

**September 2011**  
ZEN 2011 (black edition)

**LSM 700**  
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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Documentation number: M60-1-0036 e

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## How to make best use of the LSM 700 operating instructions

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 problem, 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 dealt with 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.

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**2 Setup Requirements**

The Setup Requirements section outlines the installation and supply requirements of the LSM 700 Microscope System, together with the relevant specifications.

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**3 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 graphic user environment.

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**4 Macros and Visual Basic**

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

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**5 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".

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**6 Annex**

The annex contains the Application-Specific Configurations and special notes and information for using the microscope.

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**7 Certification**

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**8 Laser Safety Warning Labels**

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**9 Introduction to Laser Scanning Microscopy**

This section contains the "LSM 700 Quick Guide". The section introduces you to the performance features of your LSM 700.

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# CHAPTER 1    NOTES ON DEVICE SAFETY

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## 1 General

The LSM 700 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).

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



Installation and commissioning of the LSM 700 system must be performed by authorized Carl Zeiss staff. The system should not be used prior to instruction by a Carl Zeiss representative.

## 2 Regulations

Extensive knowledge of the hardware/the system is indispensable for safe operation of the LSM 700.



Read these operating instructions and all device publications belonging to the system conscientiously **before** operating the LSM 700! 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 700 has 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 61326-1 "Electrical equipment for measurement, control 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 agree with the EU-regulations 2002/95/EG (RoHS) and 2002/96/EG (WEEE), if applicable for the product. The product and its accessories have been classified as instrument category 9 (laboratory equipment or comparable standard).

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.


### 3 Power Requirements

The LSM 700 system must be connected to the a.c. network via four line cables.

Any additional components (i.e. incubation systems) have to be connected separately to the a.c. network. See the individual manuals of those components for further instructions.

As shown in Fig. 1, some system components must be plugged into two (or three) multipoint connectors containing a single-phase plug. The two (or three) multipoint connectors must be connected to separate power sockets to an AC network. The used scanning stage and the used illumination unit have to be connected directly to the a.c. network.

Line voltage	220 V AC ... 240 V AC (+10 %)	100 V AC ... 125 V AC (+10 %)
Line frequency	50 ... 60 Hz	50 ... 60 Hz
LSM 700 incl. Lasers		
Max. current	1 phase at 4 A	1 phase at 7 A
Power consumption	800 VA	700 VA
Power plug	Country-specific connectors	NEMA 5/15 (only USA)
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)

The following components must be inserted in the first 3-point connector (power strip 1, see Fig. 1):

- flat screen monitor
- control computer
- microscope

The following component must be inserted in the second 3-point connector (power strip 2, see Fig. 1):

- electronics and laser module

The following components must be connected directly to the a.c. network using two line cables (see Fig. 1):

- X/Y scanning stage via controller
- HBO 100 / XBO 75 / HXP 120

If incubation components are used the following components must be inserted in the third 3-point connector (power strip 3, see Fig. 1):

- HBO 100 / XBO 75 / HXP 120
- Temp module
- Heating unit



Others than the components shown should not be connected to the multipoint connectors, due to the touch current limits of the system.



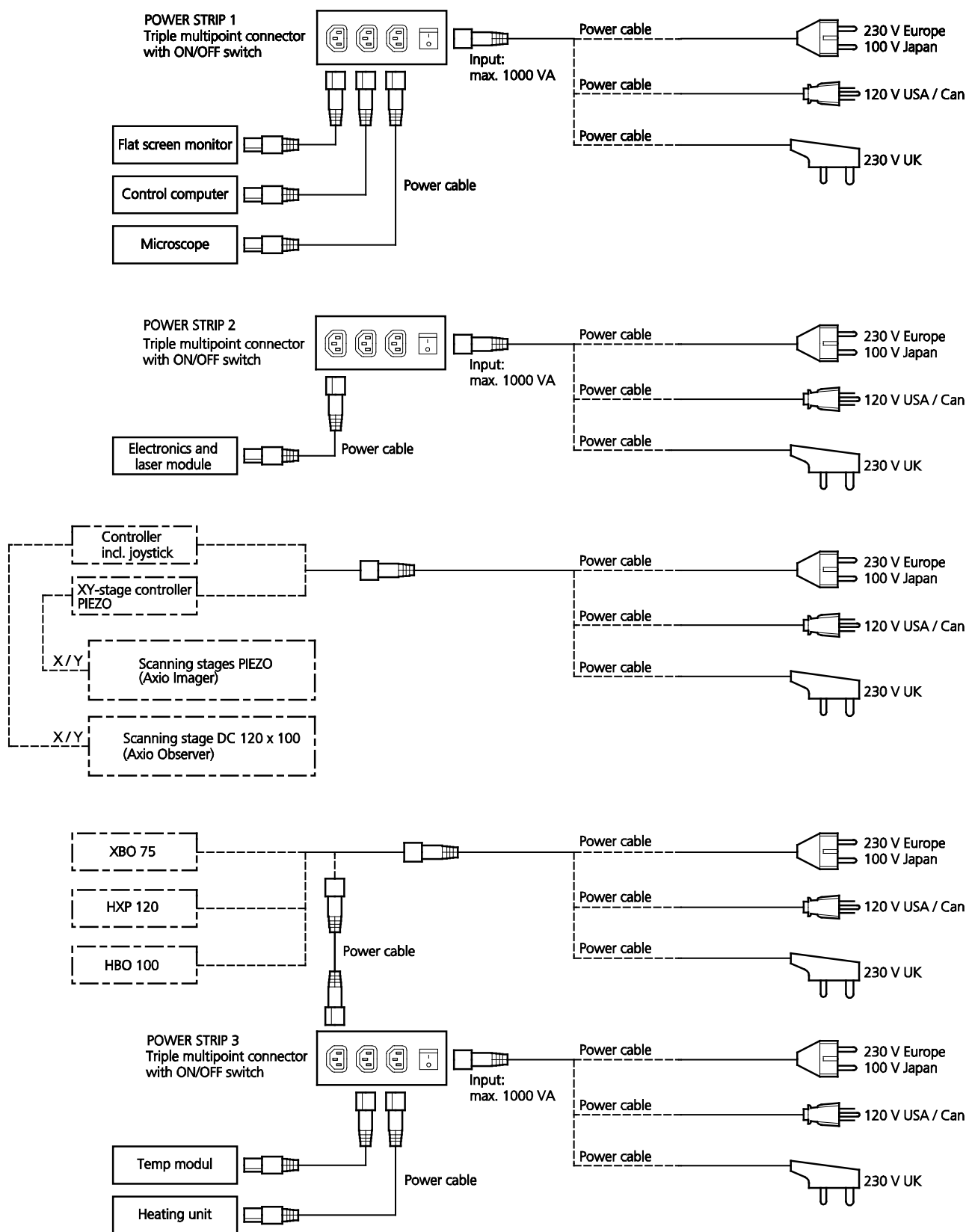
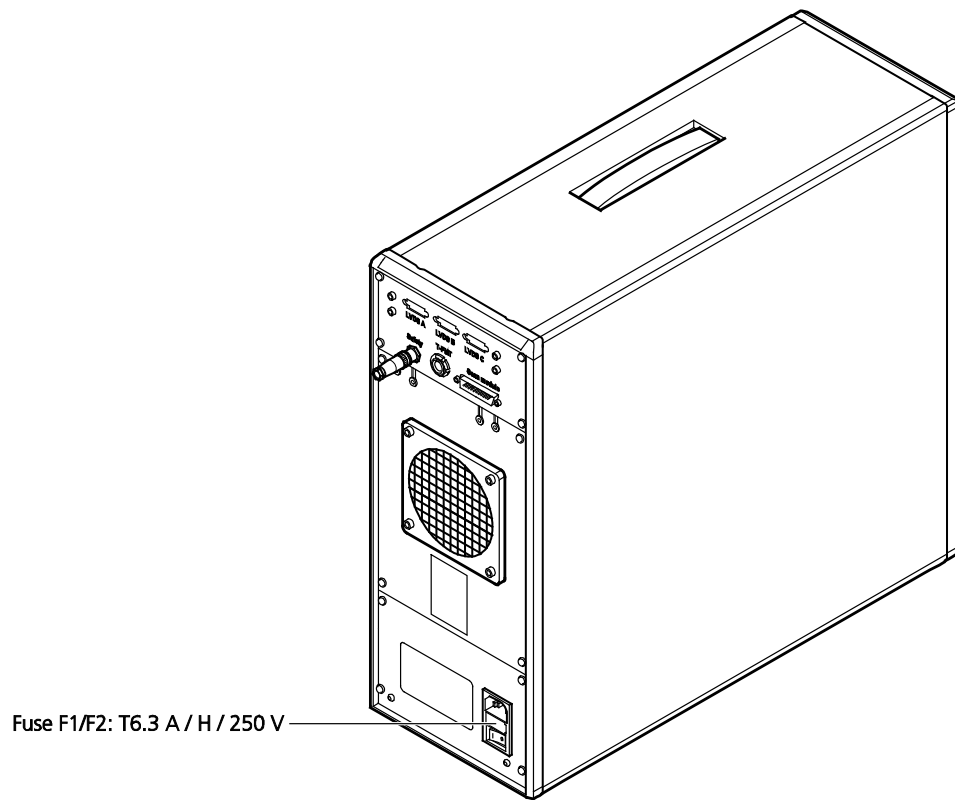


Fig. 1 Power connectors for LSM 700 system



**Fig. 2**      **Electronics and laser module for LSM 700**

## 4 Notes on Setting up the Microscope System



Installation and commissioning of the LSM 700 system must be performed by authorized Carl Zeiss staff. The system should not be used prior to instruction by a Carl Zeiss representative.

The LSM 700 laser scanning microscope is delivered in one crate.



The LSM 700 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.



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.



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 louvers on the microscope system must not be covered up. Do not position the electronics parts in a closed container

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.



Maintenance, repairs, modifications, 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 us 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 700 is a high-precision opto-electronic instrument. Inexpert handling may easily impair its function or even damage it and will invalidate any claim under warranty.



There are hot surfaces on the HBO and HAL lamp.



For reasons of laser safety, removing of T-PMT, switching mirror, or HAL by the user is not allowed!



For reasons of laser safety, removing of the tube by the user is not allowed for uprights stands!



On the Axio Imager 2 on the opening opposite to the camera laser light (class 1) can leave the system when the cap is removed.



Note that the power of the motor drives of the scanning stages DC is strong enough to squeeze your fingers e.g. between the scanhead housing and the scanning stage.



The HBO 50 and 100 W, XBO 75 W, HXP 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 filter glasses. Never remove the lamp and look direct into the emitted light.

After installation or after conversion of the LSM 700 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 700 should only be operated in conjunction with the system table (vibration damping).




It may happen that the monitor turns off automatically if interference radiation is too strong, even though being within the standard range. Then, the user must turn on the monitor again.

## 5 Warning and Information Labels



The warning and information labels attached on the LSM 700 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.

The  label means: "Caution! Laser hazard". If this label is affixed in the near of an securing screw the label means: "Do not remove securing screw as otherwise laser beam will escape."

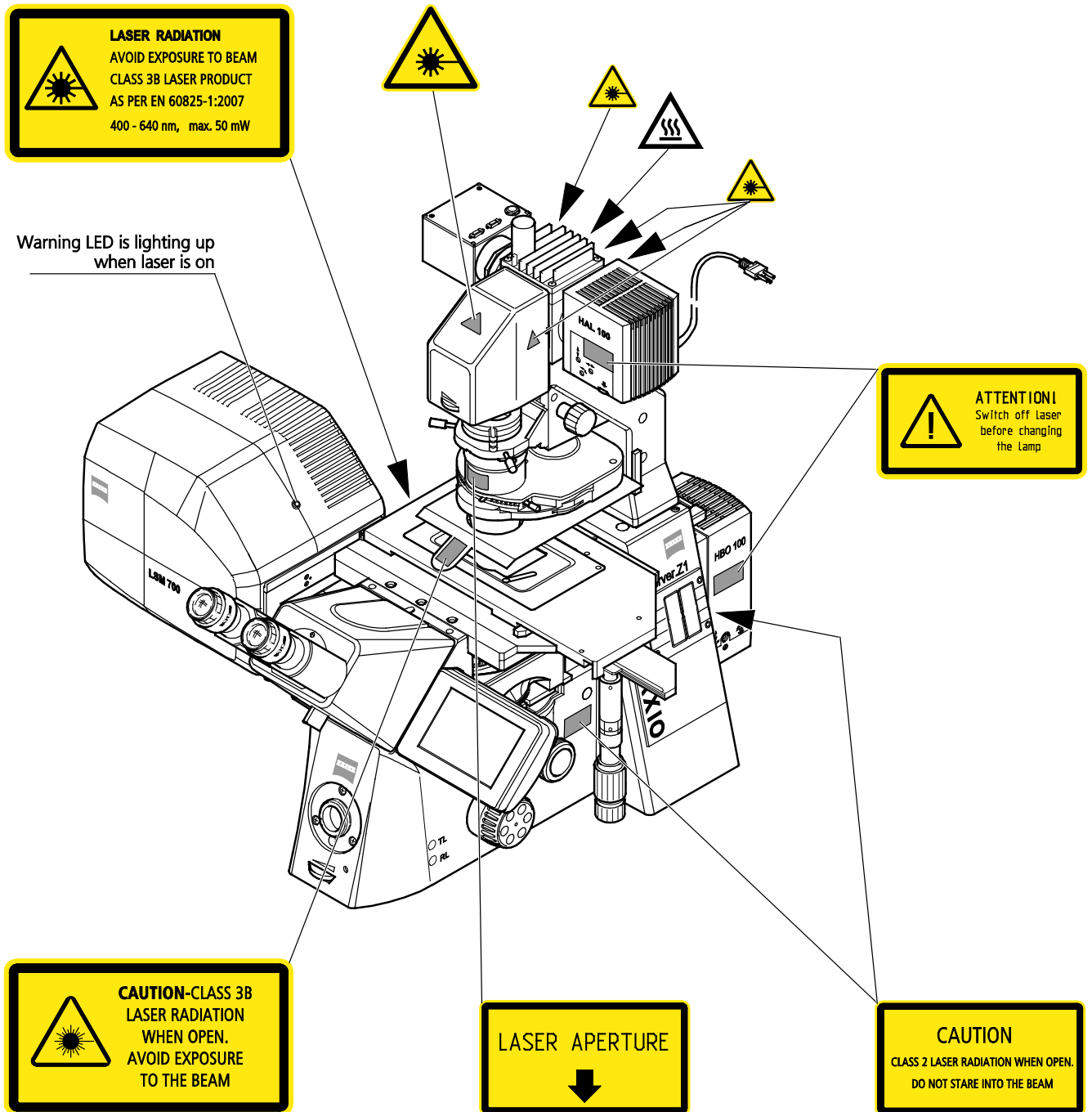


Fig. 3 Warning and information labels on the Axio Observer.Z1 microscope with the LSM 700 scanning module

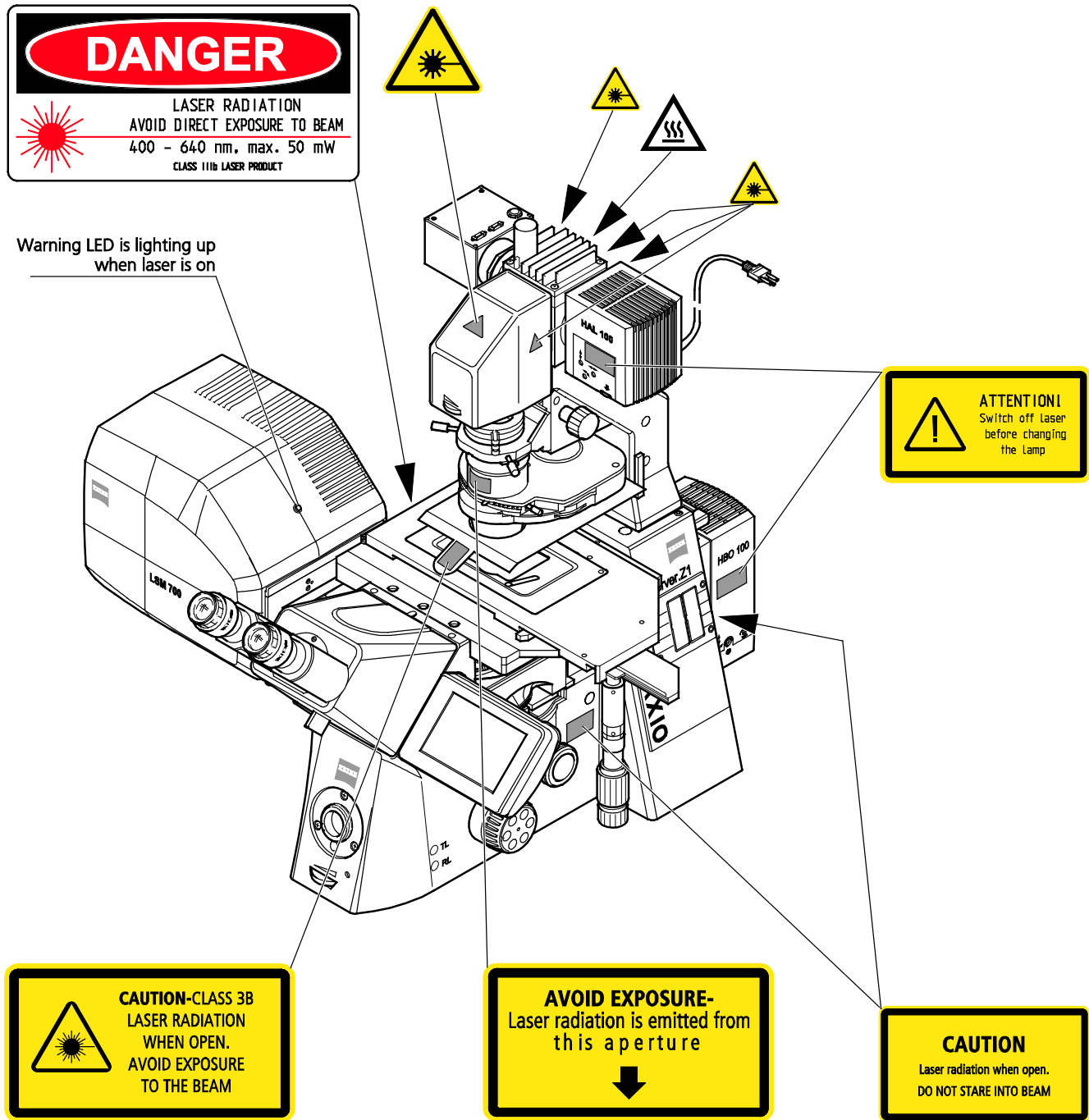


Fig. 4 Warning and information labels on the Axio Observer.Z1 microscope with the LSM 700 scanning module (US version only)

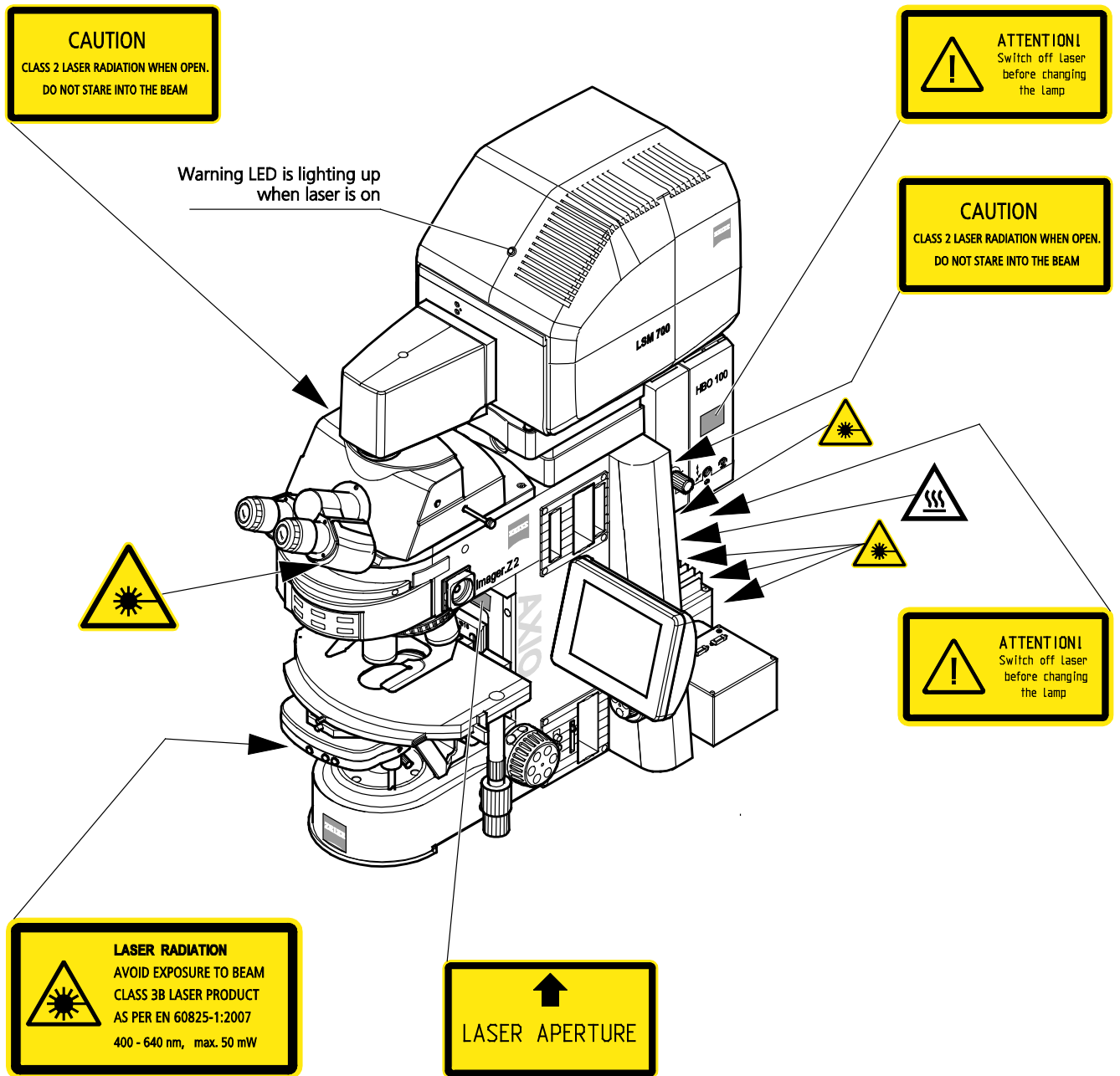


Fig. 5 Warning and information labels on the Axio Imager.Z2 microscope with LSM 700 scanning module

