unchanged. Voxels with grey values less than **Low** are highlighted in blue; those with grey values higher than **High** 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 **Low** to **High** 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**.

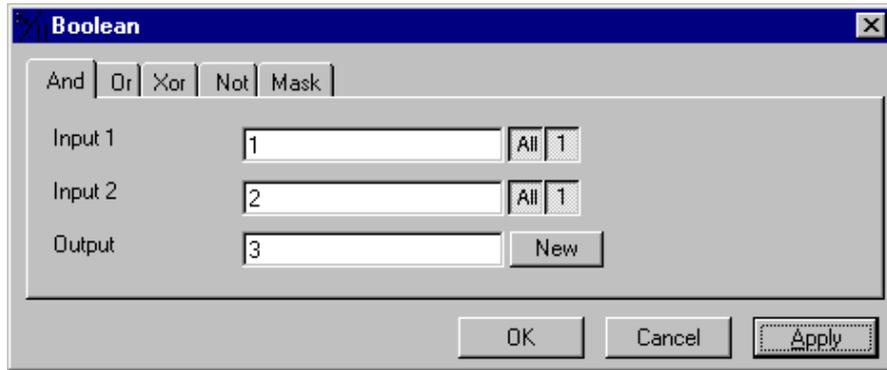


Fig. 6-26

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**.

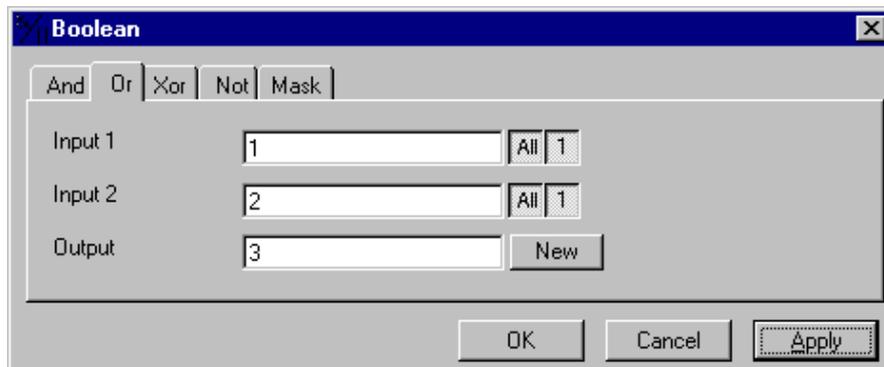


Fig. 6-27

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**.

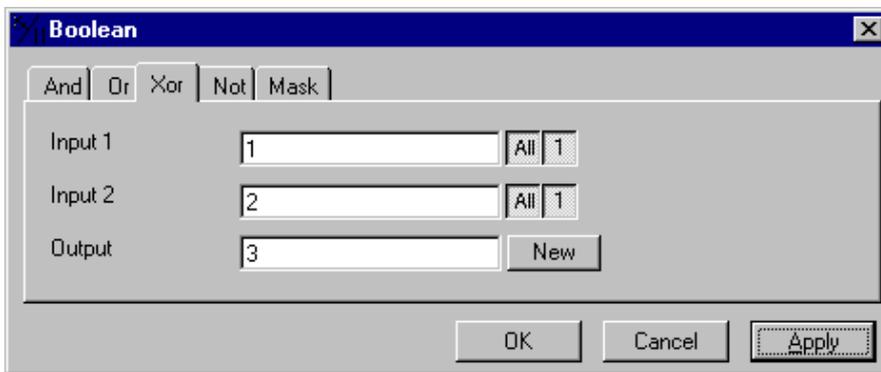


Fig. 6-28

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.

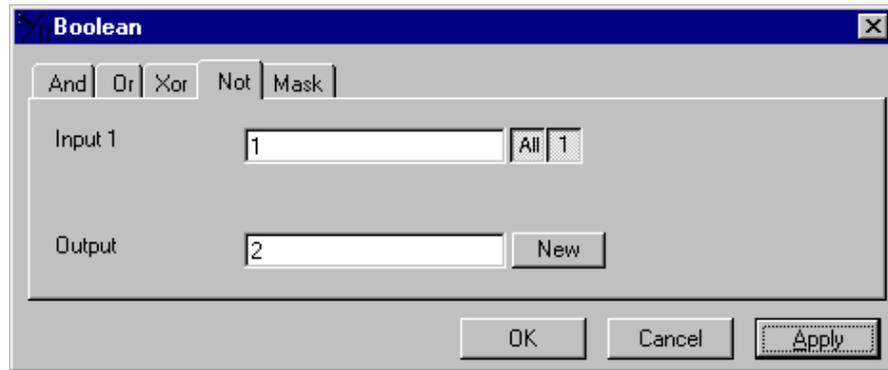


Fig. 6-29

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.

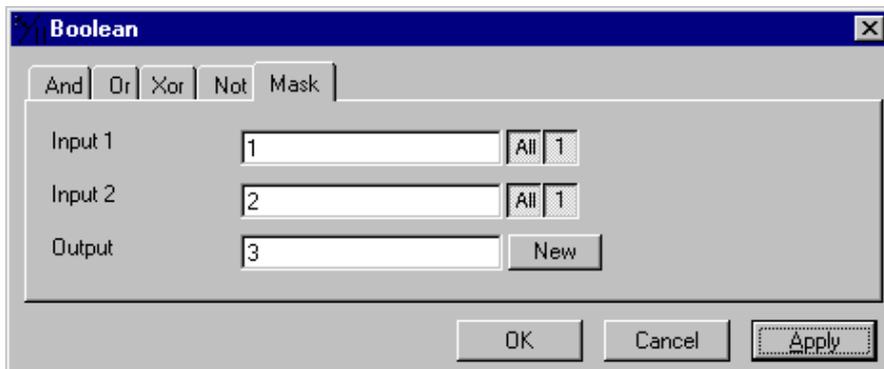


Fig. 6-30

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 1**. 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.

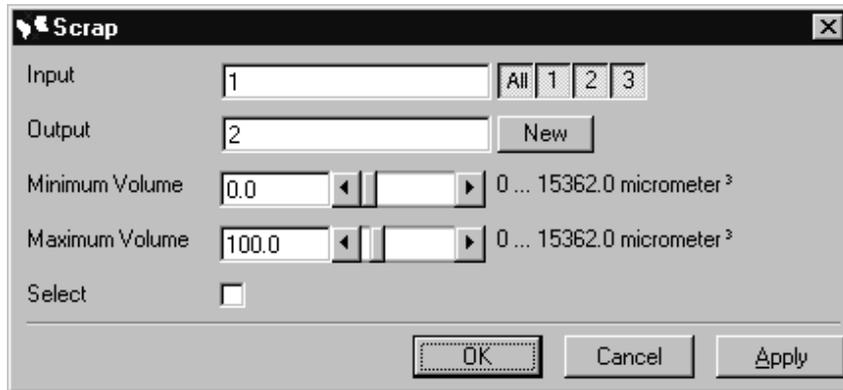


Fig. 6-31

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**.

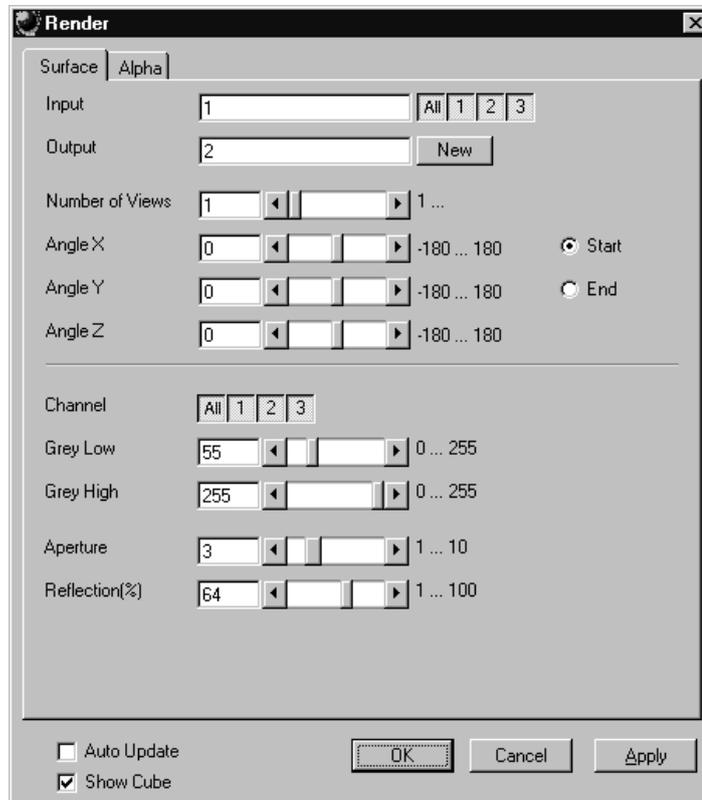


Fig. 6-33

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 diffuse 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**.