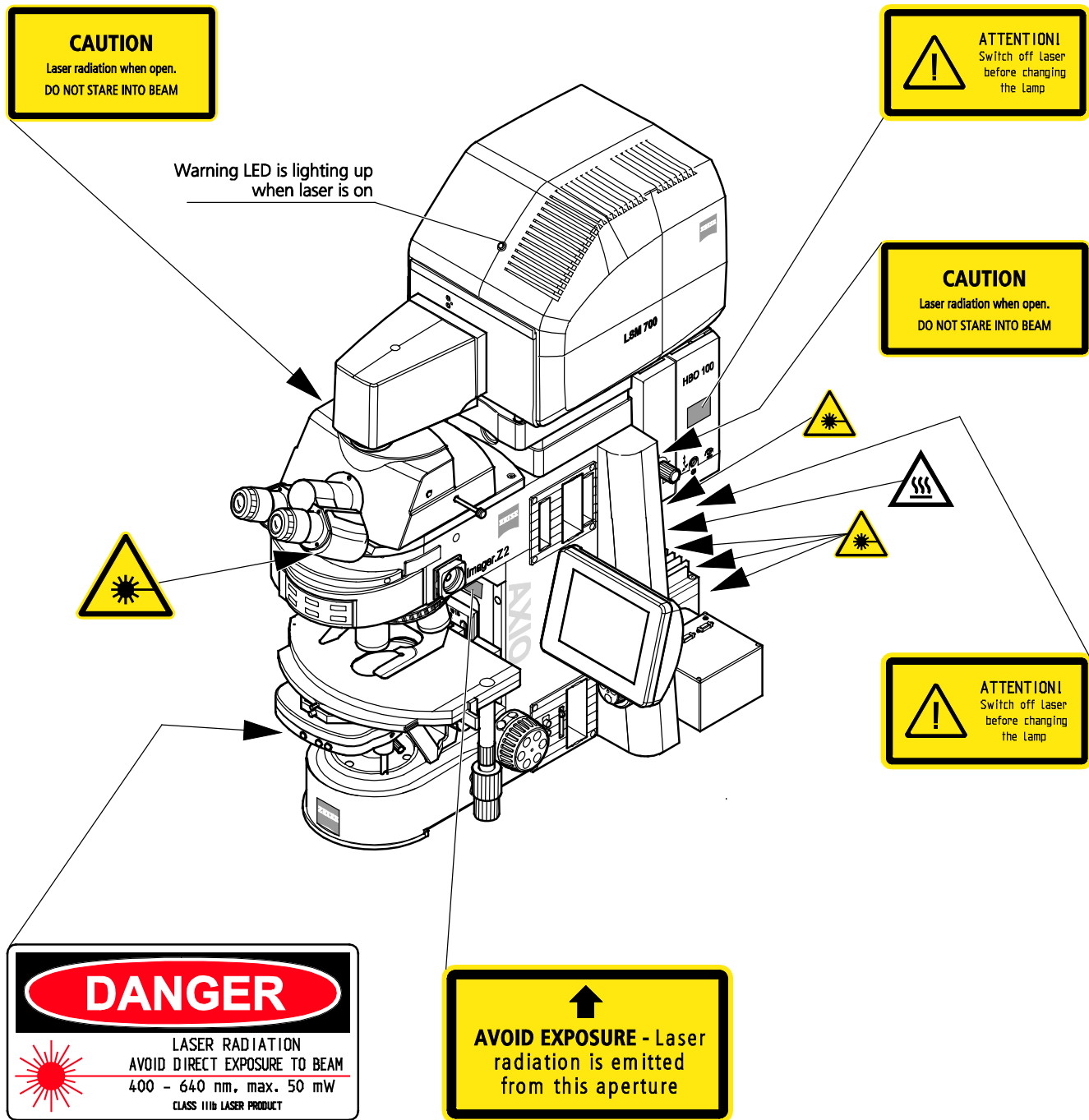


Fig. 6 Warning and information labels on the Axio Imager.Z2 microscope with LSM 700 scanning module (US version only)



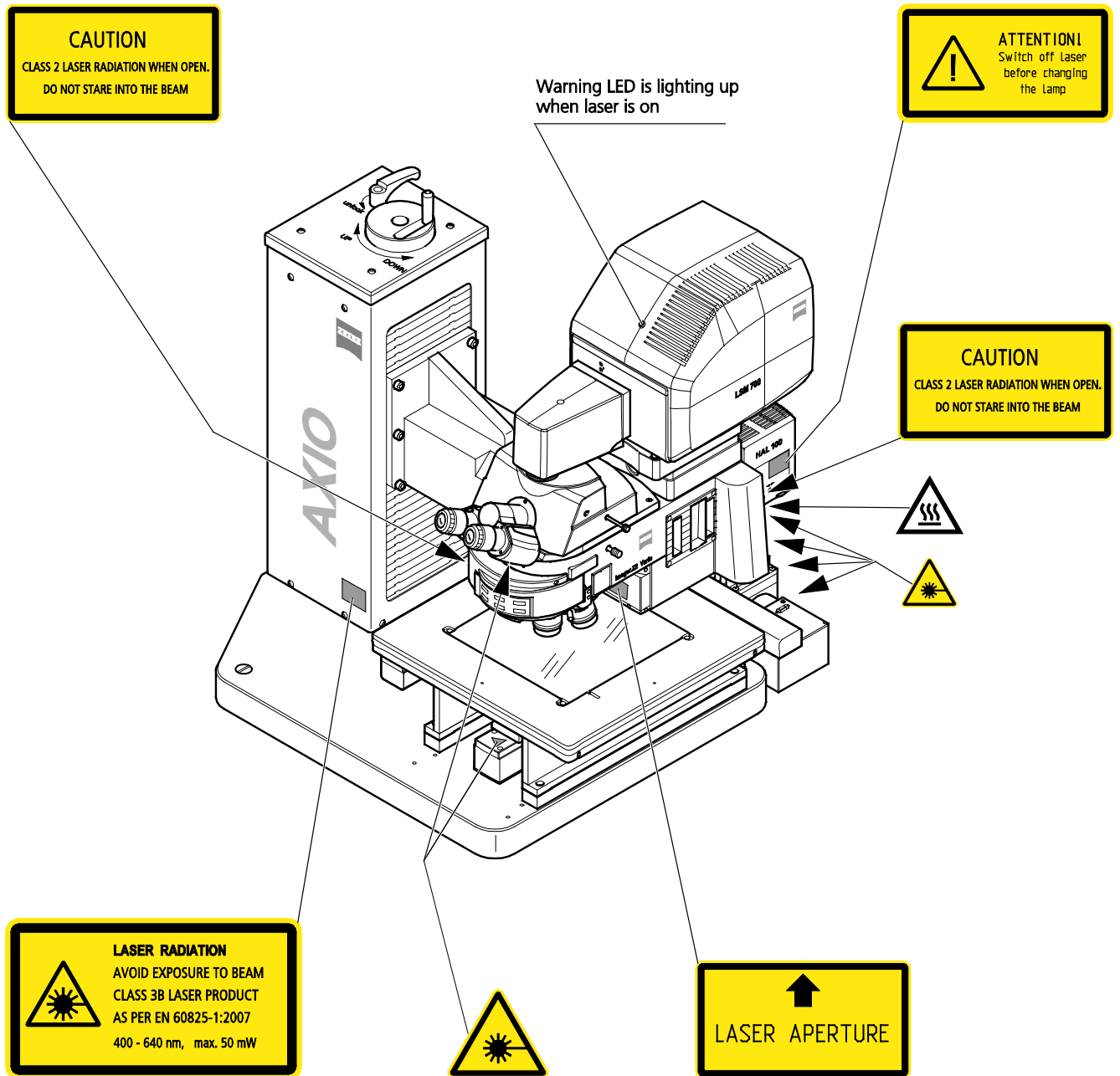


Fig. 7 Warning and information labels on the Axio Imager.Z2 Vario microscope with LSM 700 scanning module

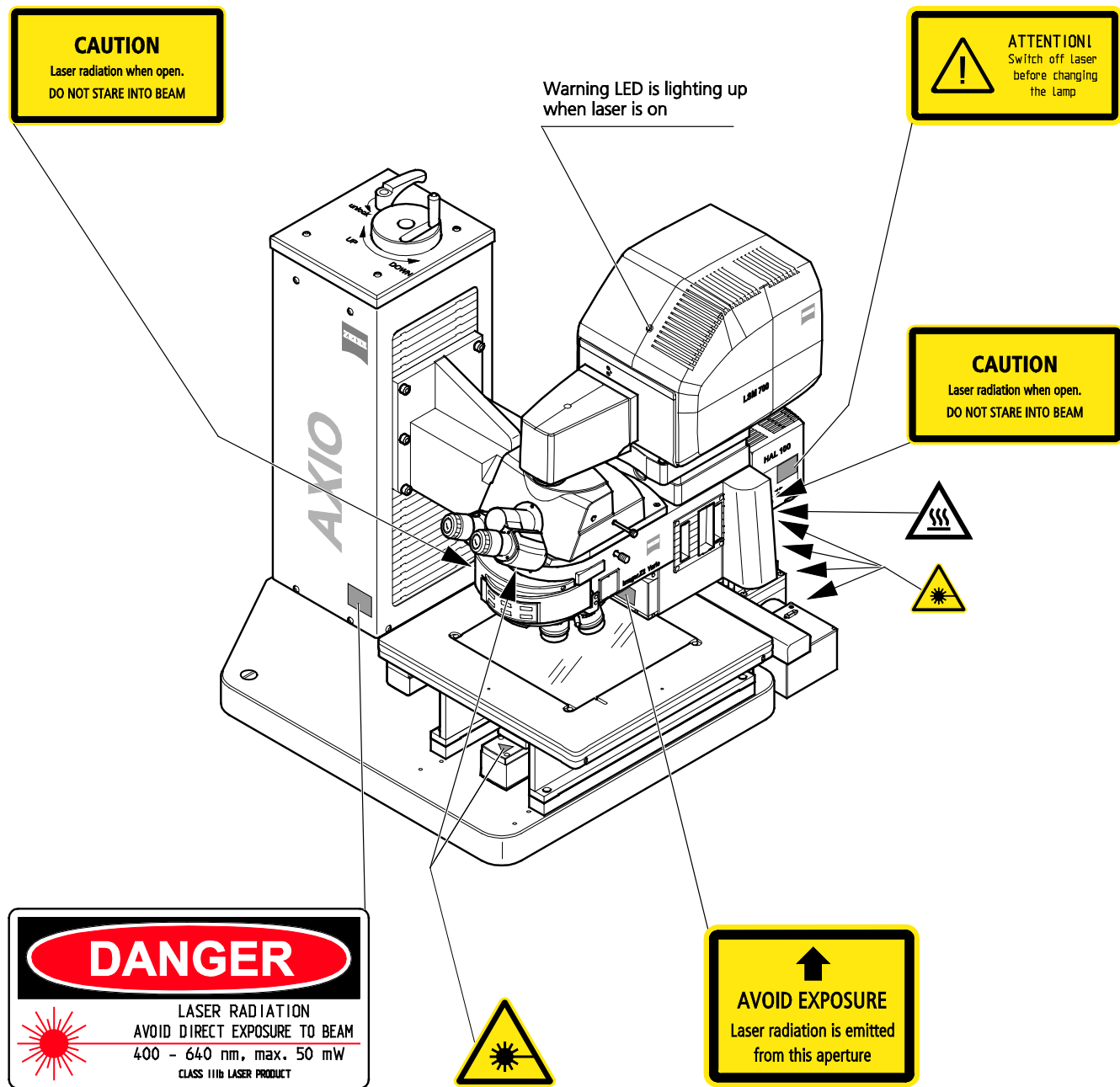


Fig. 8 Warning and information labels on the Axio Imager.Z2 Vario microscope with LSM 700 scanning module (US version only)

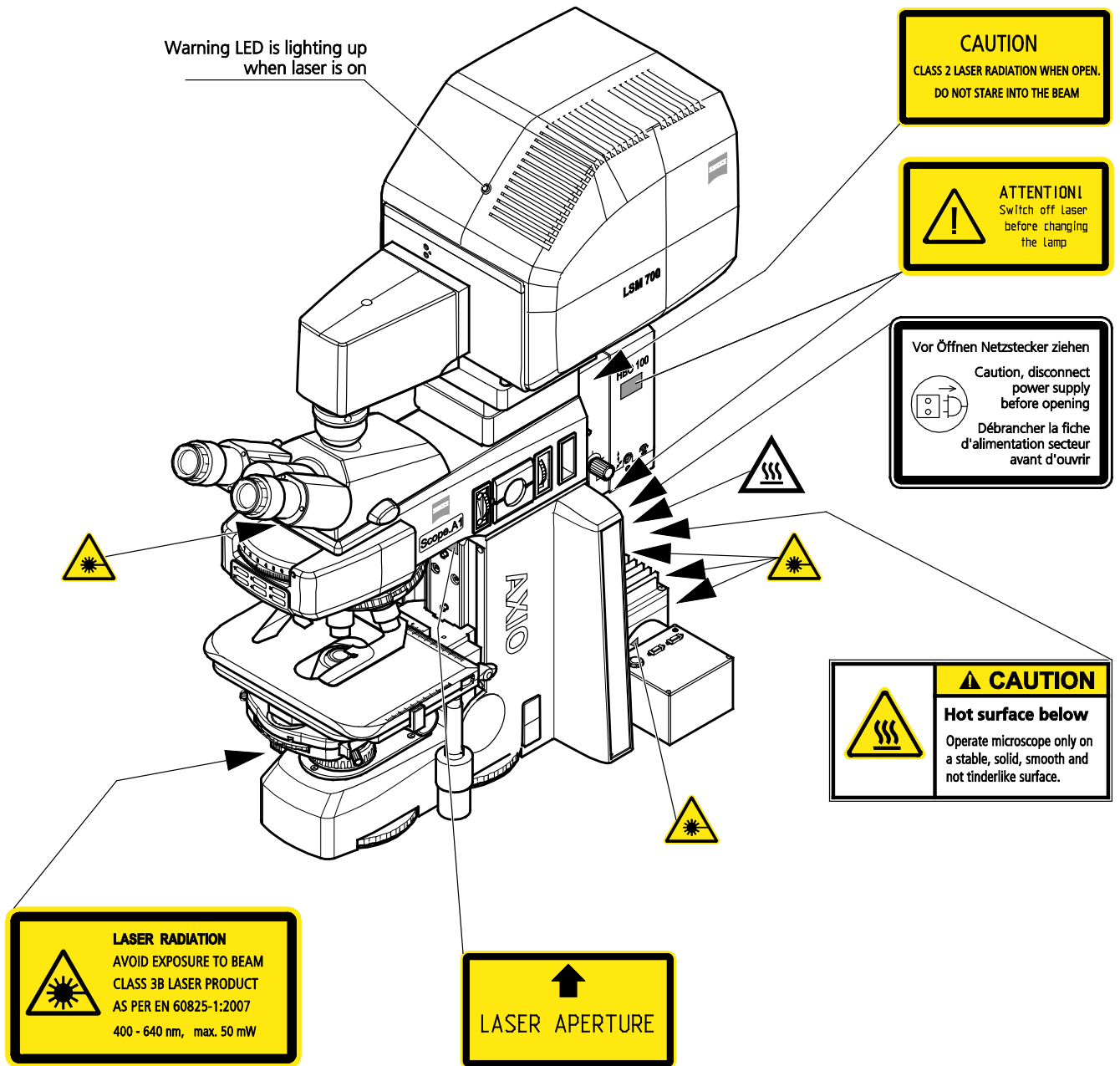


Fig. 9 Warning and information labels on the Axio Scope microscope with LSM 700 scanning module

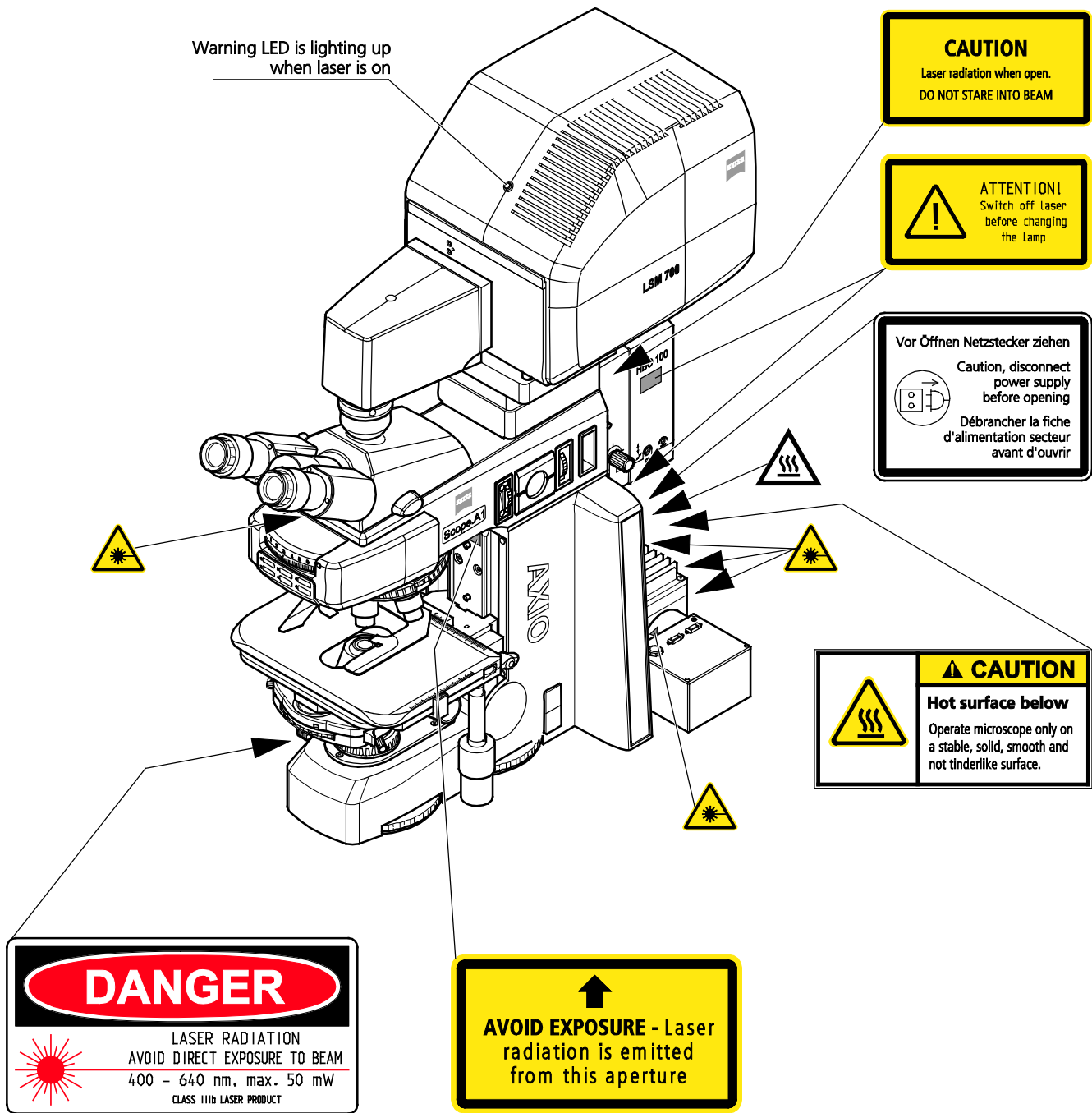


Fig. 10 Warning and information labels on the Axio Scope microscope with LSM 700 scanning module (US version only)

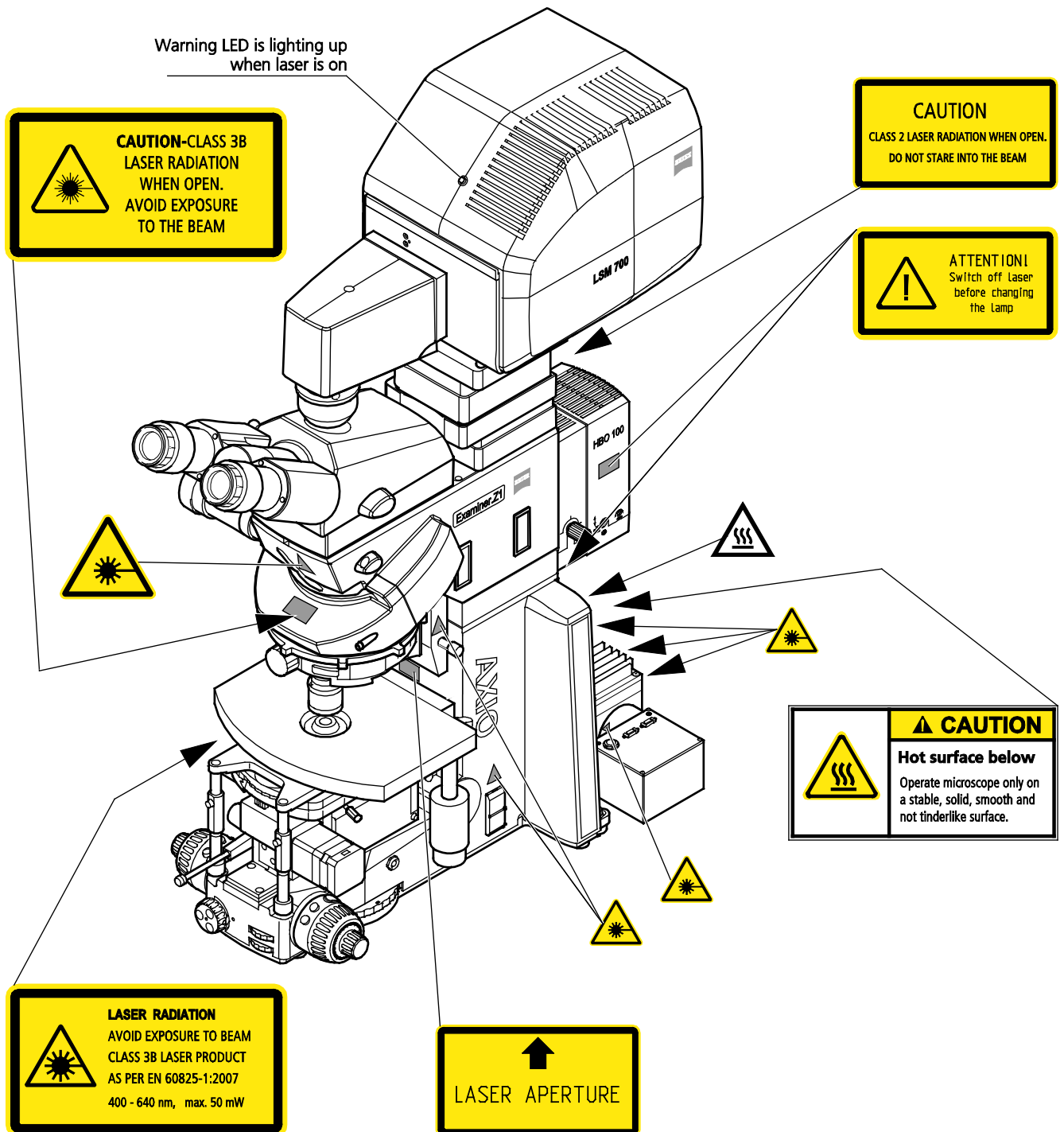


Fig. 11 Warning and information labels on the Axio Examiner microscope with LSM 700 scanning module