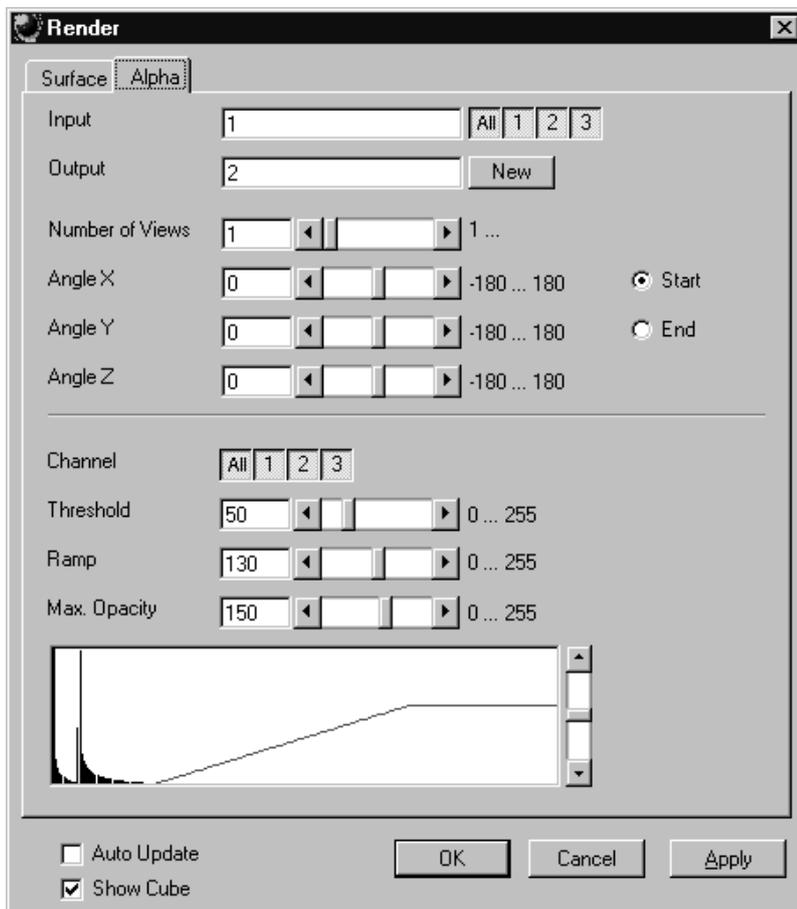


Fig. 6-34

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:

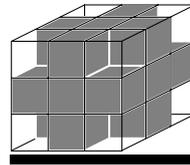
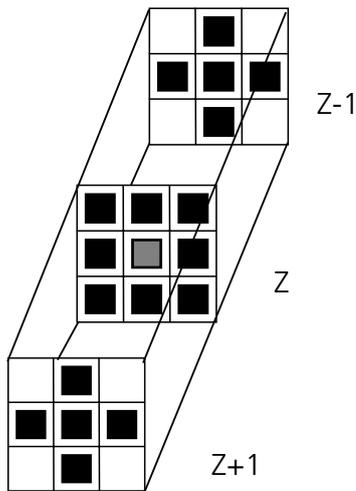


Fig. 6-35

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

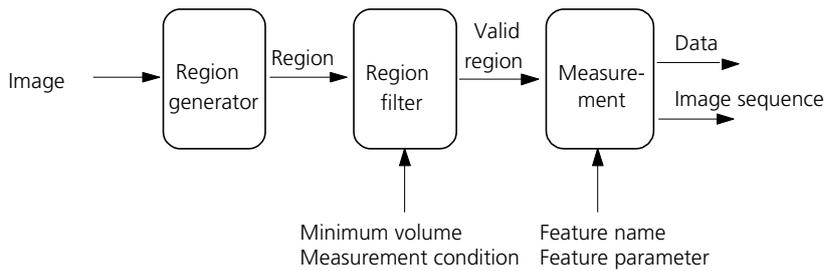


Fig. 6-36

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.