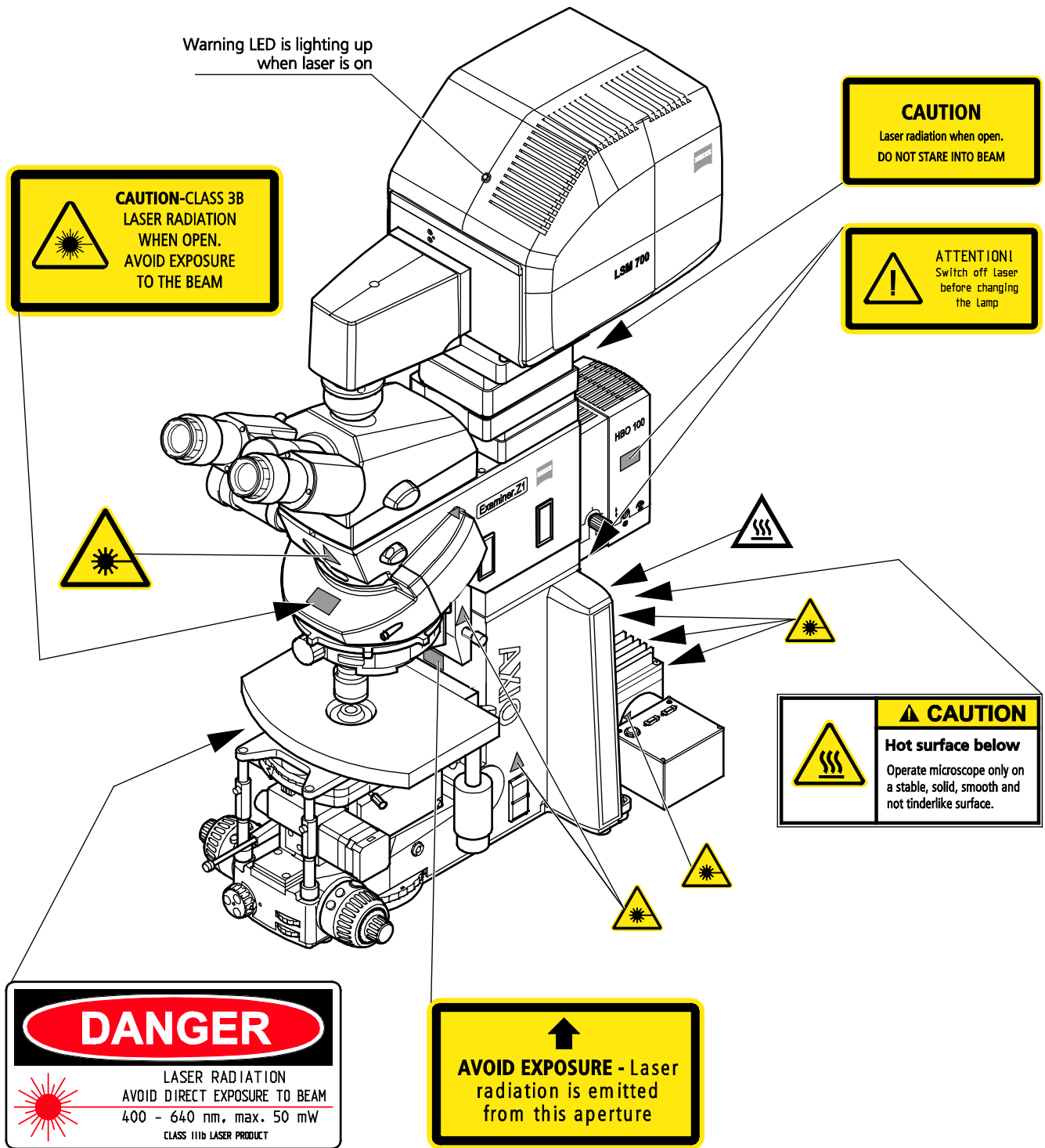


Fig. 12 Warning and information labels on the Axiio Examiner microscope with LSM 700 scanning module (US version only)

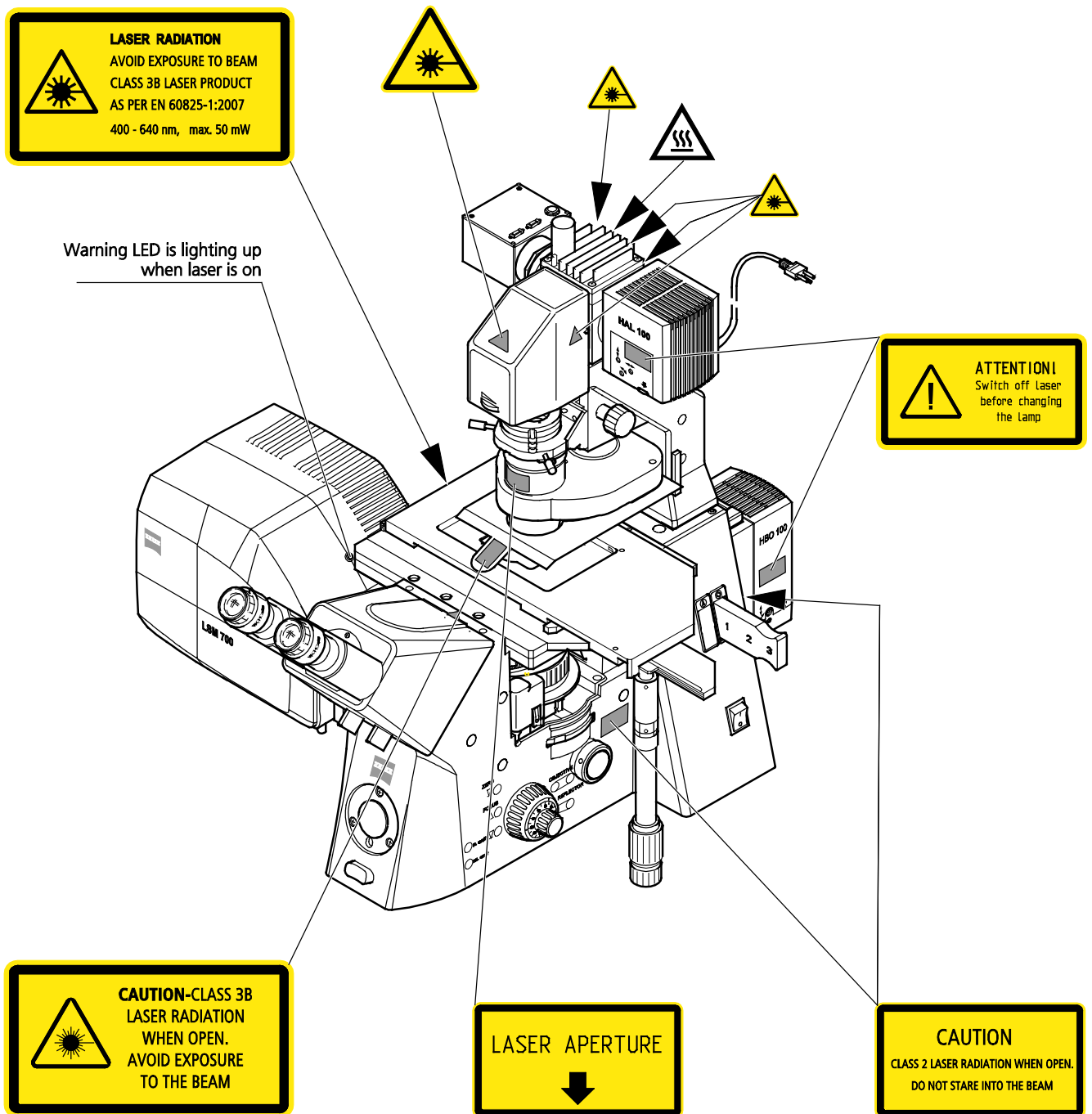


Fig. 13 Warning and information labels on the Axiovert 200 microscope with LSM 700 scanning module

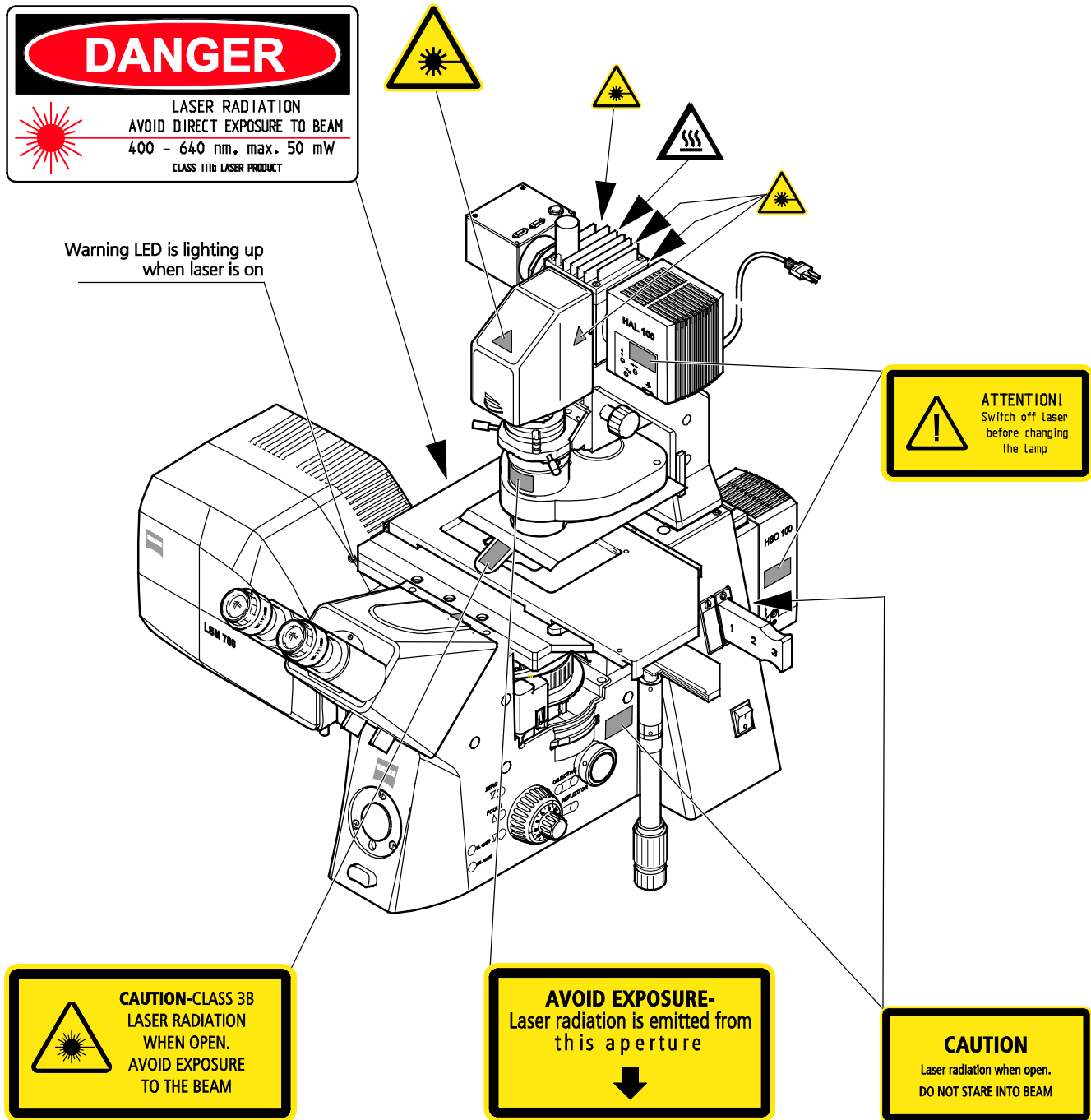


Fig. 14 Warning and information labels on the Axiovert 200 microscope with LSM 700 scanning module (US version only)



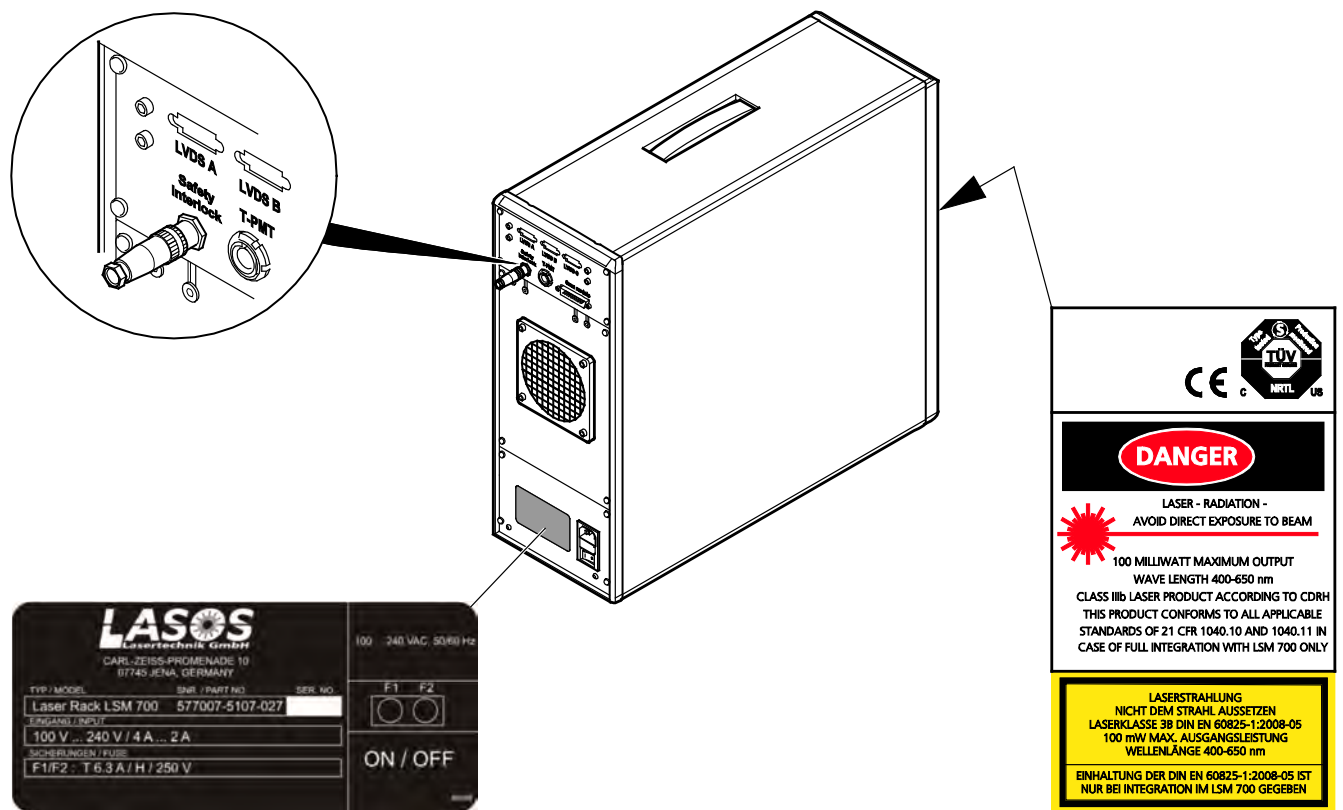


Fig. 15 Warning and information labels on laser rack

## 6 Notes on Handling the Laser Components and Illumination Systems



The LSM 700 is a laser hazard class 3B instrument and is marked as such. This moderate-risk class embraces medium-power lasers. You must take care not to expose yourself to the radiation of such lasers. In particular, never look into the laser beam!



**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 used in the LSM 700.

- 1 Laser 405 nm, 5 mW
- 2 Laser 445 nm, 5 mW
- 3 Laser 488 nm, 10 mW
- 4 Laser 555 nm, 10 mW
- 5 Laser 639 nm, 5 mW




Please contact Carl Zeiss if you intend to use a laser type with a wavelength or output power other than the ones above.

If used properly, the LSM 700 will not pose any laser radiation risks for operating staff. Nevertheless, you should observe the following warnings:



- Caution – use or adjustments or performance of procedures other than those specified herein may result in hazardous radiation exposure
- If necessary - insofar as specified by law - inform the laser protection officer before commissioning the laser.
- The laser rack is equipped with a key-interlock.
- The laser rack is equipped with an electric interlock at the back of the rack (see Fig. 15). This can be used to connect a remote interlock.
- Always store the laser rack keys where they are inaccessible to persons not authorized to operate the laser.
- An orange LED on the front of the scan head lights up when one or all of the lasers emit laser radiation.
- A yellow LED on the front of the laser rack lights up, once the lasers are turned on with the key.
- Do not place any reflecting objects into the beam path.
- Never open any covers or paneling (except the user interface described in CHAPTER 7 of this manual).
- 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 screw positions of the nosepiece uncovered.
- Suitable eye wear for laser protection should cover the following wave lengths: 405/445/488/555/639 nm and should have an optical density of 1.5 or higher.

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



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

## 7 Laser Lifetime

The following table shows the expected laser life times:

Laser wavelength in nm; Power out of fibre	Type	End of life time power spec.	typical life time (in hours)
405; 5 mW	Solid state, laser cassette	4 mW	5000
445; 5 mW	Solid state, laser cassette	4 mW	5000
488; 10 mW	Solid state, laser cassette	8 mW	8500
555; 10 mW	Solid state, laser cassette	8 mW	8500
639; 5 mW	Solid state, laser cassette	4 mW	5000

## 8 Notes on Handling the Computer and Data Media

The computer used as standard in your LSM 700 system is a high-end computer with WINDOWS operating system.

As standard, your computer has one hard disk drive and one CD-ROM drive. A DVD reader/writer is installed.



Do make sure, though, that you receive your LSM 700 system with the operating system installed, with initialization and start files set up and with the LSM program also installed.



When working with the hard disk, it is important to know that the more data it contains, the slower its operation will become. Therefore, data that you do not need permanently should be stored on a DVD, a CD-ROM, a DVD or an external HD drive.



When handling diskettes, 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 benzene, 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 exited the LSM program and run down the WINDOWS operating system. Otherwise, the program and/or data files may get lost.



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

## 9 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 also render all warranty claims null and void.

You are well advised to arrange a service agreement with your nearest 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. 2. The fuses are inserted into fuse holders (normally used for power supply units). The fuse type is given on the hardware itself and also in Fig. 2.

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

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

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



Before opening the housing of the HBO/XBO lamps, HXP light sources or HAL lamps switch off the electronics and laser module.

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. 16/1), heat sink (Fig. 16/2) and halogen lamp:

**Make sure to let them cool down sufficiently!**

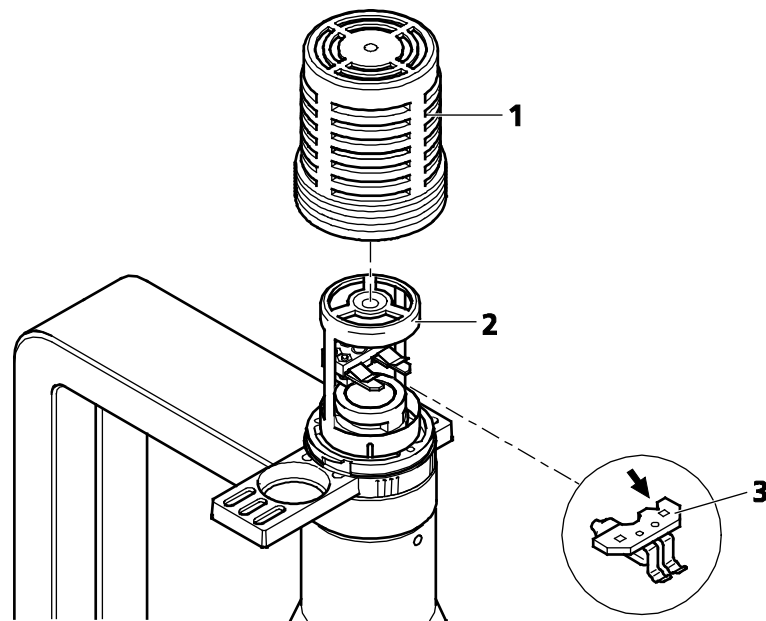


**Caution:**

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. 16/1) by slightly turning it anticlockwise.
- Remove the halogen lamp with alignment base (Fig. 16/3).
- Remove new 12 V 35 W halogen lamp with alignment 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. 16 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 hairbrush.
- Make sure, that no cleaning liquid penetrates into the system.

## 10 User Interface - Mounting and Dismounting Lamps, T-PMT and Switching Mirror



All interface ports which can potentially emit laser radiation are equipped with special TORX safety screws which must not be loosened by the user.

The following interfaces are **no** longer user interfaces when used with a LSM 700 system, even when other manuals (e.g. the stand manual) describe them as user interfaces.

- Halogen lamp housing
- Transmission PMT
- Switching mirror
- Scan heads
- Tube on upright stands
- Reflector turret in case of the Axio Scope
- Transmitted-light module in case of Axio Imager.Z2 Vario.

Other interfaces which are not described here are service interfaces and are only to be operated by authorized Carl Zeiss service personnel.

Only the following devices can be mounted and dismantled by the user. Please refer to the manuals of the microscope stand and the HBO/HXP lamp for details and safety measures.

- Halogen lamp bulb
- HBO / HXP lamp including the housing at the reflective light path
- Cameras
- Tube on inverted stands



The user interface of the reflective light path can emit class 2 laser radiation. Always switch off the system before removing the parts connected to this user interface and do not leave this user interface uncovered during operation of the system.

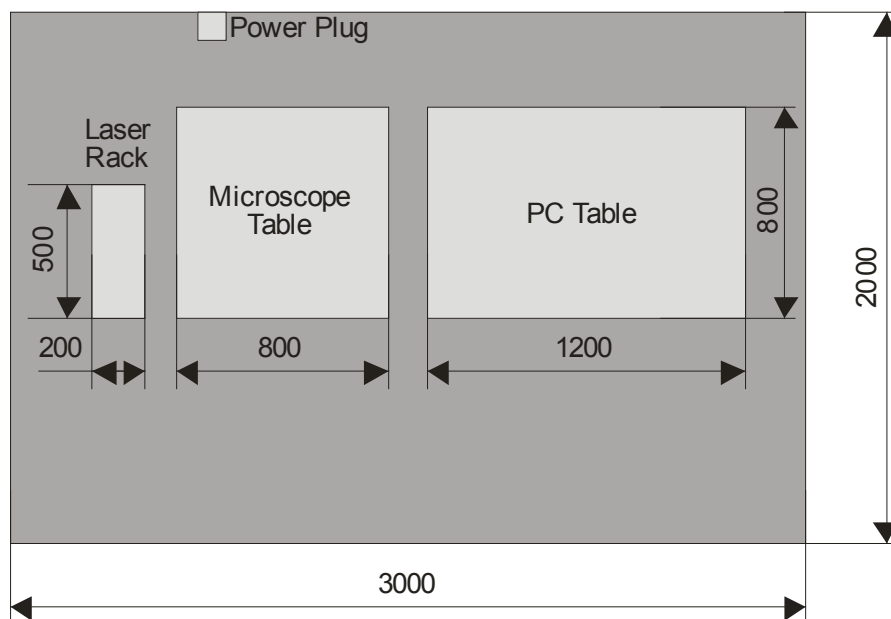
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# CHAPTER 2                    SETUP REQUIREMENTS

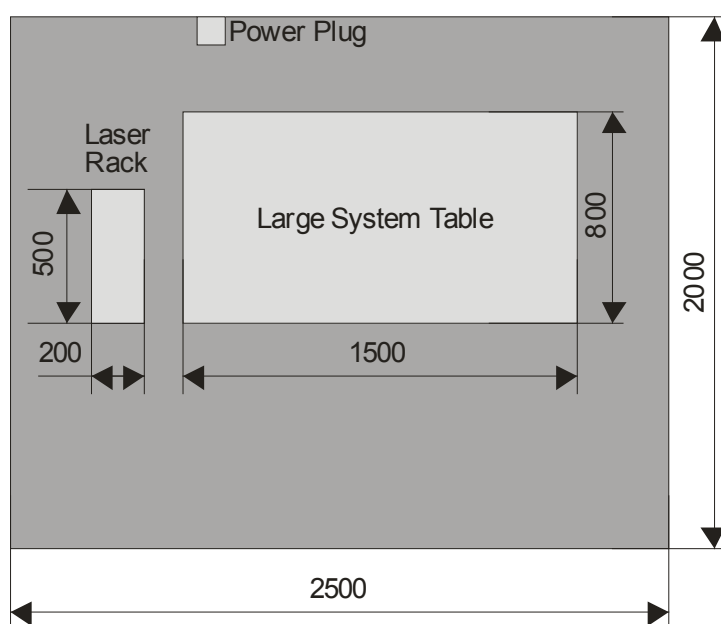
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## 1 SPACE REQUIREMENTS



**Fig. 1** LSM 700 with microscope table and PC table (dim. in mm)



**Fig. 2** LSM 700 with large system table (dim. in mm)

The laser modules and the power supply for the scan head are mounted inside the laser rack, which should be positioned beside the microscope stand on the floor.



## 2 POWER REQUIREMENTS

The LSM 700 system must be connected to the a.c. network via four line cables.

Any additional components (i.e. incubation systems) have to be connected separately to the a.c. network. See the individual manuals of those components for further instructions.

As shown in Fig. 3, some system components must be plugged into two (or three) multipoint connectors containing a single-phase plug. The two (or three) multipoint connectors must be connected to separate power sockets to an AC network. The used scanning stage and the used illumination unit have to be connected directly to the a.c. network.

Line voltage	220 V AC ... 240 V AC (+10 %)	100 V AC ... 125 V AC (+10 %)
Line frequency	50 ... 60 Hz	50 ... 60 Hz
LSM 700 incl. Lasers		
Max. current	1 phase at 4 A	1 phase at 7 A
Power consumption	800 VA	700 VA
Power plug	Country-specific connectors	NEMA 5/15 (only USA)
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)

The following components must be inserted in the first 3-point connector (power strip 1, see Fig. 3):

- flat screen monitor
- control computer
- microscope

The following component must be inserted in the second 3-point connector (power strip 2, see Fig. 3):

- electronics and laser module

The following components must be connected directly to the a.c. network using two line cables (see Fig. 3):

- X/Y scanning stage via controller
- HBO 100 / XBO 75 / HXP 120

If incubation components are used the following components must be inserted in the third 3-point connector (power strip 3, see Fig. 3):

- HBO 100 / XBO 75 / HXP 120
- Temp module
- Heating unit



Others than the components shown should not be connected to the multipoint connectors, due to the touch current limits of the system.

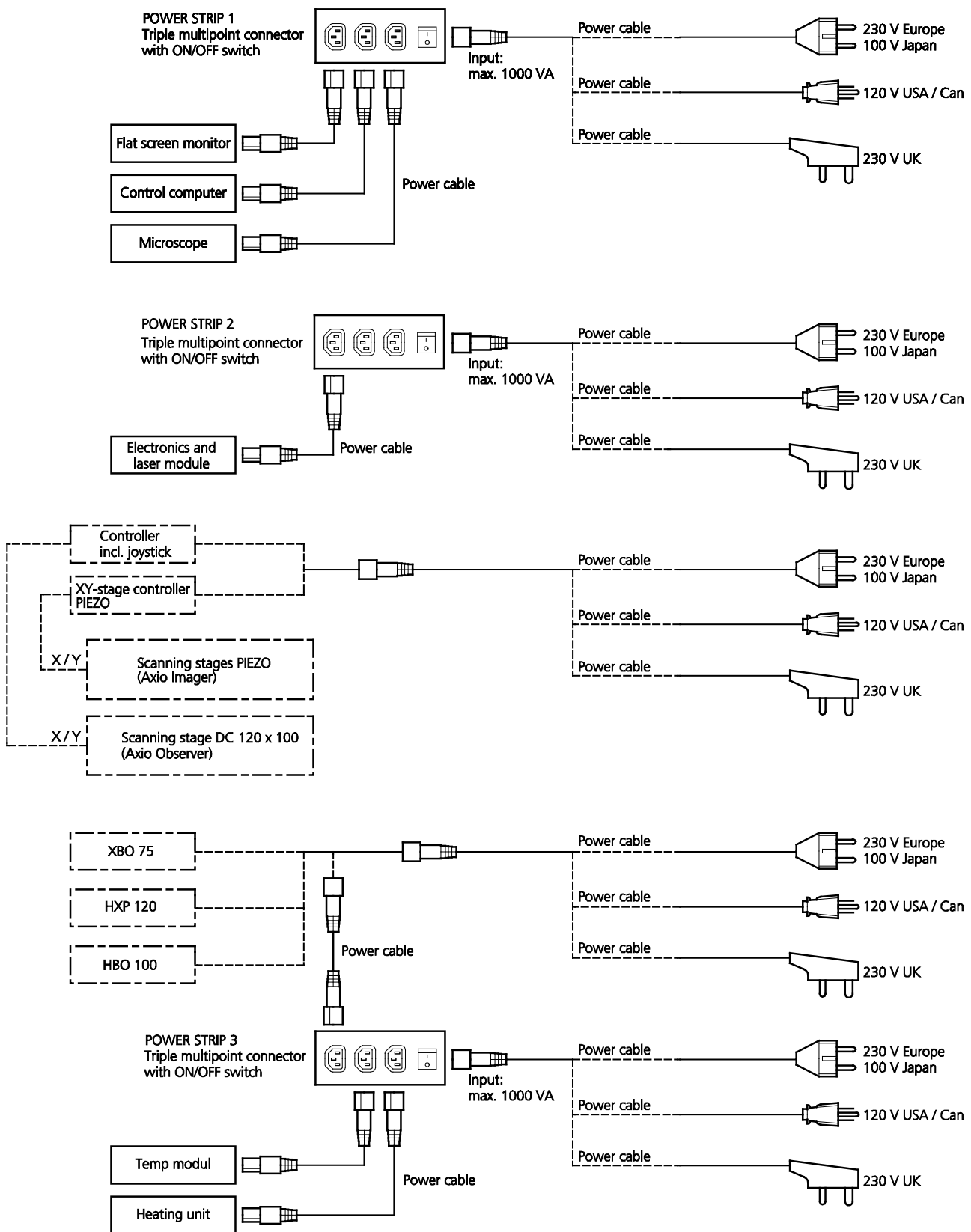


Fig. 3 Power connectors for LSM 700 system

### 3 PHYSICAL DIMENSIONS

	Length (cm)	Width (cm)	Height (cm)	Weight (kg)
<b>Small system table</b>	65	80	78	60
<b>Scanning Module</b>	27	20	23	8
<b>Active anti-vibration table</b>	130	100	75	137
<b>Microscope</b>	50	35	50	20
<b>Axio Imager.Z2 Vario</b>	71	96	70	100
<b>Laser Rack</b>	50	19	45	35
<b>Fiber optic cable, VIS(ible)</b>	250			
<b>Cables</b>	100 - 300			

### 4 ENVIRONMENTAL REQUIREMENTS

For operation the system has to be placed in a closed room.

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 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 55 °C
5. Temperature gradient	± 0.5 °C/h
6. Warm up time	1 h, for high-precision and/or long-term measurements ≥ 2 h
7. Relative humidity	< 65 % at 30 °C
8. Operation altitude	max. 2000 m
9. Loss of heat	800 W

## 5 VIBRATIONS

### 5.1 General Requirements

Vibrations under operation conditions (with system table)	Shipping shock (LSM box)
5 $\mu\text{m}$ pp at 5 Hz 10 $\mu\text{m}$ pp at 10 Hz 10 $\mu\text{m}$ pp at 20 Hz	3 g

### 5.2 Special Requirements for LSM 700 Systems with Axio Imager.Z2 Vario

LSM 700 systems including Axio Imager.Z2 Vario on system table LSM 710 passive or active air damped (order numbers 000000-1954-812 and 000000-1954-813) require this vibration environment on the floor during operation (measured peak to peak):

- < 10 nm at 3 - 10 Hz
- < 60 nm at 10 - 35 Hz
- < 100 nm at 35 - 70 Hz
- < 20 nm above 70 Hz

## 6 LASER SPECIFICATIONS

- Laser line 405 nm, 5 mW, laser class 3B
- Laser line 445 nm, 5 mW, laser class 3B
- Laser line 488 nm, 10 mW, laser class 3B
- Laser line 555 nm, 10 mW, laser class 3B
- Laser line 639 nm, 5 mW, laser class 3B

In the unlikely case of extreme fluctuations of the external power net, the laser rack will switch off or the laser power might vary. Note that this is not a defect and the laser will not be damaged.

## 7 MICROSCOPES

Inverted Axio Observer.Z1 SP left  
 Upright Axio Imager.Z2, Axio Imager.M2 or Axio Imager.Z2 Vario  
 Upright Axio Examiner  
 Upright Axio Scope mot

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

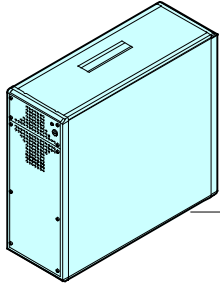
Z motor DC servomotor, opto-electronically coded  
 Least Z interval:  
     10 nm (Axio Imager.Z2, Axio Imager.Z2 Vario, Axio Observer.Z1 SP)  
     25 nm (Axio Imager.M2)  
     30 nm (Axio Examiner)  
     80 nm (Axio Scope mot)  
 measured according to VDI 3441

## 8 SCANNING MODULE

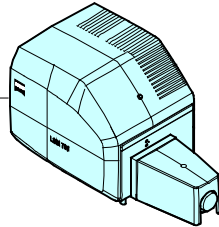
Scanners 2 individually driven galvanometric scanners  
 Scanning speed Up to 5 frames/sec (512 × 512 pixels)  
 Field resolution Max. 2048 × 2048 pixels (optional adjustable for each axis)  
 Field of view 10 × 10 mm<sup>2</sup> with a 1.25× objective  
 Zoom 0,5× ... 40×, continuous control  
 Channels Up to 3 channels simultaneously:  
     Up to 2 confocal reflection/fluorescence channels (PMT)  
     Optional 1 transmitted light channel (PMT)  
 Dynamic range 16-bit DAC for each detection channel  
 Pinholes 1 variable pinhole  
 Computer controlled automatic alignment

# 9 SYSTEM OVERVIEW

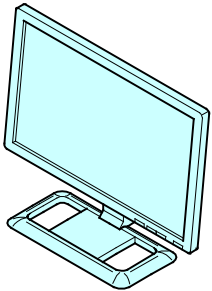
## 9.1 LSM 700 - BioMed



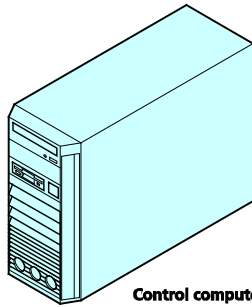
Electronics and laser module for LSM 700 (4x pigtailed laser 405-639 nm)



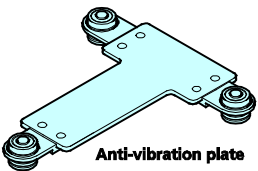
1-2-channel scanning module LSM 700



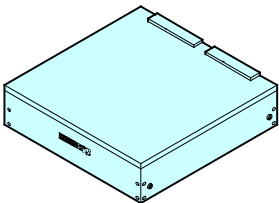
LCD TFT flat screen monitor 30"  
16:10 flat screen monitor 24"



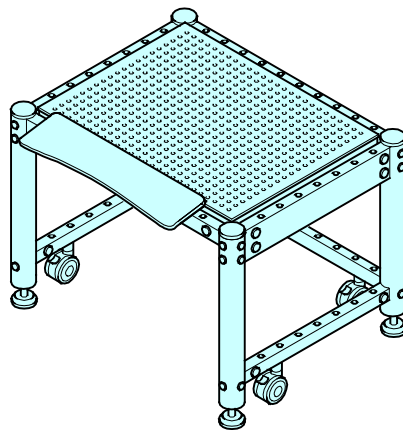
Control computer



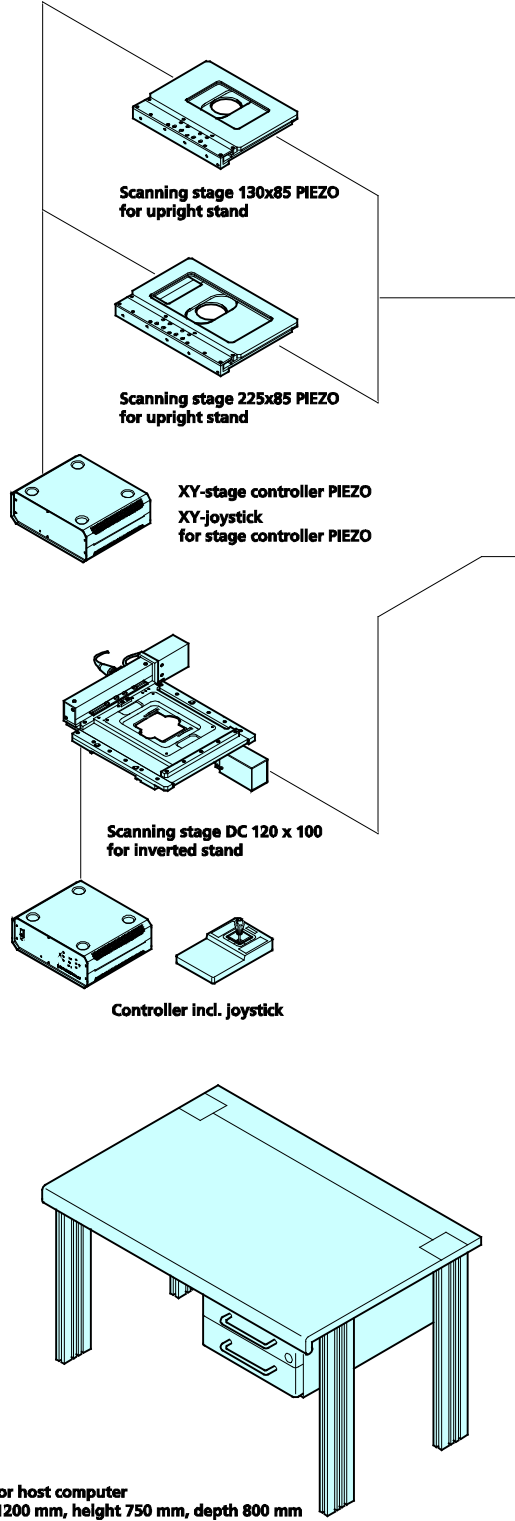
Anti-vibration plate

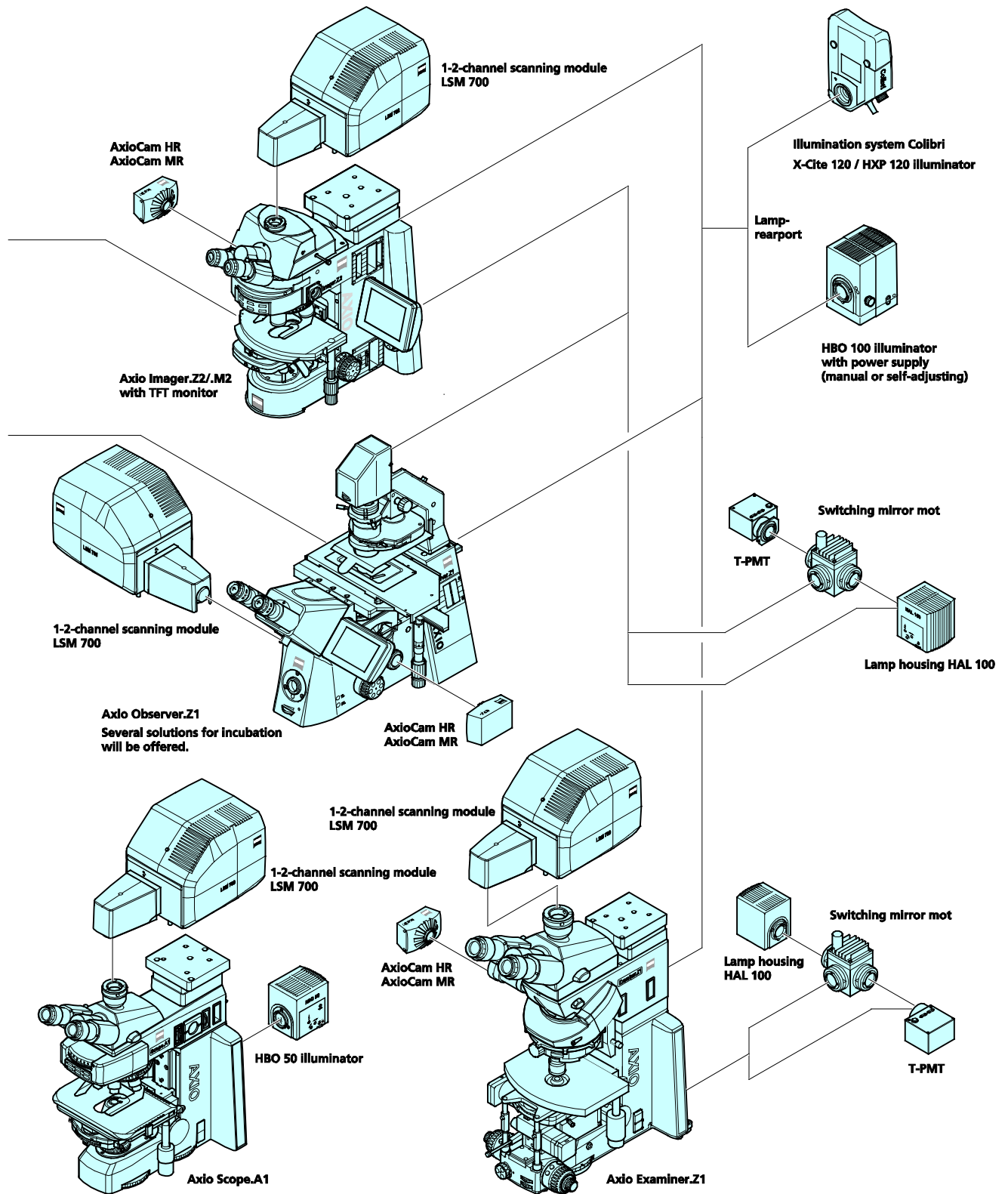


MOD-1M active antivibration system  
table surface: 40 cm x 40 cm  
MOD-1L active antivibration system  
table surface: 60 cm x 60 cm

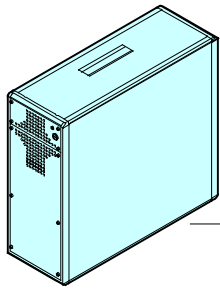


System table with breadboard  
Wide: 1000x750mm (1200x950 overall)  
Narrow: 750x1000mm (950x1200 overall)

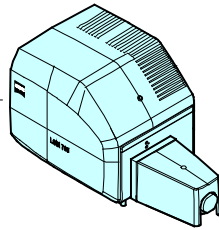




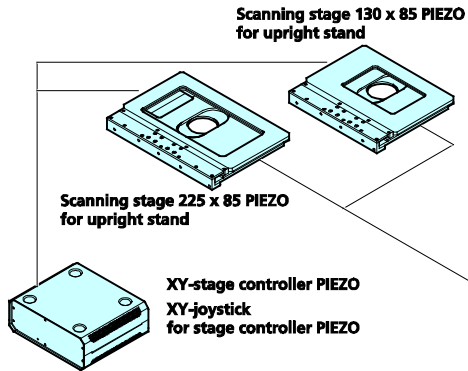
### 9.2 LSM 700 - Material



Electronics and laser module for LSM 700 (4x pigtailed laser 405-639 nm)



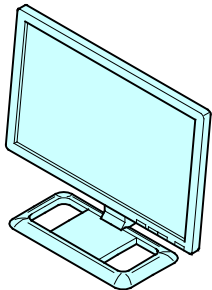
1-2-channel scanning module LSM 700



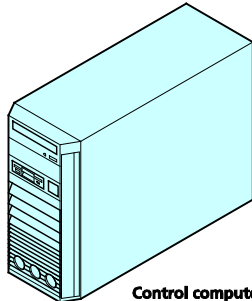
Scanning stage 130 x 85 PIEZO for upright stand

Scanning stage 225 x 85 PIEZO for upright stand

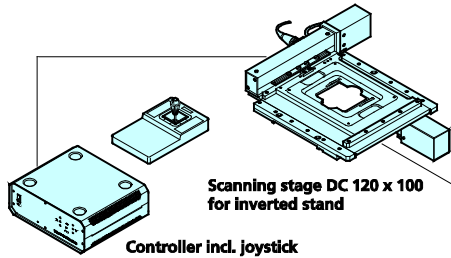
XY-stage controller PIEZO XY-joystick for stage controller PIEZO



LCD TFT flat screen monitor 30" 16:10 flat screen monitor 24"

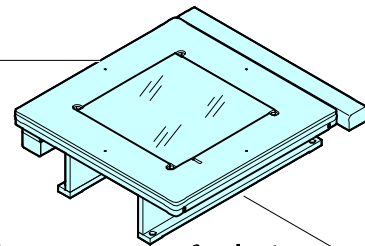


Control computer



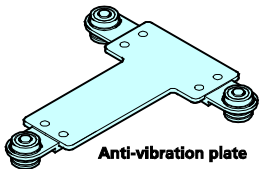
Scanning stage DC 120 x 100 for inverted stand

Controller incl. joystick

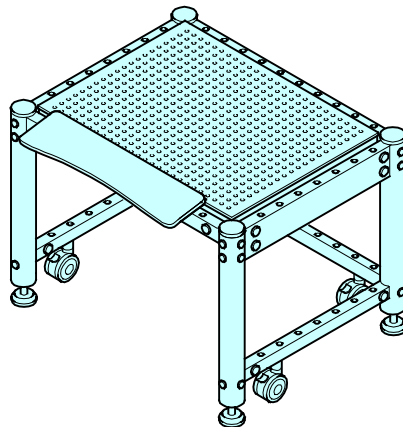


Scanning stages 200x200 STEP 300x300 STEP

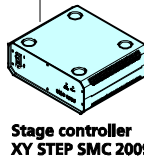
Joystick XY



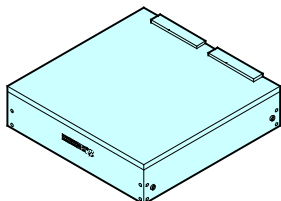
Anti-vibration plate



System table with breadboard Wide: 1000 x 750mm (1200 x 950 overall) Narrow: 750 x 1000mm (950 x 1200 overall)



Stage controller XY STEP SMC 2009



Micro 40 active antivibration system table surface: 45 cm x 40 cm  
Micro 60 active antivibration system table surface: 65 cm x 60 cm

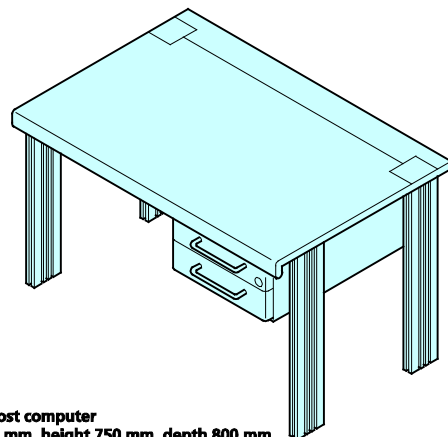
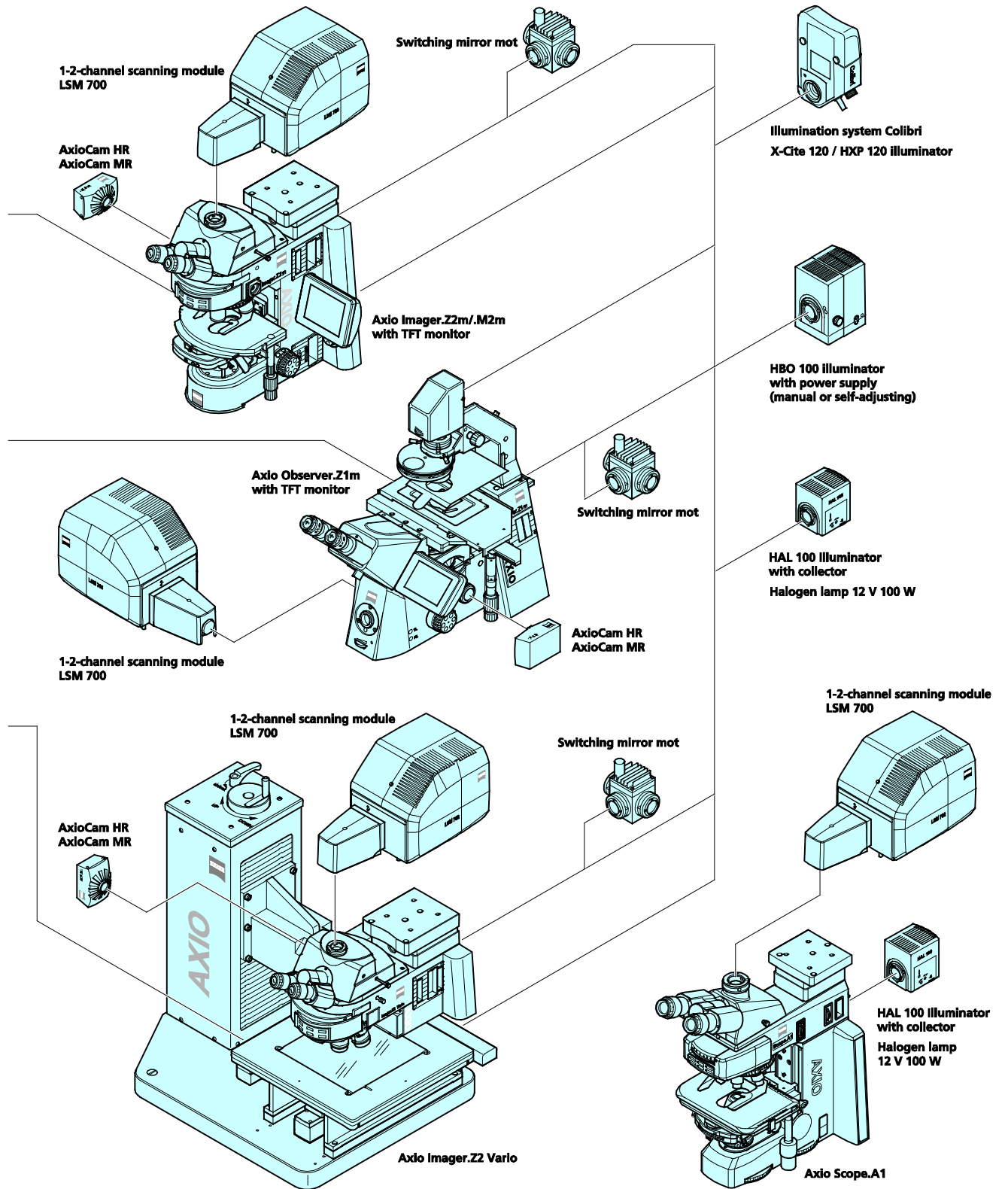