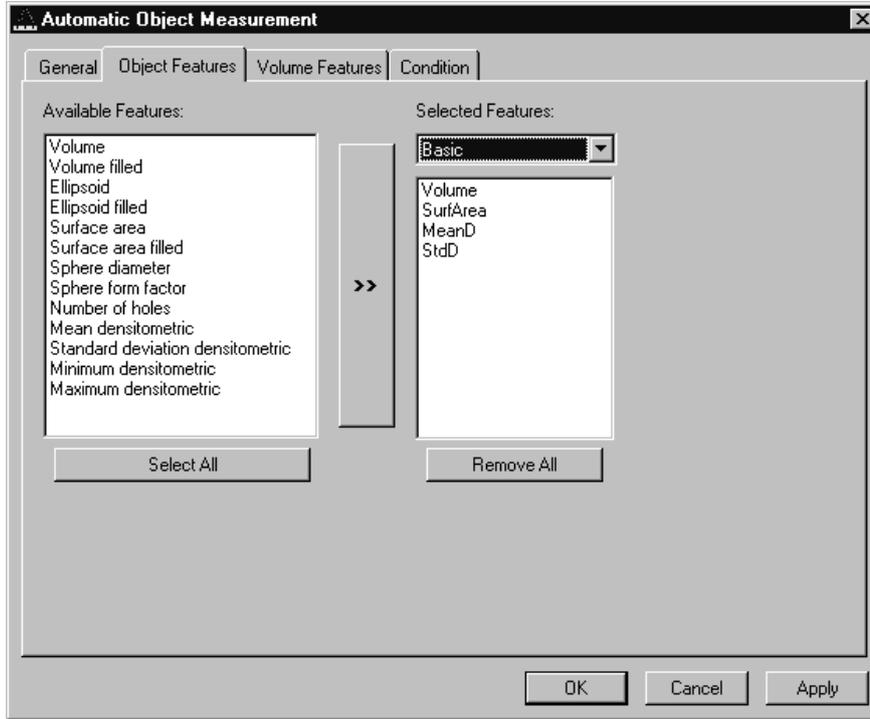


Fig. 6-37

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. $\sqrt{6 * \text{VOLUMEF} / \pi}$
Sphere Form Factor	Fsphere	Form factor of the object. $6 \cdot \sqrt{\pi} \cdot \frac{\text{VOLUMEF}}{\sqrt{\text{SURFAREA}^3}}$
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 Densitometric	MinD	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.

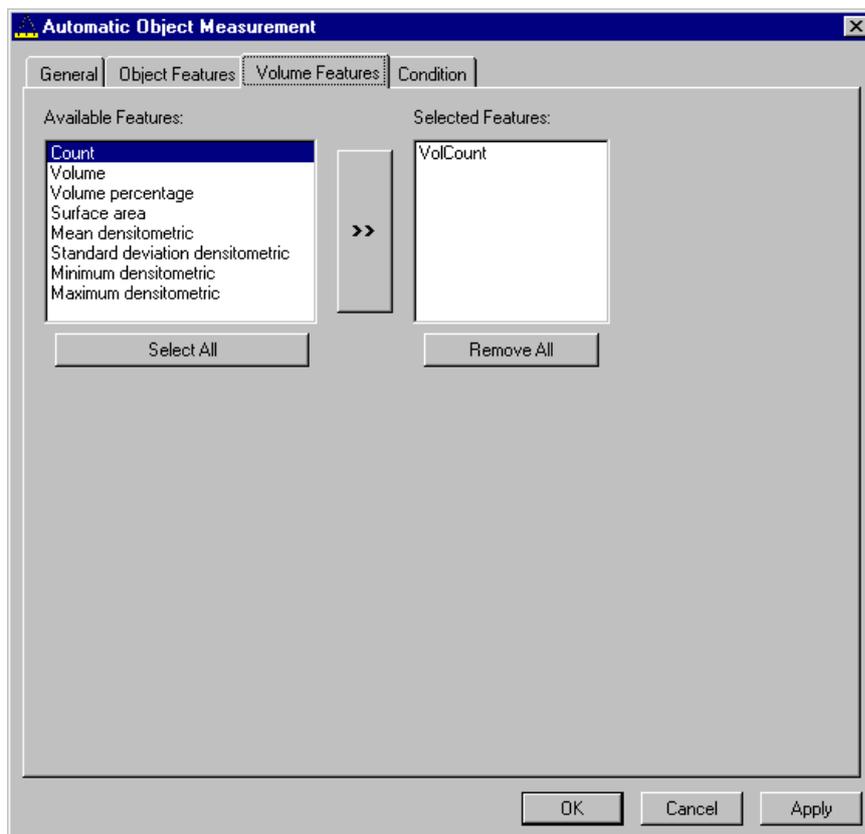


Fig. 6-38

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.