Table for host computer width 1200 mm, height 750 mm, depth 800 mm







## IMPORTANT NOTES FOR CHAPTER 3

<b>Changes to the operation system</b>	Do not update Windows, the version installed from the factory is already optimized to run with ZEN 2011. Automatic installation of patches might lead to conflicts with ZEN.
<b>Dye-spectra of deselected channels stay in the display</b>	The spectra will stay until a new dye is chosen for this channel.
<b>FCS model for PCH</b>	In the PCH model decimals can be entered into the F1, F2 and F3 input boxes the following way: for example instead of typing 0.361, type in 361, then position the cursor before the digit 3, then enter the dot.
<b>Tile scan with overlapping borders of single images</b>	When performing a tile scan with a defined overlap of > 0 the functions <b>Position</b> and <b>Stage</b> in the <b>2D View</b> tab / <b>Dimensions</b> are not available.
<b>LSM 710 and LSM 780 with 'In Tune' laser</b>	<p>While the 'In Tune' laser is busy due to a wavelength change the wavelength display is not marked in red as is indicated for NLO lasers (64-bit systems only).</p> <p>The automatic selection of the main dichroic (MBS) in basic mode of the Light Path tool window (Show all mode not activated) can give an unexpected result for the case of a line wise multitrack acquisition, where the 405nm laser, a line from the In Tune laser, and a line from one of the visible lasers are selected. The main dichroic (MBS) can block the light of the visible lasers. In this case switch to Show all mode and select the neutral density (80/20) main beam splitter. To achieve good laser suppression it is recommended to use the frame wise multi track mode.</p> <p>The automatic selection of the main dichroic (MBS) will consider the currently selected wavelength of the In Tune laser even if the laser is currently not in use. This might block the laser wavelengths of other visible light laser lines. The workaround is to tune the In Tune laser to the lowest possible wavelength.</p>
<b>Scan speed uni versus bidirectional (for all systems)</b>	For some scan speeds the acquisition with two tracks in the line wise multi-track mode is faster in the uni-directional mode than in the bi-directional mode.
<b>LSM 7 MP with two NLO lasers</b>	<p>Bleaching in simultaneous mode is slower than expected when the wavelength of the imaging laser in the Bleach tool window has a different wavelength than in the Channels tool window. ZEN will tune the laser to the selected wavelength even if the laser line is not in use.</p> <p>To achieve fast simultaneous bleaching:</p> <ul style="list-style-type: none"> <li>– Switch the laser line of the imaging laser in the bleach tool window on</li> <li>– Enter the same wavelength as for in the Channels tool window</li> <li>– Switch the laser line off again.</li> </ul>

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**NLO/MP systems with conventional and BiG NDD modules**

When cascading NDD modules, the performance is the better the closer the module is located to the microscope coupling port. This is also true when eventually using a LSM BiG as an NDD module behind a conventional PMT based NDD module. For best performance, use the LSM BiG as the exclusive NDD detector directly at the microscope NDD port.

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## 1 Purpose

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

When starting up and operating the microscope system, mind the operating instruction manuals for the Axio Imager.Z2, Axio Imager.M2 and Axio Observer.Z1, Axio Scope.A1 microscopes:

- M70-2-0020 Axio Imager.Z2 / M2, Operating Manual
- B 40-0111 Axio Observer.Z1, Operating Manual
- M60-2-0007 Axio Scope.A1, Operating Manual
- M60-2-0003 Axio Examiner, 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 64-bit Microsoft® WINDOWS 7 operating system.

Main components:

- Computers: Esprimo P7936, Z800
- Operating systems: Microsoft Windows 7, 64-bit

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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 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 Compact Flash Drive – this drive has to be connected first manually inside the computer to get access to the backup.

### 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):

- Carl Zeiss Image files: \*.CZI
- LSM Image files: \*.lsm
- Exported images: \*.\* (\*.Tiff, \*.LSM-Tiff, \*.BMP, ...)
- Palette files: ZEN\Palettes\\*.lut
- Pinhole setting files: ZEN\bin\\*.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 after pinhole adjustment: ZEN\bin\\*.txt
- Scanner files: ZEN\bin\\*.bin
- Microscope stand database: ZEN\database\\*.mdb

## 1.4 Software Operation

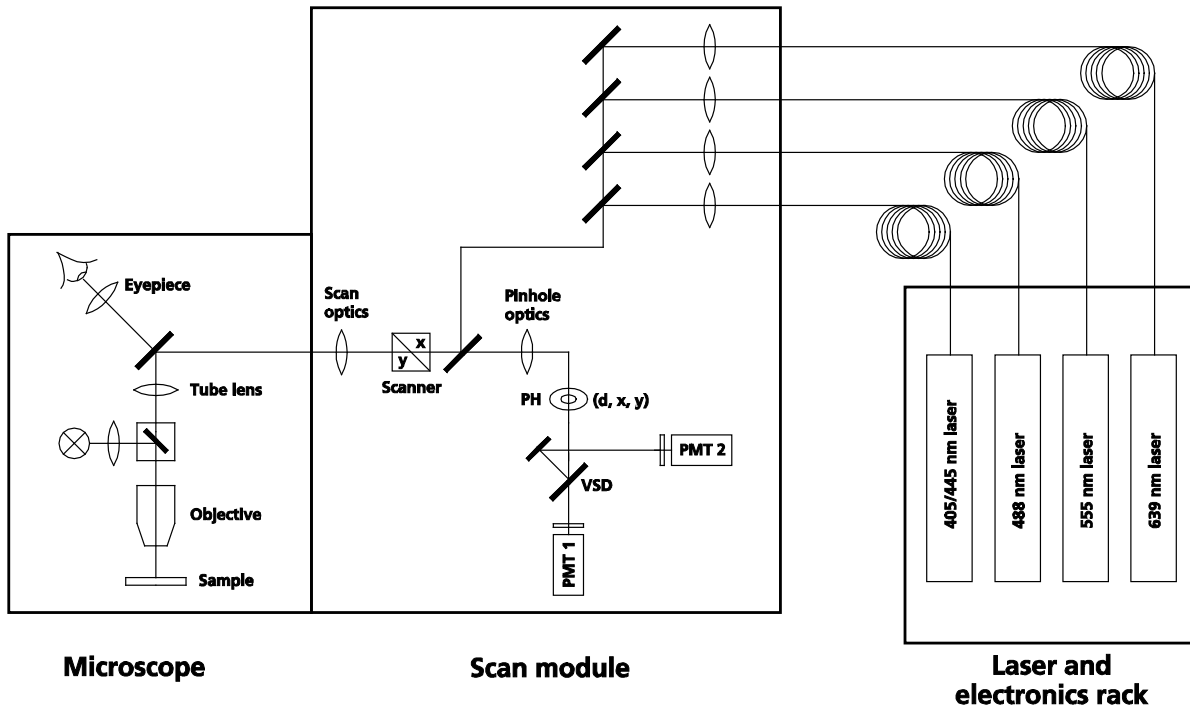
The ZEN software can be operated using the mouse, the PC keyboard, or both.

The operation of the mouse and the keyboard is identical to that of the Microsoft® 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 (Schematic)



- PH Variable pinhole
- PMT Photomultiplier
- VSD Variable secondary dichroic mirror

**Fig. 1 Optical path**

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

Laser light is focused onto the specimen through an objective in a diffraction-limited mode. Light emitted at the focal plane and at planes below and above it is directed via an XY scanner onto a main dichroic beam splitter, which separates the emissions from the excitation light.

The fluorescence Signals are separated from each other by a) the Main Dichroic Beam Splitter and b) a variable Secondary Dichroic Beam Splitter (VSD) and directed via optional emission filters to the individual photomultipliers (PMT1 and PMT2).

---

## 2.2 Performance and Features

### Optical and Mechanical Aspects

The highly integrated system design allows 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 or SP) or upright (Axio Examiner, Axio Imager.M2, Axio Imager.Z2, Axio Scope.A1) microscope.

Laser Module: the user can select from up to four lasers with wavelengths of 639, 555, 488, and 405/445 nm. Coupling of the laser light is through polarization-preserving single-mode optical fibers. One beam combiner provides optimum adaptation of the respective laser wavelength to the objective used and, thus, optimum correction for Z aberrations.

The two simultaneous image acquisition channels, usable for reflection or fluorescence, and an additional transmitted-light channel are ideal for the investigation of multiple fluorescence specimens. In the two channels, the diameters of the pinhole and its XY position can be optimized, and the desired emission filter placed into the beam path, by servo-motor control. In the simultaneous registration of multiple fluorescences, identical optical sections can be obtained in each confocal channel. This is of importance, e.g., with the FISH method (fluorescence in-situ hybridization) used for genome analysis in cytogenetic studies.

The use of the variable secondary dichroic mirror enables the user to flexibly adjust the detection bands for both of the channels.

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

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

The high-NA C-APOCHROMAT objectives especially developed for the LSM technique reach the physical limit in resolving power, and can be used throughout the 400...800 nm spectral range with the same high quality, producing brilliant images.

A two-mirror scanner system, controlled by real time electronics and a PC, offers several advantages. The large deflection angle of the scanning mirrors allows a wide area to be scanned. With a 1.25x objective, the object area scanned is 10 x 10 mm<sup>2</sup>.

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

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

Selection of the specimen detail of interest for zooming is fast and convenient, and the zoomed image is automatically centered. No centering the specimen with the microscope stage is necessary.

Using a bi-directional scanning ability will double the scanning rate to 5 frames/sec (at 512 x 512 pixels); if two different excitation 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 crosstalk between detection channels.

## 2.3 Microscope Equipment of the System

The LSM 700 system is equipped either with the inverted Axio Observer.Z1 SP microscope or with the upright Axio Imager.Z2, Axio Imager.M2, Axio Examiner or Axio Scope.A1 microscopes.

Referring to the delivered operating manual of the microscope stand, only differences to this manual will be explained.

### (1) Stand

- The motorized objective nosepiece 5x H DIC is firmly fixed to the stand. Operation will be done entirely LSM software controlled. The "Restriction of revolver height to protect the objectives when changing the objectives motorized" 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 6 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 clipped.
- 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, this can be entered in the **LSM Options** tool on the **Maintain** tab of ZEN.
- The stand features a power supply for the internal motors and stand electronics. The power supply can be switched separately. To power up the stand use the On/Off Button of the Microscope. External power supply units will be used for the mercury vapor short arc 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 LSM-mode the analyzer must **not** be located in the beam path; the analyzer rod must be pulled out.

### (2) Specimen stages and fine focus drives

- Mechanical stage  
The stage must be mounted with the coaxial drive on the right side of the stand.
- Scanning stage
- Piezo objective focus drive
- Z-Piezo stage insert for scanning stage

**(3) Transmitted-light illumination**

- The illuminator support contains a **metal plate**, ensuring that **no laser light escapes the specimen area on the microscope**. This is true also when the support is moved to the back. There are **no** additional electric shutters interrupting the laser light when the support is moved to the back.
- The illuminator support is equipped with a rotatable polarizer. The Axio Observer.Z1 description contains the adjustment for DIC mode during conventional observation.
- For scanning 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 is not allowed be located in the beam path because the laser light already is polarized.
- On the illuminator support as an option there is a software controlled switching mirror fully motorized. Alternatively the light is directed to the T-light detector or enables conventional transmitted-light observation.
- The focusing screw for conventional transmitted-light is located in a support in front of the halogen lamp housing.
- Further information to halogen lamp and condensers can be found in the Axio Observer.Z1 operating manual.

**(4) Reflected light fluorescence**

All Axio Observer.Z1 fluorescence accessories except the reflector slider can be used.

Further information can be found in the Axio Observer.Z1 operation manual.

**(5) Imaging optics**

Optovar sliders are not usable in LSM mode.

The analyzer for 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**

Microscope cameras, as described in the Axio Observer.Z1 operation manual, can be used.

**(7) Camera adaptation**

The left side camera port and the tubes can be used as described in the Axio Observer.Z1 and LSM 700 operation manual.

## **2.4 Computer Hardware and Software**

The LSM 700 is controlled through a standard high-end PC. The link with the electronic control system is done via Gigabit Ethernet interface. The PC comes with the WINDOWS 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; this way, complete application programs can be loaded and executed by pushbutton control.

Conversion of the light signals into a digital image is effected by means of 3 16 - bit A/D converters, each of which can generate up to 65536 brightness levels.

The software provides a wide range of image processing functions, including all standard 2D/3D (stereo, projection) functions as well as sophisticated 3D reconstruction capabilities (surface and transparency 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

##### 3.1.1 Switching on the Laser Module

- Use the main power switch (Fig. 3/2) on the back of the laser module to turn on the power of the module.
- Turn the key (Fig. 2/1) on the front side of the laser module to switch on the lasers. A yellow LED will indicate that the lasers are operational.
- Since the laser module contains only solid state lasers the system needs no warm up time.
- On the back of the system there is a plastic screw (Fig. 3/1). This is a laser interlock which can be used for coupling the laser module e.g. to doors. If this screw is **unscrewed** the lasers will **not** turn on!
- If you interrupt your work with the LSM 700 for a break, the "laser life extender" function of the ZEN software is switching off the lasers automatically in order to extend the lifetime of the lasers. This happens if the system is not used for more than 15 min. The lasers are switched on again automatically when the system is used.

##### 3.1.2 Switching on the Microscope and the Computer

- Switch on the power supply of the microscope and the microscope itself.
- Switch on peripheral components and controllers like a motorized XY stage or the power supply of an Excite Lamp.
- Switch on the computer

##### 3.1.3 Switching on the HBO 100

- If the HBO 100 is required, switch it on via the toggle switch of the HBO 100 power supply.

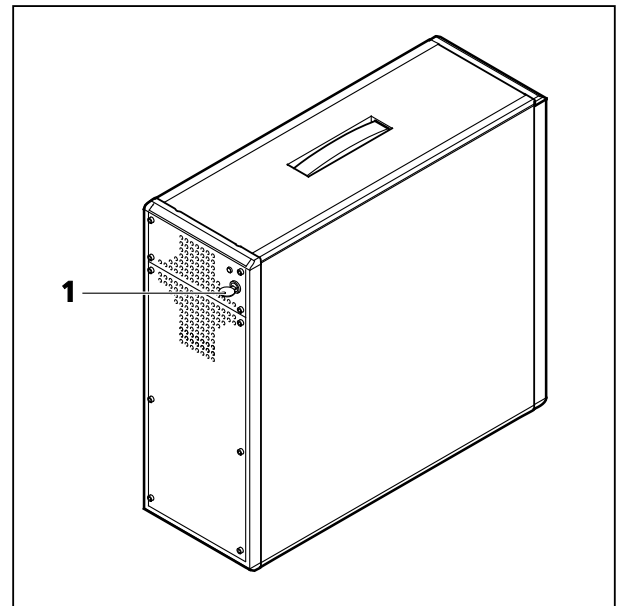


Fig. 2 Laser module (front)

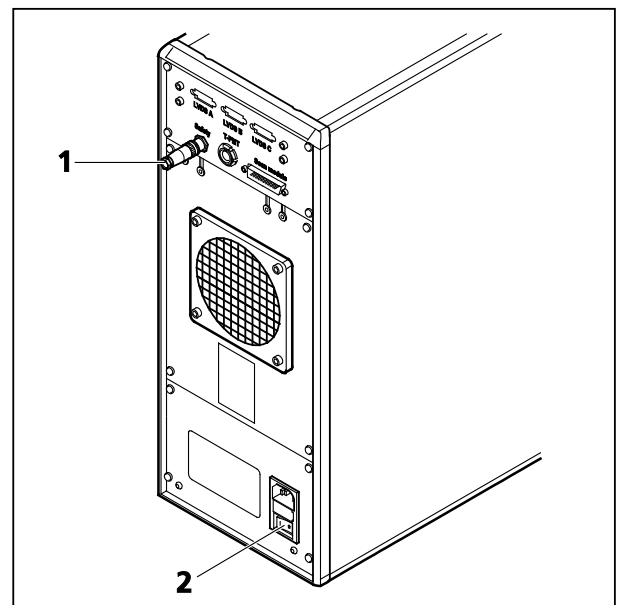


Fig. 3 Laser module (back)

### 3.1.4 Starting ZEN



#### 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 5: TOOLS, ADDITIONAL SOFTWARE.



#### ZEN icon

Start ZEN software for operating the LSM laser scanning microscope.

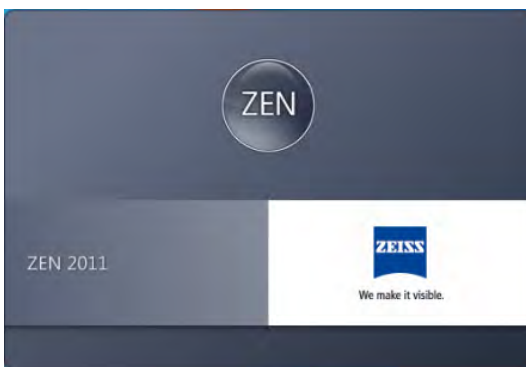


Fig. 4 Starting the ZEN software – The Loading "ZEN" window

- To start the software double click the **ZEN** icon on your desktop!
  - During loading the program the **ZEN Access** window will appear (Fig. 4). It displays the ZEN symbol in the upper part, the designation of the current ZEN software version (lower left) and the Zeiss icon (lower right).
  - Once the program is loaded the **Login ZEN** dialog will appear (Fig. 5) in front of the ZEN interface. The number after ZEN designates the current version.
- By pressing the **? (question mark)** symbol, the context sensitive **Help menu** will appear (Fig. 6). Close the menu with the Windows commands if you do not want to search the help database by pressing the **x (cross)** symbol.
- Pressing the **x (cross)** symbol, the **Login ZEN** dialog will be closed and the ZEN software will not be started.



Fig. 5 Starting the ZEN software – The Login "ZEN" dialog

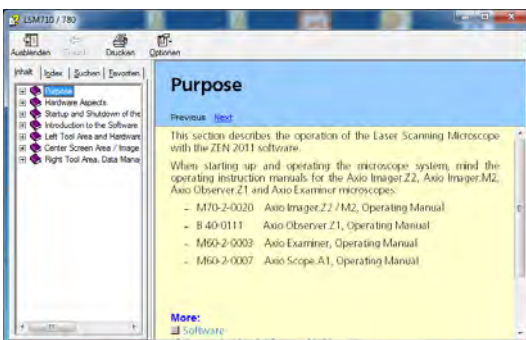


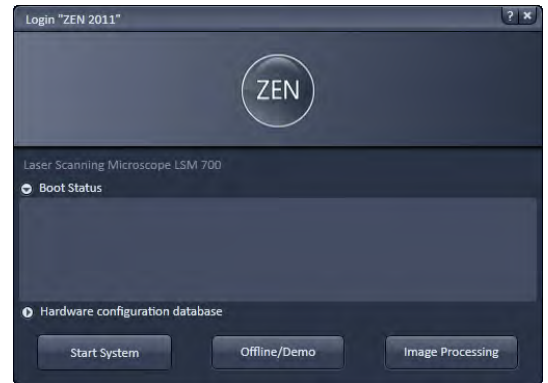
Fig. 6 The Help menu dialog

The **Login ZEN** dialog allows you to boot the Software in different modes. If the **Boot Status** is closed, the options are **Start System** and **Image Processing**.

A third option, **Offline/Demo**, becomes available (Fig. 7), once the **Boot Status** field is expanded by pressing the **expansion** symbol (triangle).

You can collapse the field by pressing the expansion symbol again. Select:

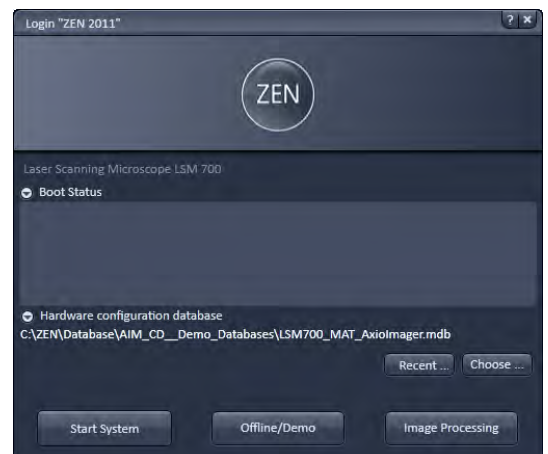
- **Start System** for system control, data / image acquisition / recording and analysis. The whole microscope system will be initialized. The progress of the boot process will be displayed by the blue time tab. During booting the status of the system will be displayed in the **Boot Status** window and the single components will be listed which have been or are currently being checked during the boot process (Fig. 7).
- **Offline/Demo** for data analysis and simulation. **Offline/Demo** 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" This option should be chosen when working at an offline station and if you want to test functionalities of the system. The progress of the boot process will be displayed by the blue time tab. The single components will be listed, which are checked during the boot process. Note that the software will not control the hardware in this mode.
- **Image Processing** for data analysis only. This mode ignores the hardware and activates only data handling and image processing functionality to analyze stored images. Note that only those software tools will be displayed that are needed for processing and analysis of the data. No hardware control tools will be shown.
- **Cancel** to abort the booting process and to leave the dialog. Note that the **Cancel** button will appear in the place of the **Image processing** button only if **Start System** or **Offline/Demo** was selected (Fig. 8).
- You can leave the software by choosing the **File** menu from the main tool area and selecting **Exit**.
- You can select a database before booting, if you wish to change the current one. To change the database open the **Hardware configuration database** expansion field (Fig. 9). The expansion field displays the currently loaded database and its location on the hard disc.



**Fig. 7 Starting the ZEN software – The Login "ZEN" dialog with Boot Status field expanded**



**Fig. 8 Starting the ZEN software – The Login "ZEN" dialog with Boot Status field expanded and during the process of booting**



**Fig. 9 Hardware configuration database - The Login "ZEN" dialog allows you to boot the Software in different modes. If the Boot Status is closed, the options are Start System and Image Processing.**

## 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.

### 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.
- About 20 seconds after WINDOWS is shut down your computer turns off.

### Turning off the System

Turn off all the power switches (Microscope, lamps, laser module, xy-stages. Alternatively use the switch on the multipoint connector in order to power all components down at once. Make that the computer is already turned off if you do so.

- This puts your LSM 700 microscope system, including the computer, off power.

### Turning off the HBO 100

- Switch off the HBO 100 via the toggle switch of the HBO 100 power supply.