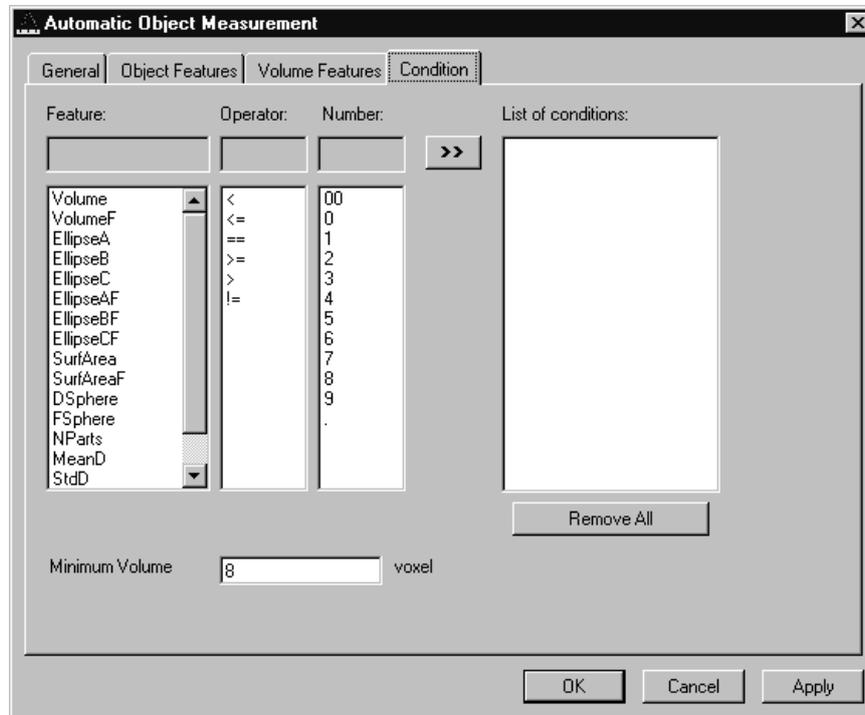


Fig. 6-39

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.

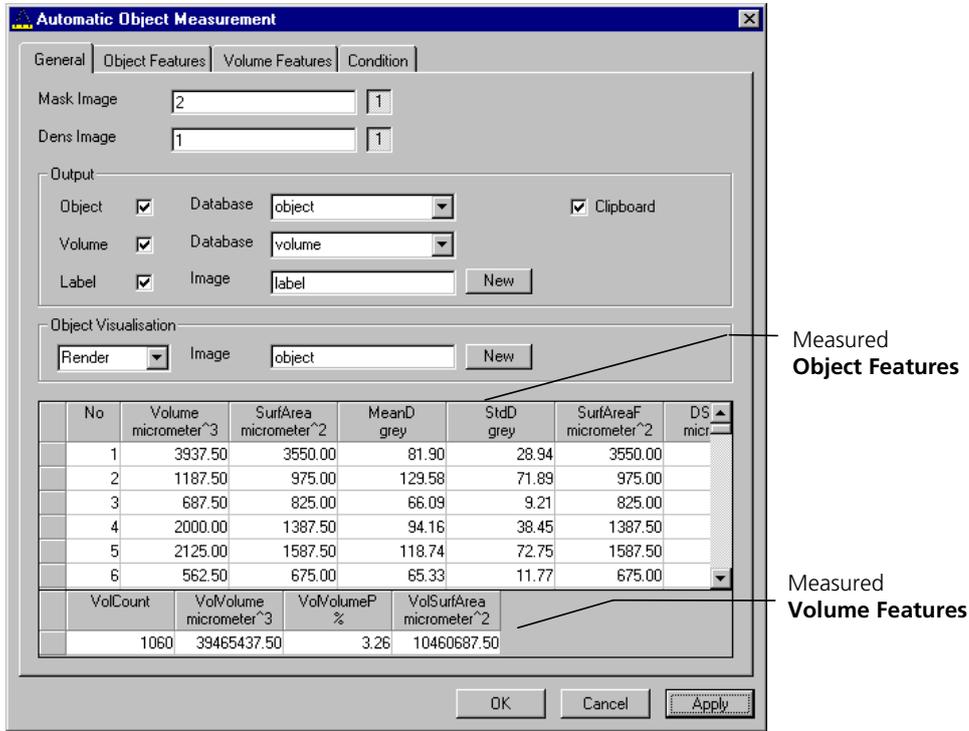


Fig. 6-40

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 spread 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).