## 4 Introduction to the Software Application Layout

### 4.1 Overview on the Screen Layout

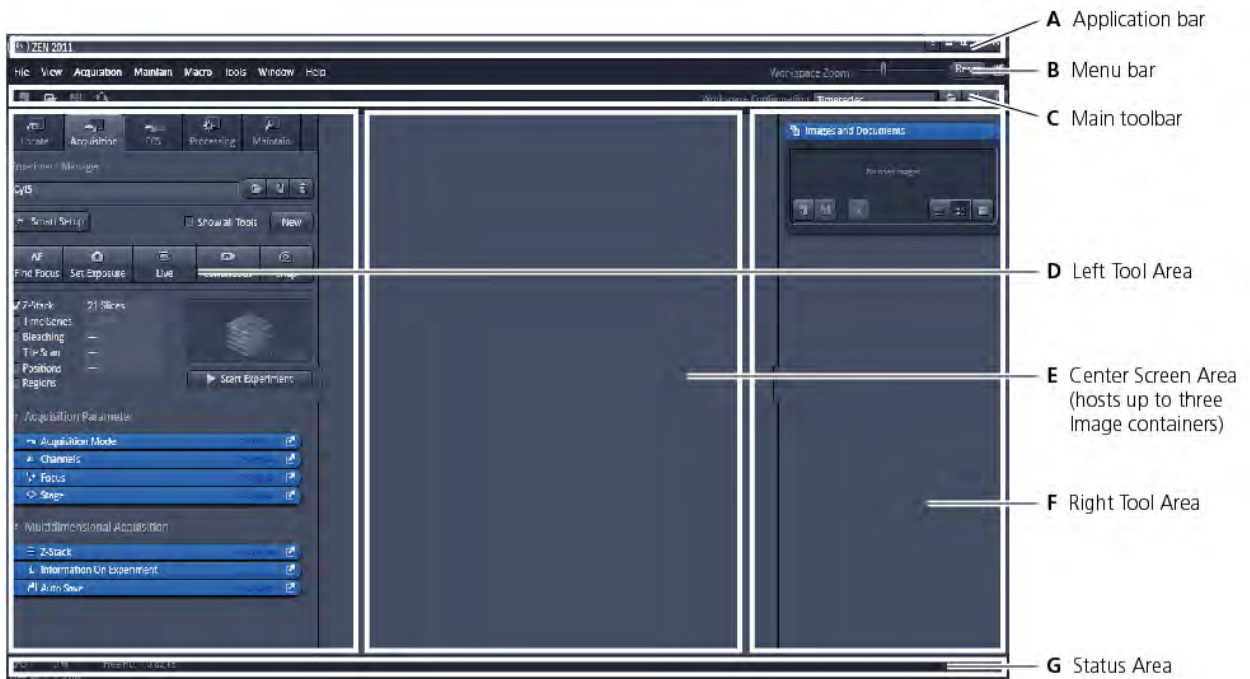


Fig. 10 ZEN Main Application window after Startup with empty image container

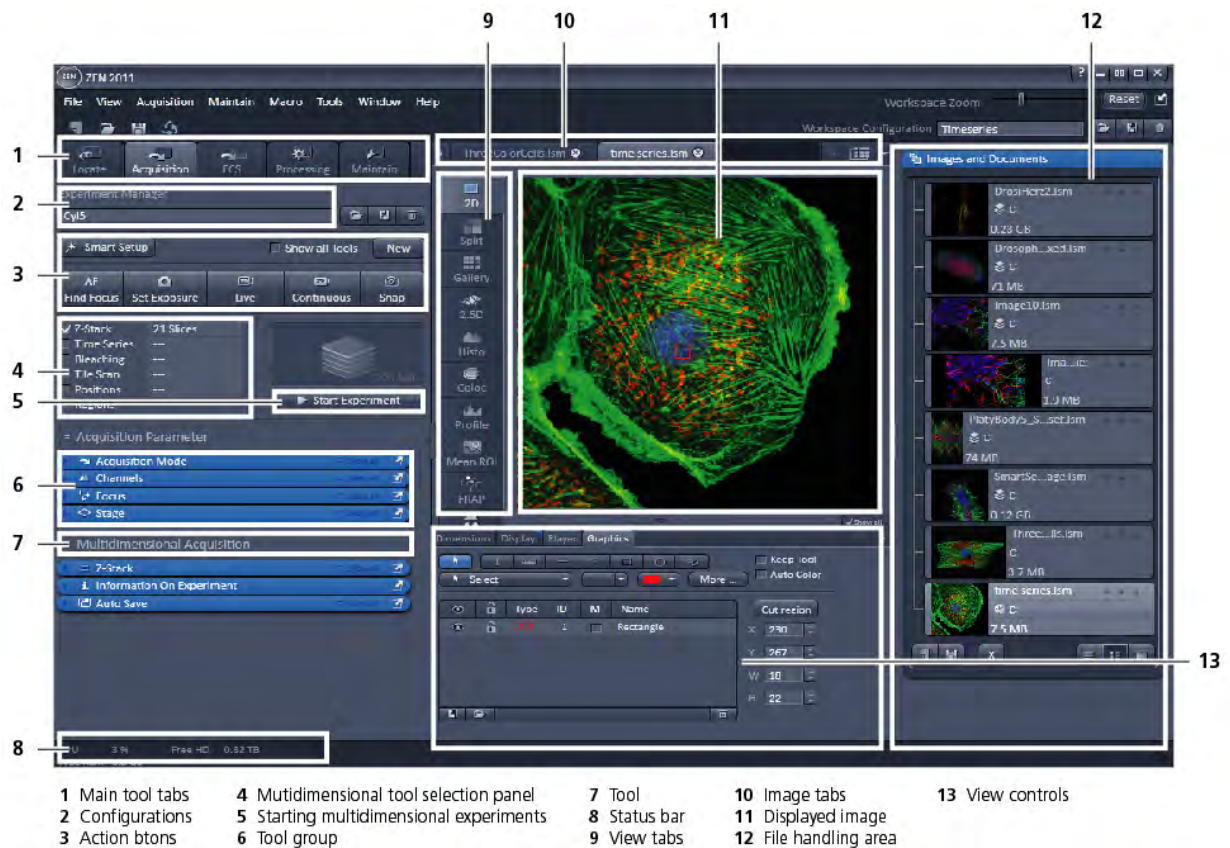


Fig. 11 ZEN Main Application window after Startup with several images loaded

## 4.2 Introduction to ZEN

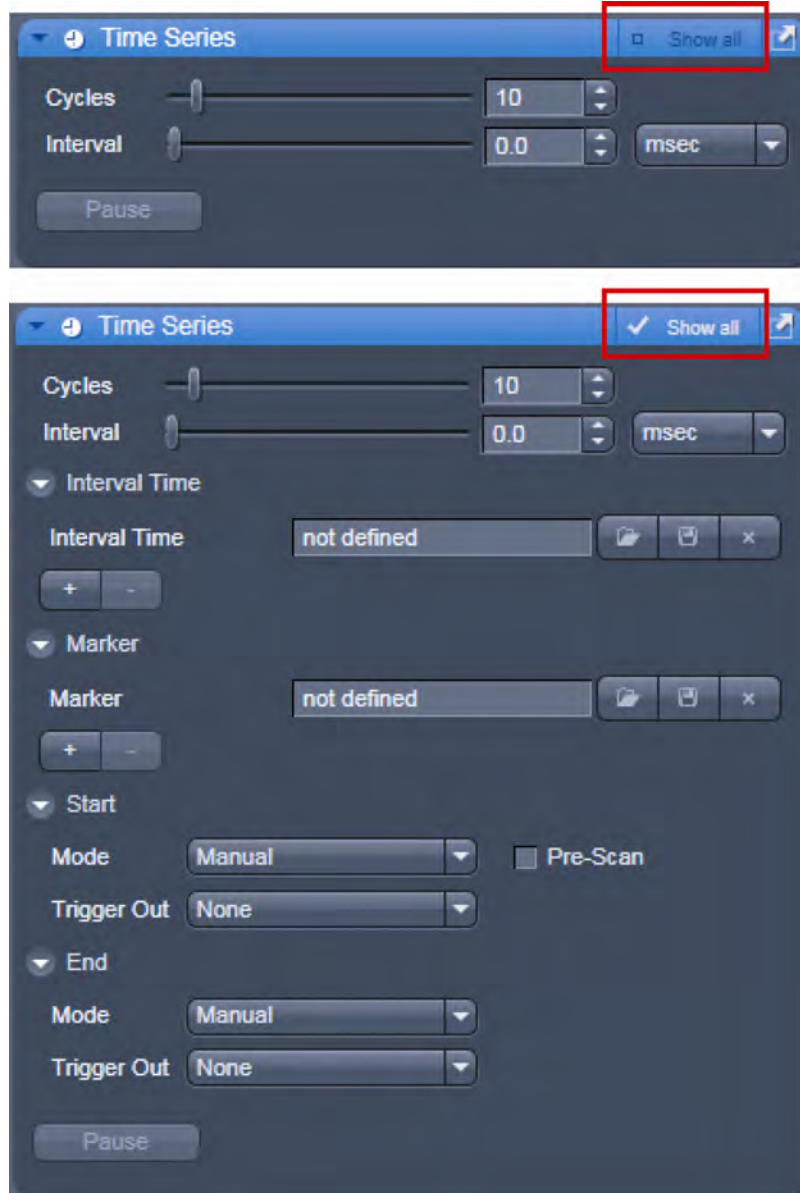
**ZEN - Efficient Navigation** - is the new software for the LSM Family from Carl Zeiss. With the launch of this software in 2007 Carl Zeiss sets new standards in application-friendly software for Laser Scanning Microscopy.

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. 10/D) the user finds the tools for image acquisition, image processing and system maintenance, easily accessible via 4 **Main** tabs (Fig. 11/1). All functions needed to control the microscope can be found on the **Locate** tab, to acquire images use the **Acquisition** tools (Fig. 11/3 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. 10 and Fig. 11).

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. 12** 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. 12).

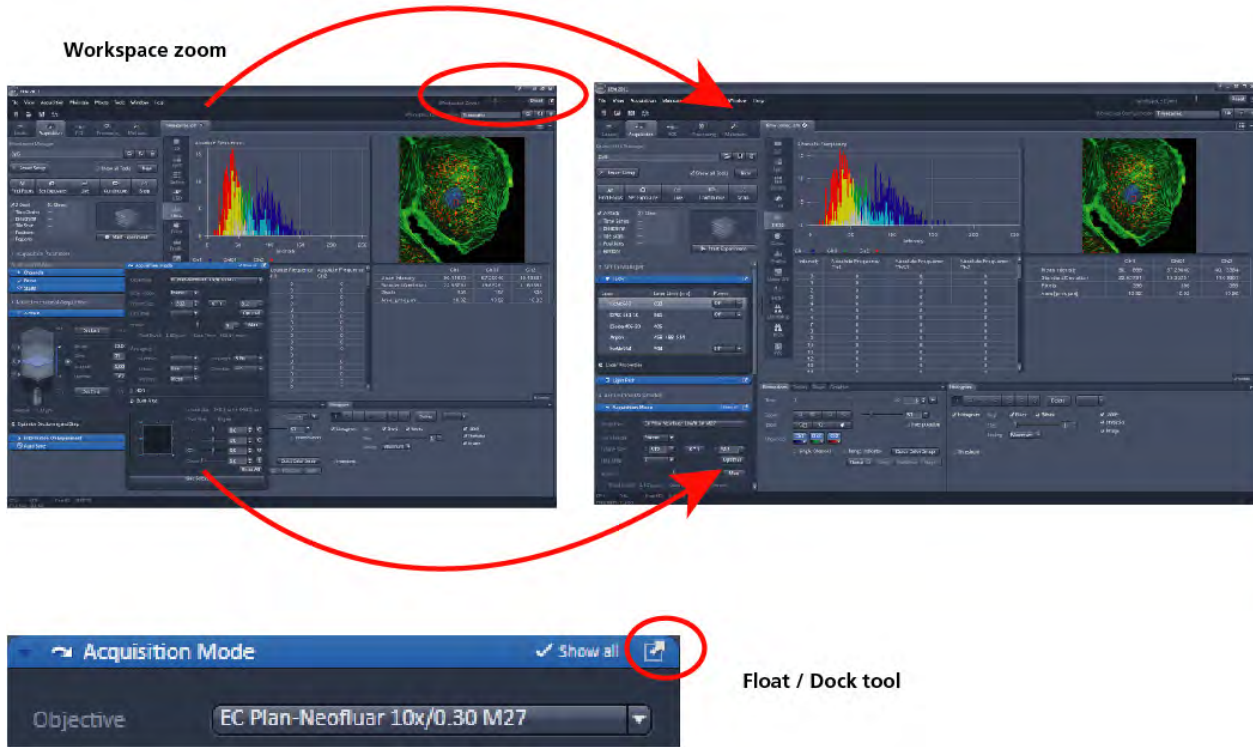


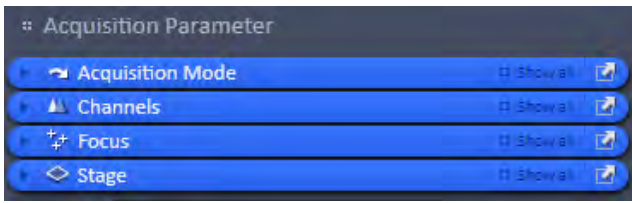
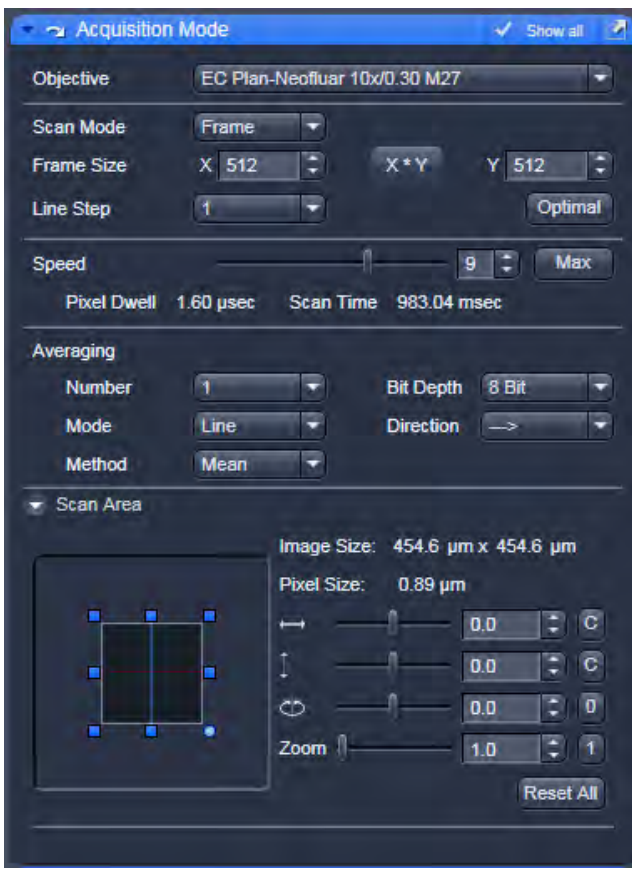
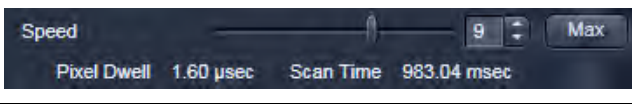
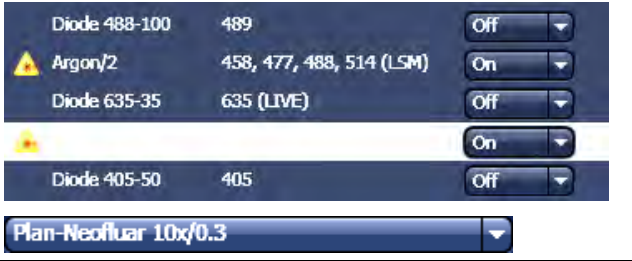
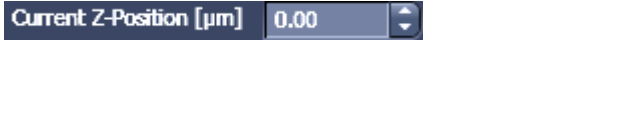
Fig. 13 ZEN window Layout configuration



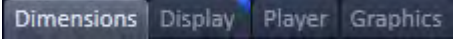


More features of ZEN include:

- 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. "Acquisition Parameter") 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. 13).
- 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. 13).
- 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
	<p>Tool group</p>
	<p>Tool (closed) or tool window (opened)</p>
	<p>Panel (e.g.: <b>Speed</b> panel)</p> <ul style="list-style-type: none"> <li>- Field with a subset of tools of a tool window</li> </ul>
	<p>List box or selection box</p> <ul style="list-style-type: none"> <li>- Selection of one of the displayed options via a mouse click. The selected option is highlighted.</li> <li>- Open the box by clicking on the arrow button.</li> </ul>
	<p>Input box with arrows ("spin box")</p> <ul style="list-style-type: none"> <li>- Input of text or numeric values via the keyboard or using the arrows.</li> </ul>

	<p>Slider with input ("spin") box and arrows</p> <ul style="list-style-type: none"> <li>– 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 <b>Shift</b> or <b>Ctrl</b> key while clicking on the arrow button to change the numeric values in coarse or fine steps.</li> </ul>
	<p>Button</p> <ul style="list-style-type: none"> <li>– Selection / performance of a function via mouse click.</li> </ul>
	<p>Tab</p> <ul style="list-style-type: none"> <li>– Selection of functions of a tab via mouse click (e.g.: <b>Dimensions</b> or <b>Display</b> tab).</li> </ul>
	<p><b>Load – Save – Delete</b></p> <ul style="list-style-type: none"> <li>– Buttons to load, save or delete a configuration, overlay set, track setting etc.</li> </ul>
	<p><b>New – Save – Close</b></p> <ul style="list-style-type: none"> <li>– Buttons to open a new acquisition document, save an image document or close it.</li> </ul>

## 4.4 Application Bar

The application bar includes the following control elements of the ZEN software application window:

-  starts the online help
-  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**.

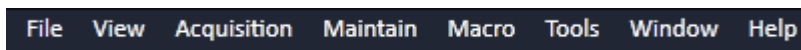


Fig. 14 Menu bar, left side

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. 15 Menu bar, right side

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

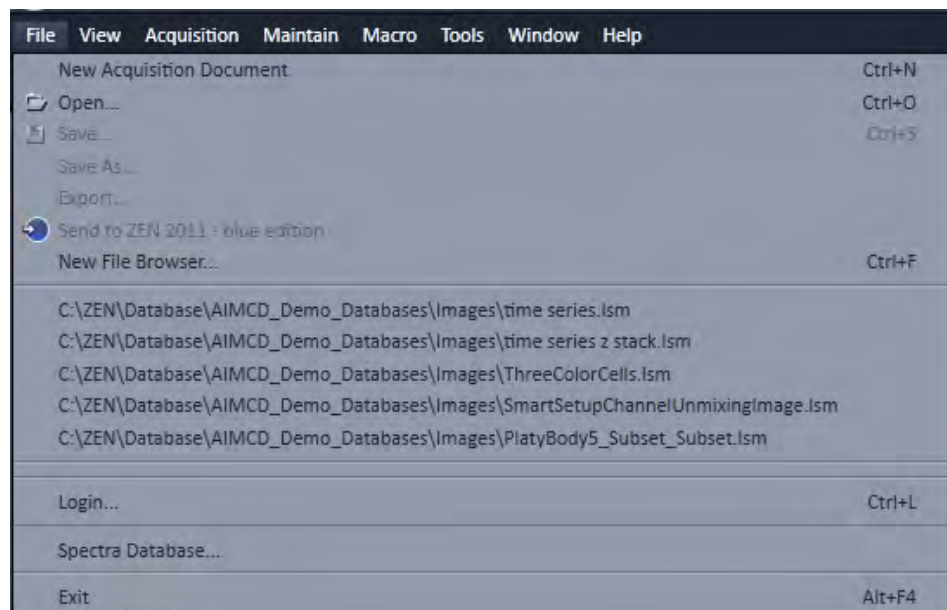
**Menu overview**

<b>File</b>	<b>View</b>	<b>Acquisition</b>	<b>Maintain</b>	<b>Macro</b>	<b>Tools</b>	<b>Window</b>
<p><b>New Acquisition Document</b> Opens a new and empty image document.</p>	<p><b>Text View</b> Displays the image tabs with text only.</p>	<p><b>New Acquisition Document</b> Opens a new and empty image document.</p>	<p><b>Set Spline</b> Opens a dialog for setting the parameters.</p>	<p><b>Macro</b> Opens the Macro Control window for creating, loading or handling macros</p>	<p><b>VSTA IDE</b> Future control of non-VBA macros, currently inactive.</p>	<p><b>Close</b> Closes the active window.</p>
<p><b>Open</b> Opens an existing document in the active image container.</p>	<p><b>Small Thumbnail View</b> Displays the image tabs with small thumbnails.</p>	<p><b>Set Exposure</b> Starts the Find procedure. This balances Gain settings.</p>	<p><b>Camera</b> Opens the Camera Color Adjustment tool.</p>	<p><b>Visual Macro Editor</b> Opens the Visual Macro Editor</p>	<p><b>VSTA Macros</b> Future control of non-VBA macros, currently inactive.</p>	<p><b>Close All</b> Closes all windows, a separate save dialog will be displayed if images have not been saved.</p>
<p><b>Save</b> Saves the active document.</p>	<p><b>Large Thumbnail View</b> Displays the image tabs with large thumbnails.</p>	<p><b>Find Focus</b> Starts the auto focus. A Line / Z Scan is performed and the focus position is determined upon the contrast values.</p>	<p><b>Hardware Administrator</b> For service only.</p>			
<p><b>Save As</b> Saves the selected image with entering new formats.</p>	<p><b>1 Container</b> Sets ZEN to work only with one image container.</p>		<p><b>Test grid</b> For service only.</p>			<p><b>Help</b> <b>Help</b> Opens the ZEN help.</p>
<p><b>Export</b> Exports the currently active document using various image formats.</p>	<p><b>2 Containers</b> Sets ZEN to work with two image containers.</p>	<p><b>Live</b> Starts the fast scanning procedure, scans with lower resolution and higher speed.</p>				<p><b>About</b> Opens a panel with information about the ZEN release.</p>
<p><b>Open Containing Folder</b> Opens the folder, where the image file is saved.</p>	<p><b>3 Containers</b> Sets ZEN to work with three image containers.</p>					
<p><b>Send to ZEN 2011 - Blue edition</b> Sends the currently active document to ZEN blue edition.</p>	<p><b>Automatically layout container</b> The size of the containers is automatically adjusted depending on the screen space available.</p>	<p><b>Countinuous</b> Scans an image continuously with the currently active scanning parameters.</p>				
<p><b>New File browser</b> Opens a document containing a ZEN File Browser.</p>	<p><b>Shared view Controls</b> Shares the view controls below the images between containers.</p>	<p><b>Snap</b> Scans one single image.</p>				
<p><b>Login</b> Opens the Login ZEN dialog to change the software operation mode.</p>	<p><b>Separate view Controls</b> Each container is displayed with a separate view control panel.</p>	<p><b>Starts Experiment</b> Starts a multidimensional experiment (Z, T, ...).</p>				
<p><b>Spectra Database</b> Opens the Spectra Database.</p>	<p><b>"Show all" (global)</b> Sets all windows, tools, and control panels to "Show all", all controls are always visible in all tools.</p>	<p><b>Stop</b> Stops the scanning immediately.</p>				
<p><b>Exit</b> Closes the ZEN software.</p>						

-  For detailed information about the image acquisition functions see section **Acquisition Tab**.
-  The functionalities of the **Macro** menu are described in CHAPTER 4: MACROS AND VISUAL BASIC of the printed manual.

#### 4.5.1 File

- In the menu bar, click on **File**.
  - This displays the menu shown in Fig. 16.

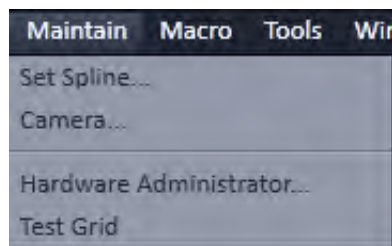


**Fig. 16 File menu**

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 dialog used to handle previously recorded reference spectra.

-  See also section **Right Tool Area, Data Management and Storage** for image file handling.



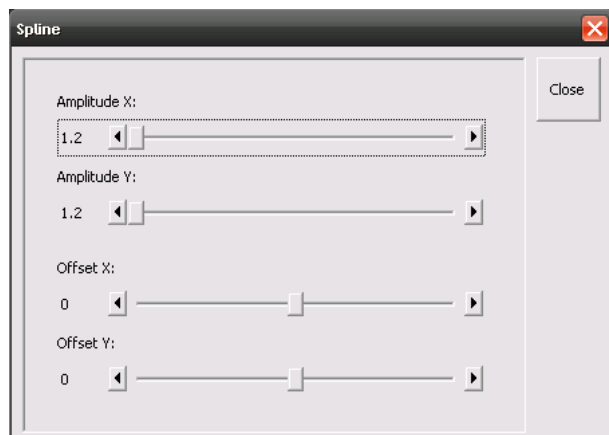
**Fig. 17** Maintain list

## 4.5.2 Maintain

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. In the menu bar, click on **Maintain**. This opens the Maintain list (Fig. 17).

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.

 See also section **Maintain Tab** for further information.



**Fig. 18** Spline window

### 4.5.2.1 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. 18).
- 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.

#### 4.5.2.2 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. 19).

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.

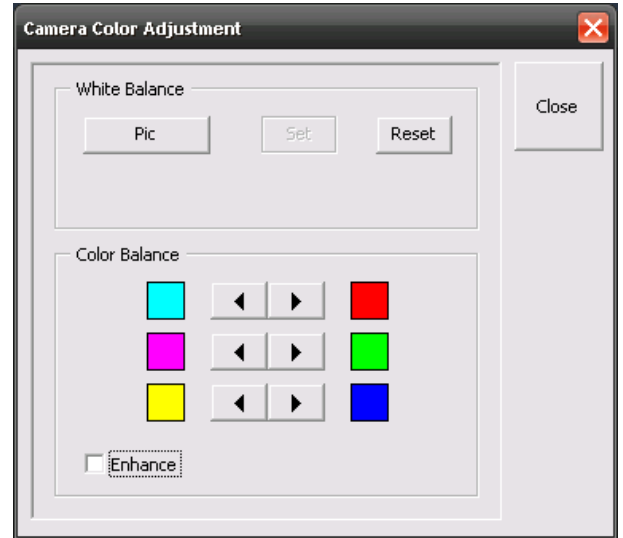


Fig. 19 Camera Color Adjustment window

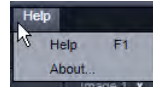
#### 4.5.2.3 Hardware Administrator

The **Hardware Administrator** function is for servicing purposes and may only be used by authorized service personnel. Its access is therefore password-protected.

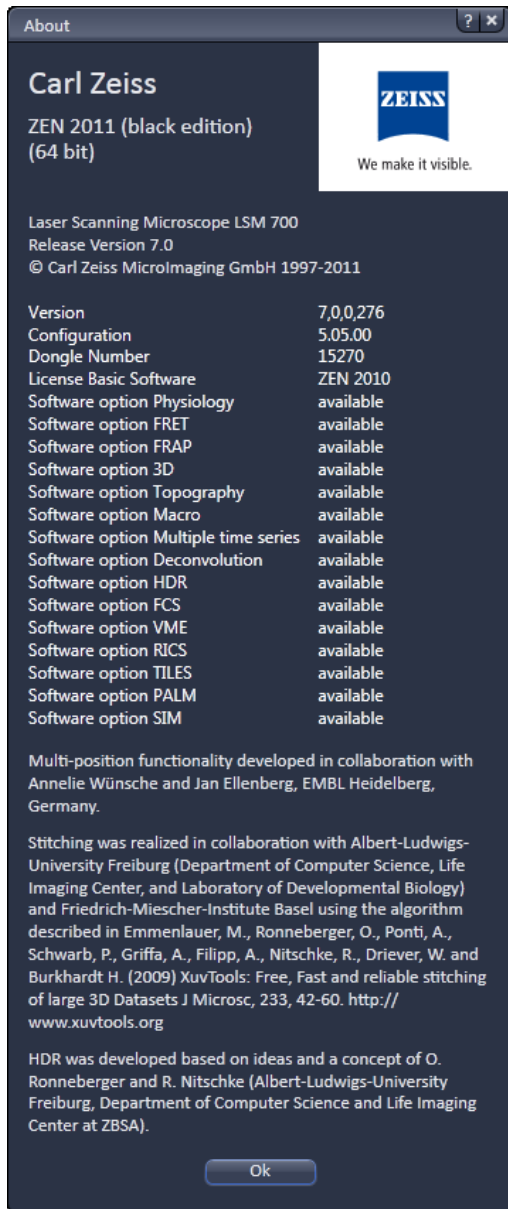
#### 4.5.2.4 Test Grid

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

### 4.5.3 Help – About



This panel can be accessed via the Help item in the menu bar clicking about opens the following dialog:

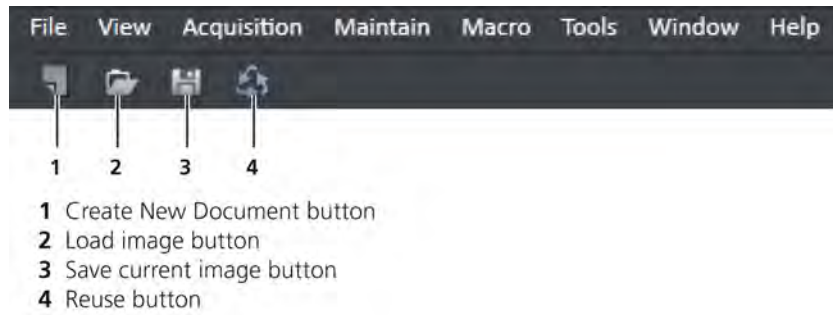


**Fig. 20 About window**

The panel hosts important information about the software version number, the license number and the available software modules.



## 4.6 Main Toolbar



- 1 Create New Document button
- 2 Load image button
- 3 Save current image button
- 4 Reuse button

**Fig. 21 Main toolbar, left side**



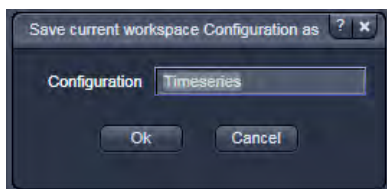
- 1 Workspace Configuration display box
- 2 Load Workspace Configuration button
- 3 Save Workspace Configuration button
- 4 Delete Workspace Configuration button

**Fig. 22 Main toolbar, right side**

### Workspace Configuration

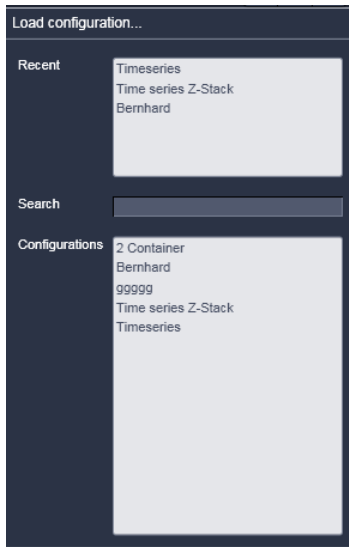
This function allows loading, saving or deleting a workspace configuration. These workspace configurations are saved settings that allow to restore a pre-defined layout of the whole ZEN application window, including status, size and position of tools and windows, workspace zoom, number of tool columns.

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



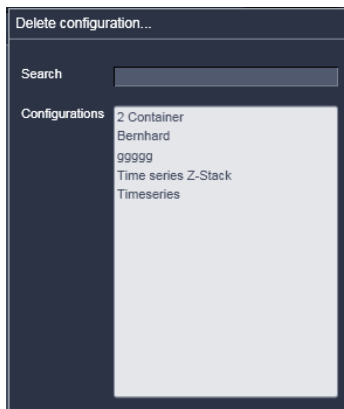
The name of the workspace configuration can be entered. Clicking **OK** saves the configuration.

- To load a workspace configuration, click on the  button. The following list box will appear:



Selecting one 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. 23** 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. 24** Progress bar

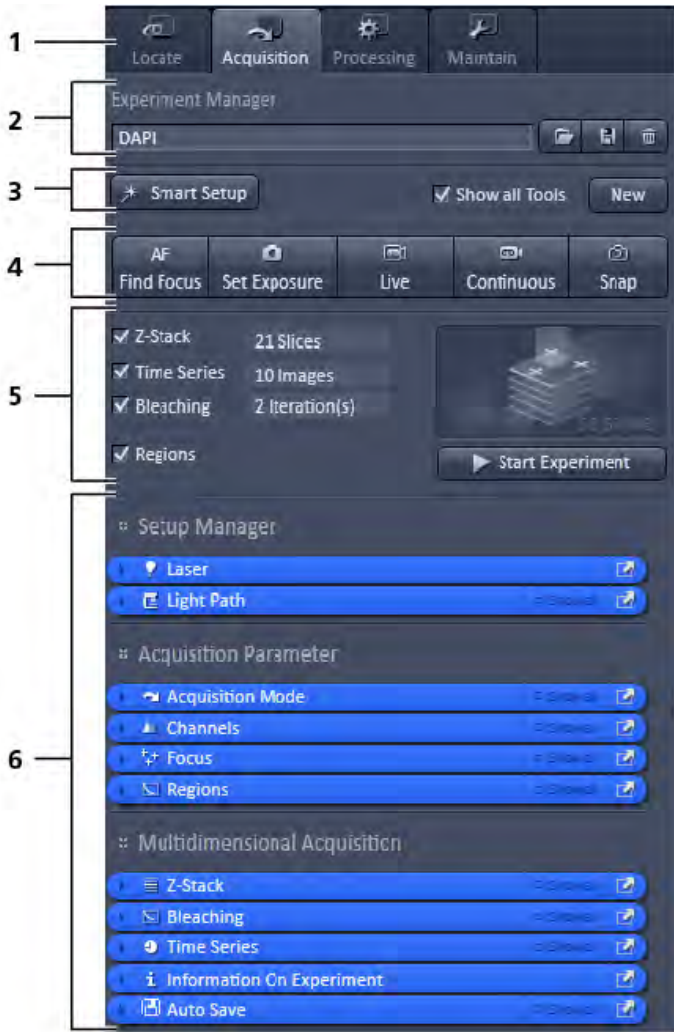
### System information

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.



- 1 Main tool tabs
- 2 Experiment Manager
- 3 Smart Setup
- 4 Action buttons
- 5 Selection panel for multidimensional acquisition
- 6 Tool groups

Fig. 25 Left Tool Area



Fig. 26 Expand slider with arrow button pointing left to collapse and right to expand

## 4.8 Left Tool Area

### Main tool tabs

Switches between the **Locate**, **Acquisition**, **Processing** and **Maintain** main tools to operate the included tools.

### Action buttons


Action buttons are only available for the **Acquisition Main** tool to control the image acquisition process.

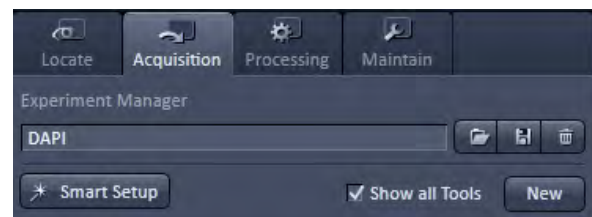
### Tool groups

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

### Setup Manager

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

 The tools **Laser** and **Light Path** are not displayed by default in the software. They can be shown if the checkbox **Show all tools** is checked.



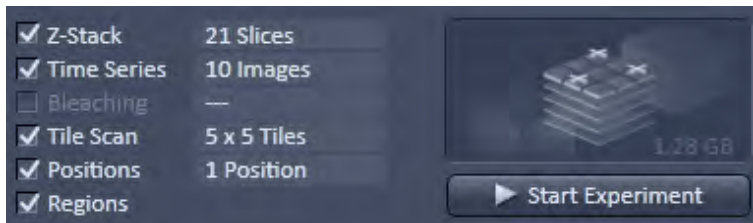
### Acquisition Parameter

Tools continuously needed for all acquisition procedures.

### Multidimensional Acquisition:

Tools for all multidimensional imaging

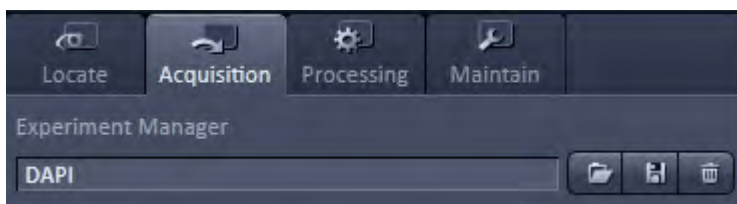
The tools of this tool group will only appear if selected in the selection panel below the start buttons. Once selected by the check box, the tool will appear in the multidimensional acquisition tool group. The tool is then active and will be used after hitting the **Start Experiment** button. The experiment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



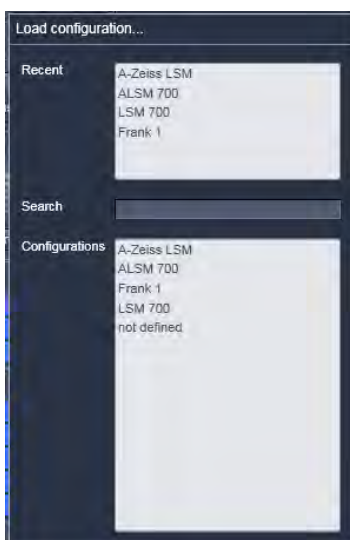
### Tools / tools window

Used for setting the microscope and software functions.

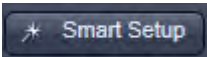
### Experiment Manager



This function allows selecting an Experiment Manager 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 to undock and move one or several tool windows from its home position in the Left Tool Area and further drag 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 the screen gets too crowded and one 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

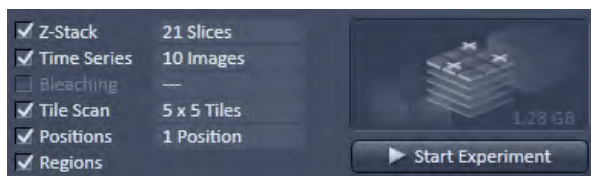
Clicking on the  button shows or hides additional functions of the tool.


 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.

**Find Focus** starts the Auto focus. A Line – Z Scan is performed and the focus position is determined upon contrast values.

**Set Exposure** starts the **Find** procedure. This balances Gain settings.

**Scan Overview Image ...** opens the **Scan Overview Image** dialog to set the parameters for an overview scan. Clicking the **Scan** button starts the procedure.

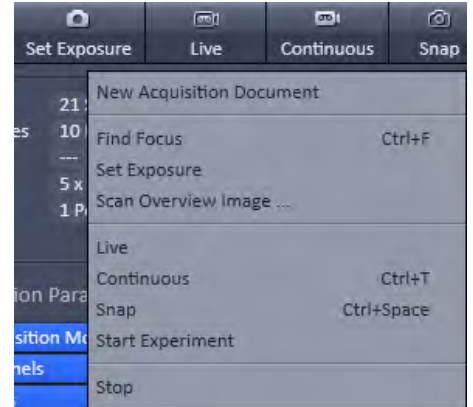
**Live** starts the fast scanning procedure – scans with lower resolution and higher speed.

**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

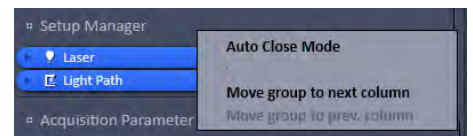


**Fig. 27** Context menu of multidimensional acquisition area

**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.

**Move tool group to next column** shifts an entire tool group to the adjacent column.



**Fig. 28** Context menu of the tool groups area

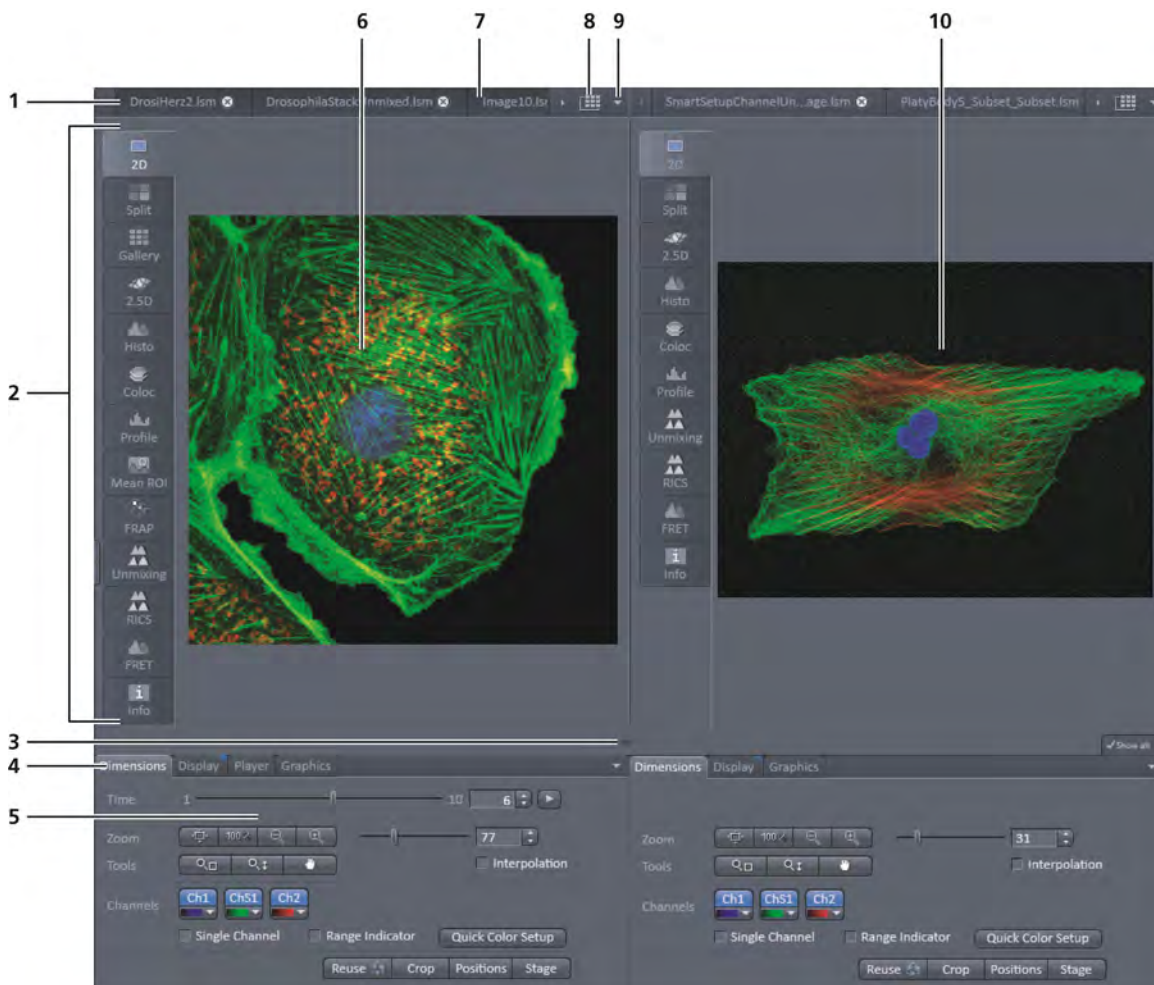
## 4.9 Center Screen Area

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



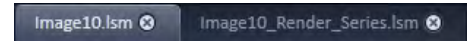
- 1 Image tabs with scroll arrow buttons left and right of container 1
- 2 Image view tabs of container 1
- 3 Arrow down or arrow up button to hide or show the image control area
- 4 Image view controls
- 5 Image control area
- 6 Image container 1 (with opened image window)
- 7 Background area of container 1
- 8 Expose mode button (overview)
- 9 Arrow down button for fast image selection
- 10 Image container 2

**Fig. 29** Center screen area



### Image tabs

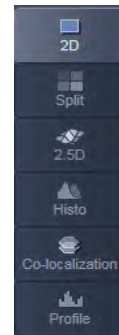
For each opened image one image tab is shown in the header of the actual container. Three modes can be selected using the context menu of the **Center Screen Area** (see also section **Context Menu of the Center Screen Area**).



**Fig. 30** Image tabs

### Image view tabs

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



**Fig. 31** 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.

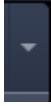


**Fig. 32** Image control area

### 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.

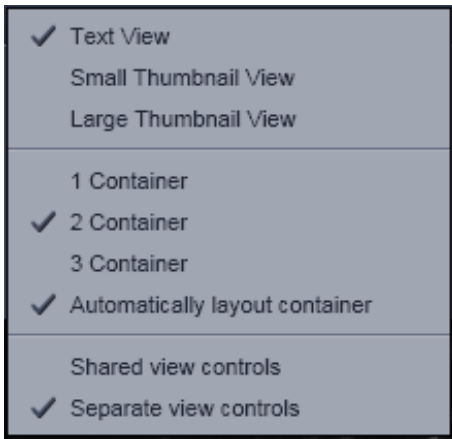


Fig. 33 Context menu

### 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, 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

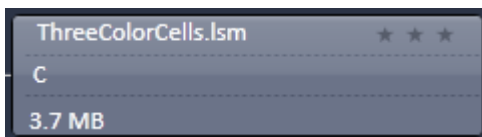
Opens an empty image document in the active container.

### Close button

Closes the selected image. The **Close image** dialog appears to close the image with or without saving or to cancel the closing.

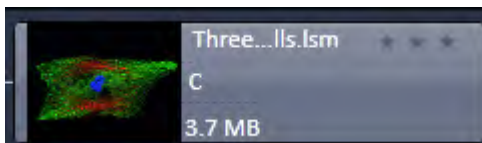
### Textual View button

Shows images only with the textual characters.



### Thumbnail View button

Shows images with the textual characters and an image thumbnail preview.

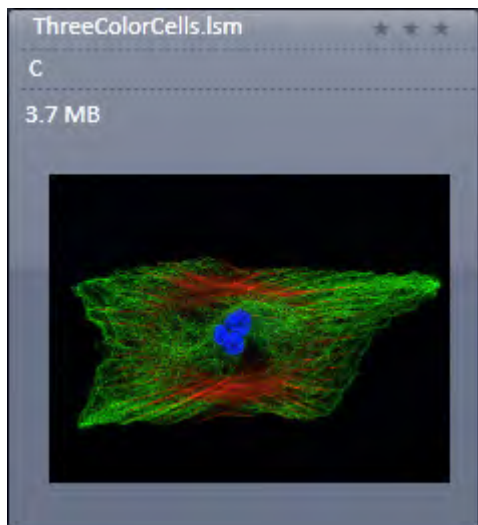


- 1 Expand slider
- 2 New image document button
- 3 Save button
- 4 Close button
- 5 Textual view button
- 6 Thumbnail view button
- 7 Big view button
- 8 Save status button
- 9 Image information field

Fig. 34 Right Tool Area (big view selected)

**Big View button**

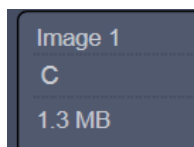
Shows images with the textual characters and a big image preview.

**Save status icon**

This icon appears in the image that is not saved yet.

**Image information**

Displays the name, type and file size of the image.



## 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 Main tool tabs **Ocular**, **Acquisition**, **Processing** and **Maintain**. This tool area always keeps its position on the monitor.

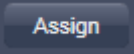
### 5.1 Locate Tab

On the **Locate** 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 **Locate** tab is set in the active mode (**Online**) again. When leaving the **Locate** tab the software remembers the state of the Online / Offline buttons.

Pressing the **Off / On** buttons for transmitted or reflected light opens or closes the appropriate light path.

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 pressing 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.