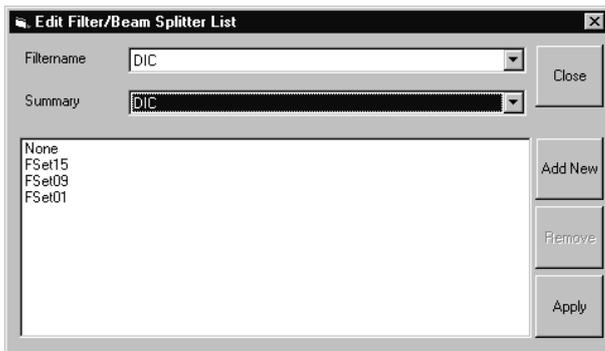


Fig. 6-41 Edit Filter/Beam Splitter List window

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 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 **Filename** 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 **Summary** 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.

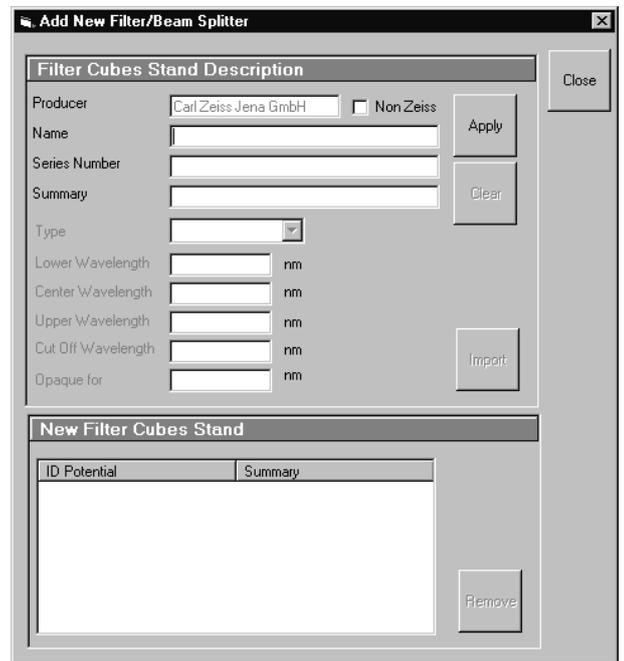


Fig. 6-42 Edit Filter/Beam Splitter List window

- 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.

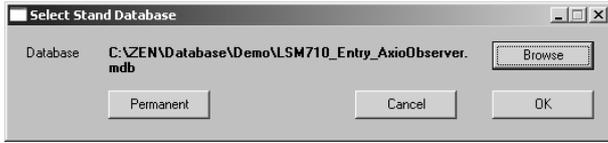


Fig. 6-43 Select Stand Database window

6.3 Stand Select

The **Stand Select** tool permits a new or updated database to be assigned to the ZEN software program. This function should preferably be performed by authorized service personnel.

If this is not possible, proceed as follows:

- Close the 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.

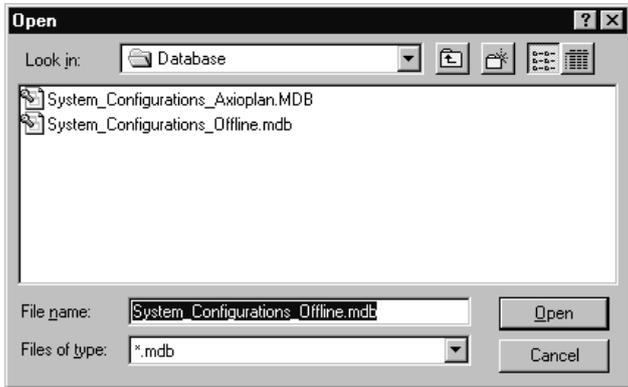


Fig. 6-44 Open window

- 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.

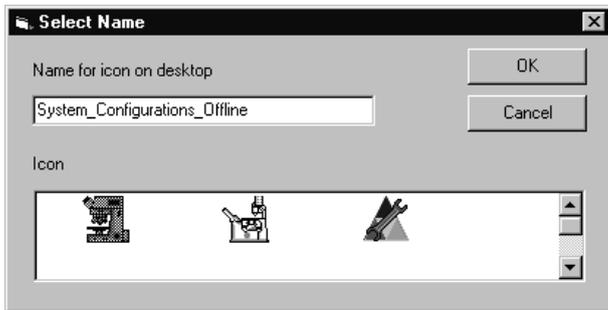


Fig. 6-45 Select Name window

- 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 ZEN software, the new database will be automatically loaded.

6.4 Maintenance Tool

With a calibration objective and correct system configuration, the maintenance tool allows convenient self adjustment of the LSM 710 system. The optical beampath, relative pinhole position and scanner adjustment can be set and checked automatically on the LSM 710.

6.4.1 Calibration objective

Observe the following notes:

- The calibration objective is not suitable for NLO, there is a risk of bubble formation in the substrate.
- Using the HAL, the IR protection filter must be used, and the intensity of the HAL should only briefly be set to more than 70 %.
- With strong heat there is a risk of deformation of the substrate in the calibration objective.
- The lateral screws need to be tightened carefully without applying too much force.
- The calibration objective needs to be entered in the system database as "APO Calibration LSM" with SAP no. 420639-9000-700. It needs to be entered at ZEN \ Maintain \ Objectives.
- Calibration objectives with other samples than shown in Fig. 6-46 shall not be used. The cap with the calibration sample can be obtained separately under SAP-No. 420639-9900-700.

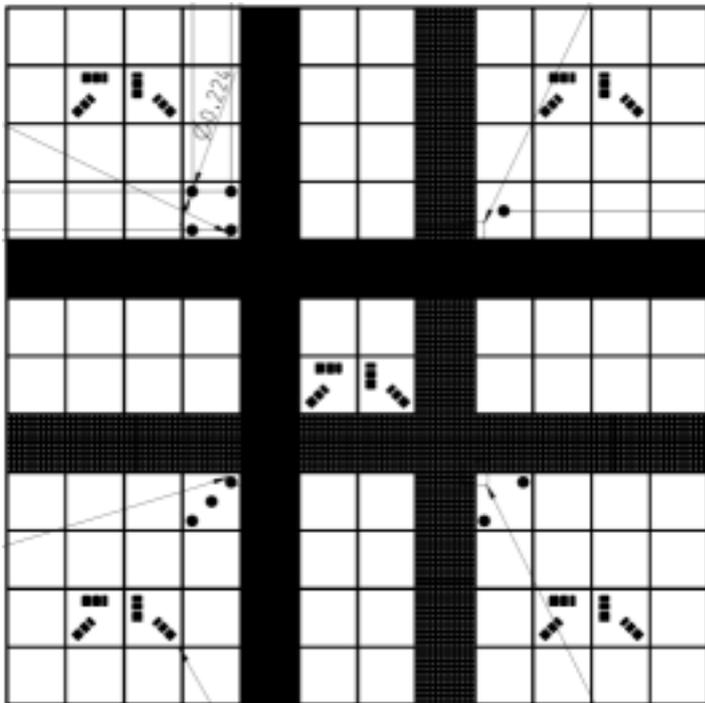


Fig. 6-46 Calibration objective

6.4.2 Starting the Maintenance Tool

Before starting the maintenance tool, the system hardware must be fully activated.

With ZEN 2009, check the following settings:

- Correct entry of all existing lenses; the calibration objective needs to be entered as "APO Calibration LSM".
- For systems with a 405 nm laser, an approximate collimator position should be saved for the calibration objective.
- The focus plane needs to be approximately correct
- All light sources (Hal, LED's, lasers) need to be activated
- After start, choose "Log in... Customer".

6.4.3 General operation of the Maintenance Tool

The desired task can be chosen from the list and started with the start button.

The calibration result is displayed with a traffic light type green/yellow/red indicator.

- If yellow is indicated, repeat the task and try to optimize the settings (e.g. check focus, KÖHLER lamps, lasers (no standby) etc.
- If red is indicated, repeat the test one more time; if it still fails, contact your local LSM service technician.

 On LSM 7 *DUO* systems, the maintenance tool calibrates only the components of the LSM 710 scanhead, not the LSM 7 *LIVE* scanhead.

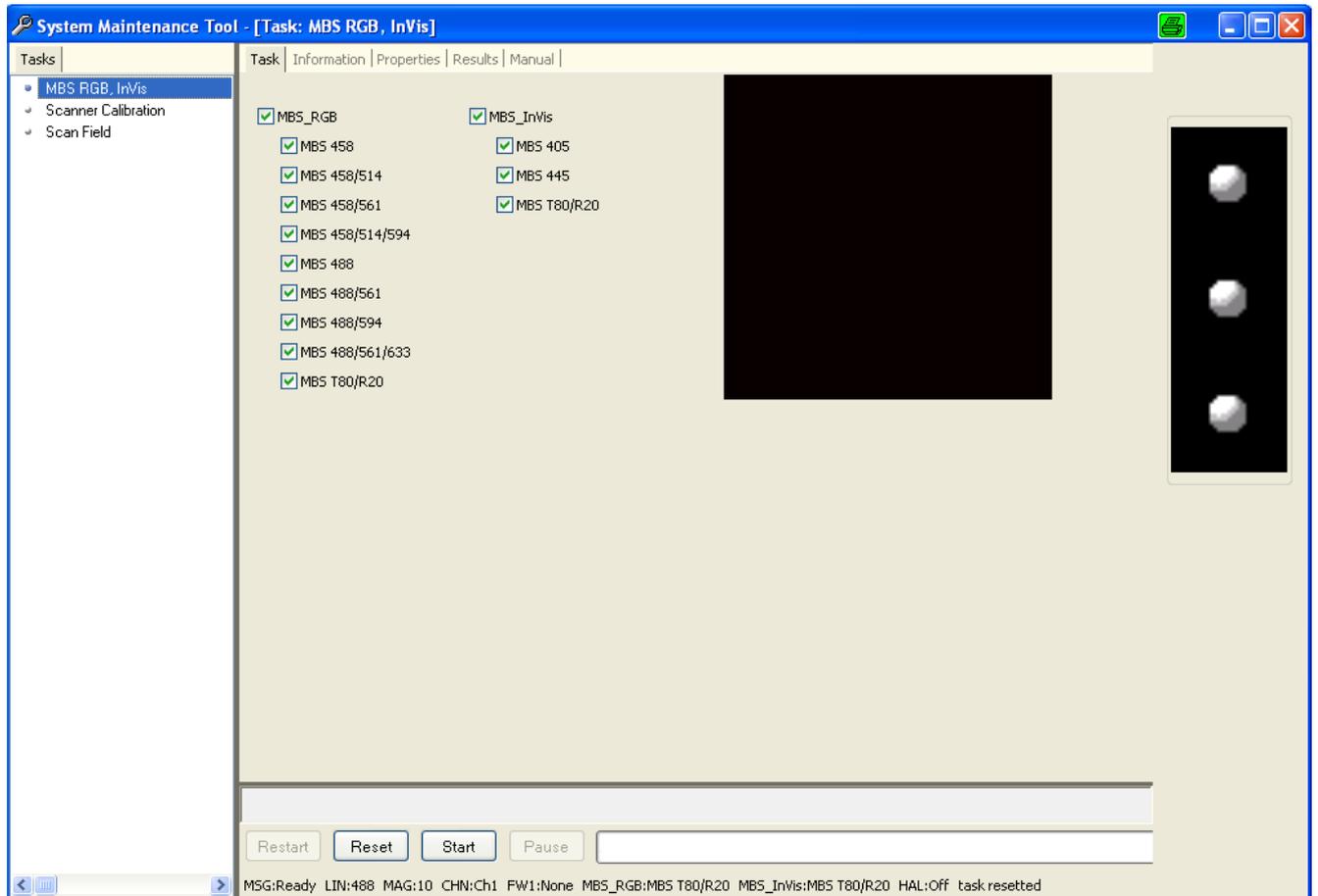


Fig. 6-47 Maintenance Tool main window

Main beam splitter (MBS) calibration, RGB, InVis:

Allows to automatically adjust the optimal beampath between the MBS, the scanning mirrors and the master pinhole on all LSM 710 systems. On LSM 710 systems with additional 405, 440 or IR (NLO) lasers, make sure the respective lasers are checked.

- => Test tool: calibration objective (turret will be set automatically)
 - KÖHLER illumination for transmitted light must be properly set

The calibration data will be included in the file "... \ bin \ PH1_6.pos".

Scanner calibration

- => Test tool: calibration objective (turret will be set automatically)
 - The scanner calibration requires a properly focused optics (see section 6.4.2).



There is no longer a difference between electrical and optical scanner calibration (as it was with earlier LSM systems).

Scanner linearity test (Scan field):

=> Test tool: calibration objective (turret will be set automatically)

Subset of task scan field checks the scanner linearity and runs much faster than scanner calibration.

Resolution test

=> Test tool: calibration objective (turret will be set automatically)

Checks the resolution of the fine structures on the calibration tool. If the resolution test is not successful, this hints on wrong collimator (and eventually pinhole) positions.

Illumination (optional):

Illumination test (fluorescence) for homogeneous coverage

=> Test tool: Delta Vision Slide orange.

After each call the image exposure needs to be set, so that no magenta-colored pixels can be seen.



This task requires a service login.