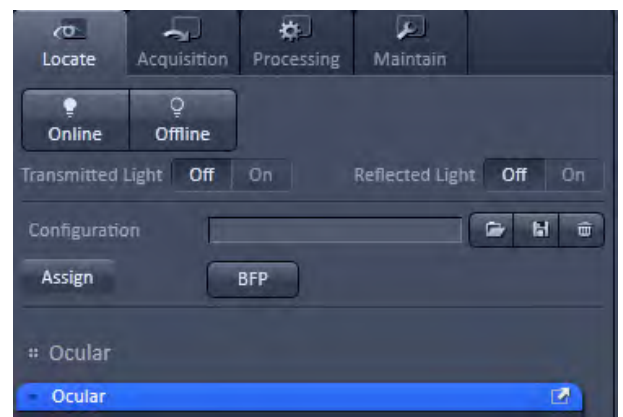


Fig. 35 Locate tab

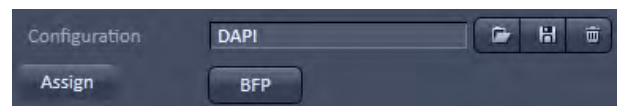


Fig. 36 Configuration panel



Fig. 37 Assign panel

---

### 5.1.1 Controls for Axio Imager.Z2

- Click on **Locate** tab in the **Left Tool Area**. Press **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:

<b>Reflected Light HBO Lamp</b>	This function controls the reflected light illumination source, e. g. 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.
<b>Shutter Reflected Light</b>	This turns the rear port mirror to guide the light to the specimen or to block it from the specimen.
<b>Tube Lens</b>	This function allows switching between available tube lenses. The appropriate lens is automatically set when switching between Ocular and LSM in the Main toolbar.
<b>Reflector</b>	Push and click reflector cube can be selected via graphical pop-up menu.
<b>Objective</b>	Objectives can be selected via graphical pop-up menu.
<b>Condenser</b>	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).
<b>Field Stop</b>	Opening of luminous-field diaphragm (transmitted light) can be set via input box or slider.
<b>Filter</b>	Transmission values for attenuation filter (transmitted light) is set via input box or slider for the front or rear filter wheel in accordance with the available filter steps.
<b>Transmitted Light Lamp</b>	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.
<b>Shutter Transmitted Light</b>	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 on the **On** button for transmitted light.
- Pull out the push rod (Fig. 39/8) to switch the beam path of the tube in LSM position.
- Control the brightness of the halogen lamp with the potentiometer (Fig. 39/4) 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 **Locate** tab in the Left Tool Area. Press **Online** if needed.
- Place specimen on microscope stage.
  - The cover slip must be facing up.



**Fig. 38** Axio Imager.Z2 controls in the Light Path tab

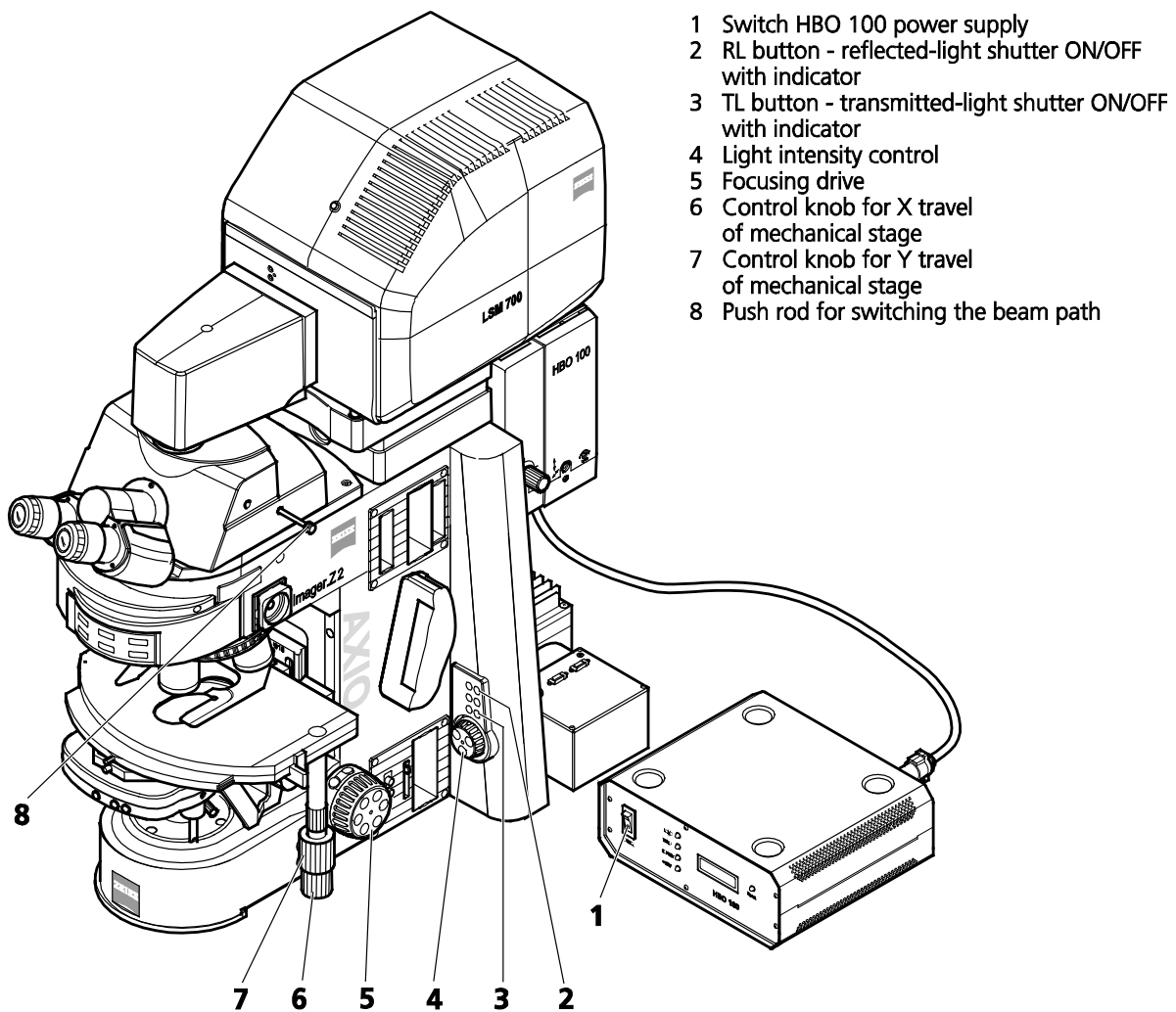


Fig. 39 LSM 700 with Axio Imager.Z2


- 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. 39/5) 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. 39/6 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. 39/1).
- **Click on the RL reflected light button.** The shutter opens.

 To avoid excessive bleaching of biological samples, expose the specimen to the minimum possible irradiation, i.e. keep the irradiation time as short as possible. For this, use the slider in the Ocular panel to attenuate the illumination of the HBO / X-Cite.


- By clicking on the reflector turret button, select the reflector module (filter sets) to suit the type of fluorescence excitation. Proceed as follows:
- Click on the reflector turret button.
- Click on the desired reflector module.
  - The reflector turret moves the selected reflector module into the beam path.

 The FITC filter set consists of an excitation filter for the 450 - 490 nm spectral range, an FT color splitter for 510 nm and an LP long pass filter, which passes emission light wavelengths greater than 510 nm (FSET 09 = FITC, FSET 15 = Rhodamine, FSET 01 = DAPI).  
Other filter sets:

DAPI: BP 365 FSET01  
FT 395  
LP 397

Rhodamine: BP 546 FSET15  
FT 580  
LP 590

 The filter sets described in this section are examples; other sets are available on request.

 The aperture setting on the condenser of the Axio Imager.Z2 is performed in fixed steps.

### 5.1.2 Controls for Axio Observer.Z1

- Click on **Locate** 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:

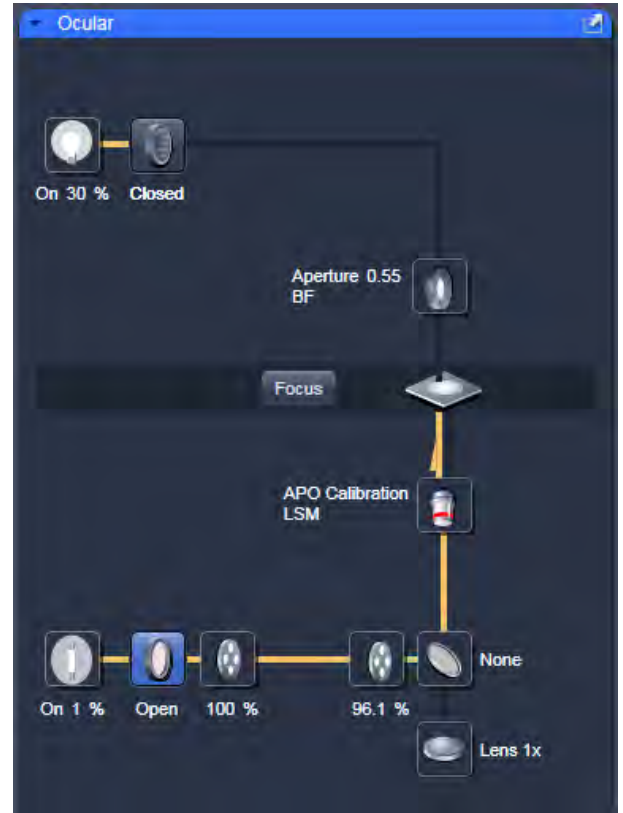
<b>Transmitted Light</b> Lamp	Transmitted light is switched on / off via ON button in the Transmitted Light frame, setting of light intensity can be varied via input box or slider. 3200 K color temperature for photo documentation can be switched on via 3200 K button in the Transmitted Light frame.
<b>Condenser</b>	Numerical aperture of the condenser is set via input box or slider. Turret position selected from graphical pop-up menu (only for motorized condensers).
<b>Objective</b>	Objective can be selected via graphical pop-up menu.
<b>Reflector</b>	Push and click, reflector cube can be selected via graphical pop-up menu.
<b>Tube Lens</b>	Push and click, tube lens can be selected via graphical pop-up menu.
<b>Shutter Reflected Light</b>	This turns the rear port mirror to guide the light to the specimen or to block it from the specimen.
<b>Reflected Light</b> HBO Lamp	This function controls of 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 X-Cite 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 on the **Locate** tab in the **Left Tool Area**.
- 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. 41/5) 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. 41/3 and 4).



**Fig. 40** Axio Observer.Z1 controls in the Light Path tab

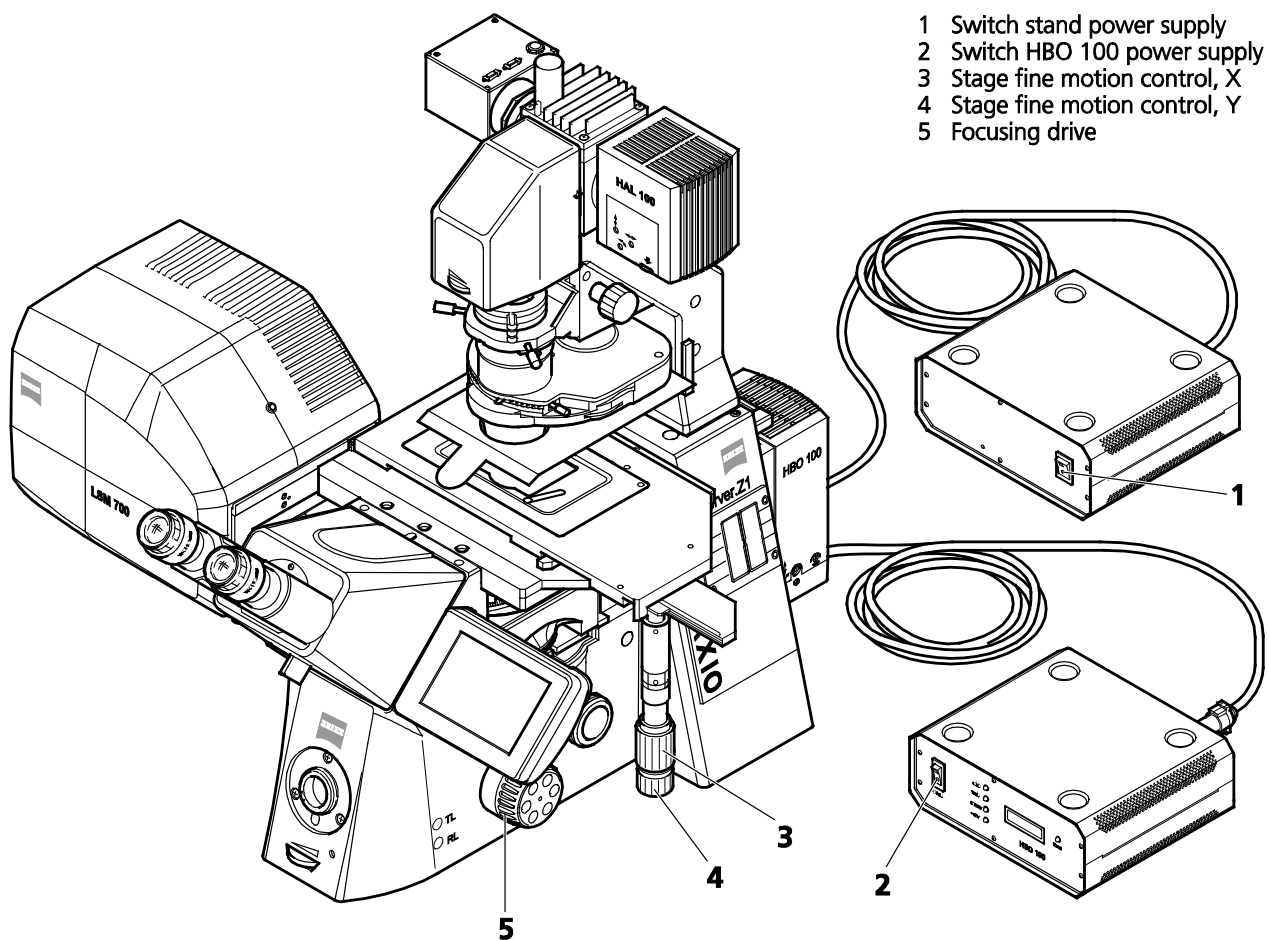


Fig. 41 LSM 700 with Axio Observer.Z1

## (2) Microscope settings on Axio Observer for transmitted-light observation

- Click on the **Transmitted light** button. Click on 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. 41/2).
- 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.

#### 5.1.3 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 Axio Examiner is not motorized (except the Z drive and the condenser carrier), all microscope settings have to be made manually.

Especially, the objectives are changed manually. The used objective must be set in the **Acquisition Mode** tool.

#### 5.1.4 Controls for the Axio Scope with motorized Z-drive

The Axio Scope 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.

#### 5.1.5 Ocular Tool – Incubator Control

From ZEN 2011 the incubation settings can be set within the main software. The settings for Temperature (Ch1 and Ch2) and CO<sub>2</sub> can be set by using the input boxes. The measured values are displayed next to the input boxes.

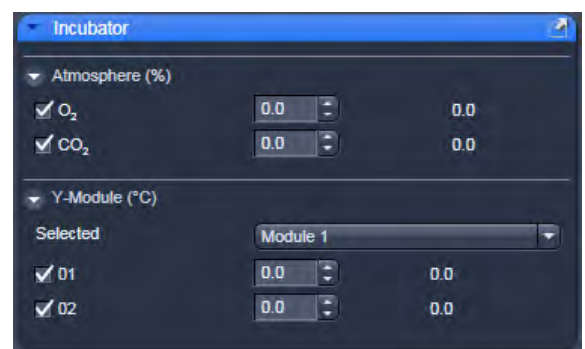
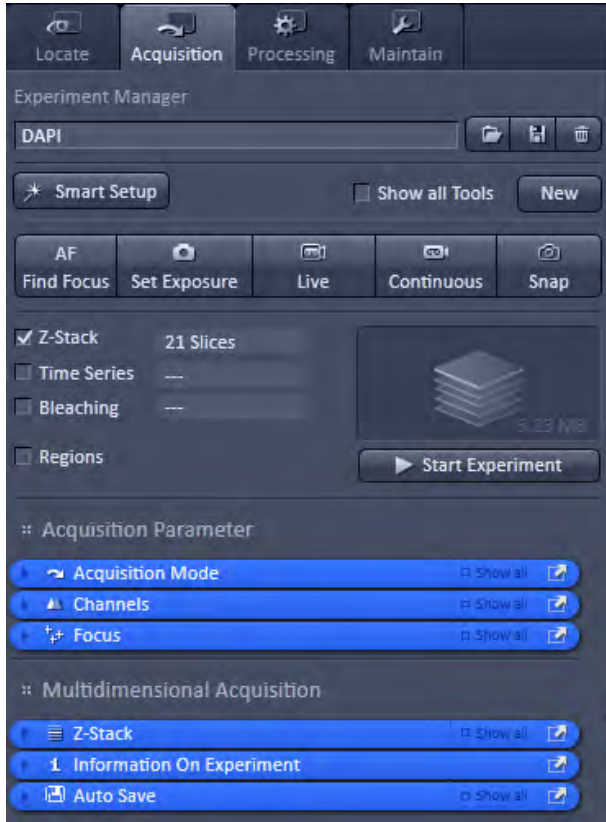
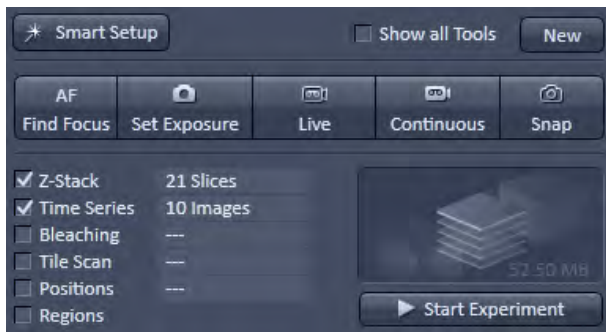


Fig. 42 Ocular tool, incubator control



**Fig. 43 Acquisition tool tab**



**Fig. 44 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.

When **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.

**AF Find Focus** starts the auto focus function.

**Set 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.

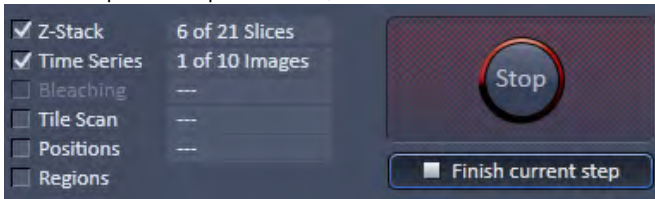
**Start Experiment** will start the image acquisition taking into account all settings including those for time series, bleaching, Z-Stack acquisition, and tile scan.

**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**

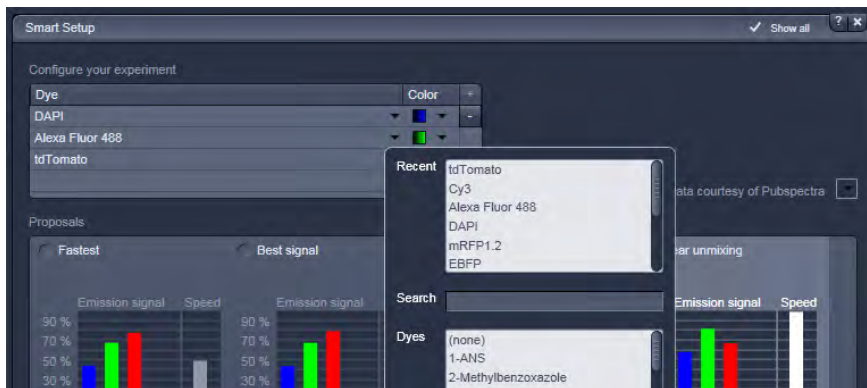
The tool **Smart Setup**, is an intuitive, user-friendly interface which can be used for almost all standard applications. It configures all the system hardware for a chosen set of dyes.



**Fig. 45 Smart Setup button**

**Configure your experiment (Fig. 47/1):**

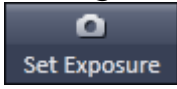
- Simply choose the dye(s) used from the list dialog, and assign signal display color(s), see also Fig. 46.



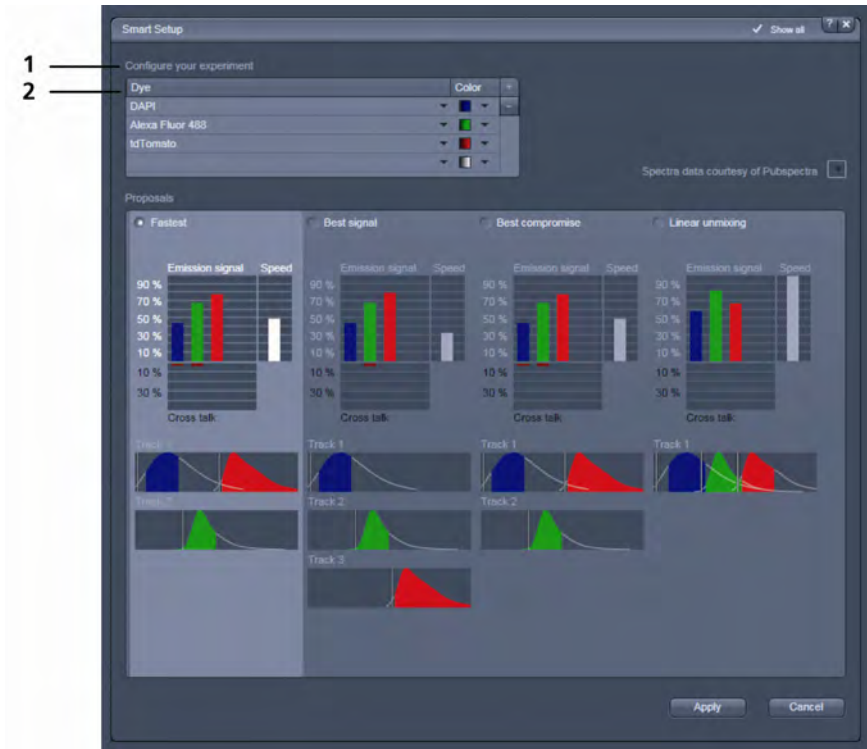
**Fig. 46 Configure your experiment**

**Dye** (Fig. 47/2):

- 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.
- Pressing **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. Pressing the **Set Exposure**



**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.




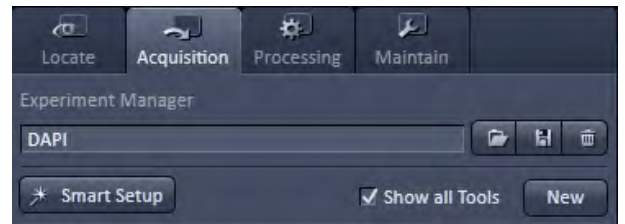
**Fig. 47 Smart Setup menu**

For questions or suggestions send an e-mail to [smartsetup@zeiss.de](mailto:smartsetup@zeiss.de).



### 5.2.3 Setup Manager – Laser

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

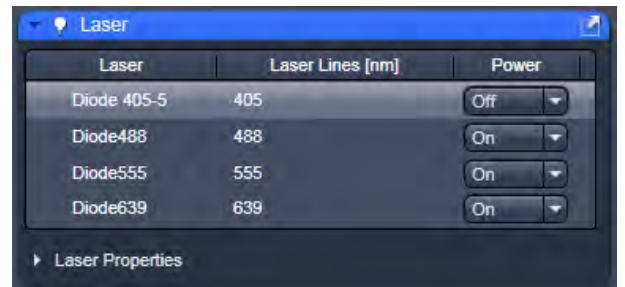


**Fig. 48** Show all tools function

The **Laser** tool shows the types of **Lasers**, the **Laser Lines**, and **Power** status of all lasers available.

The additional laser information panel shows the relevant and currently set **Wavelength, output power (out of fibre) and status** values of the currently highlighted laser. Click the arrow next to Laser Information to open this part of the window. Depending on the type of laser the provided information may vary.

On LSM 700 systems the solid state lasers are automatically turned **on** if the desired line is used anywhere in the software by the user or smart setup. The inbuilt software function **Laser life extender** is switching off all lasers after 15 min. if the system is not used. This extends the long lifetime of the lasers even further. All lasers can be switched off also manually when not in use.



**Fig. 49** Tool group Setup Manager: Laser tool

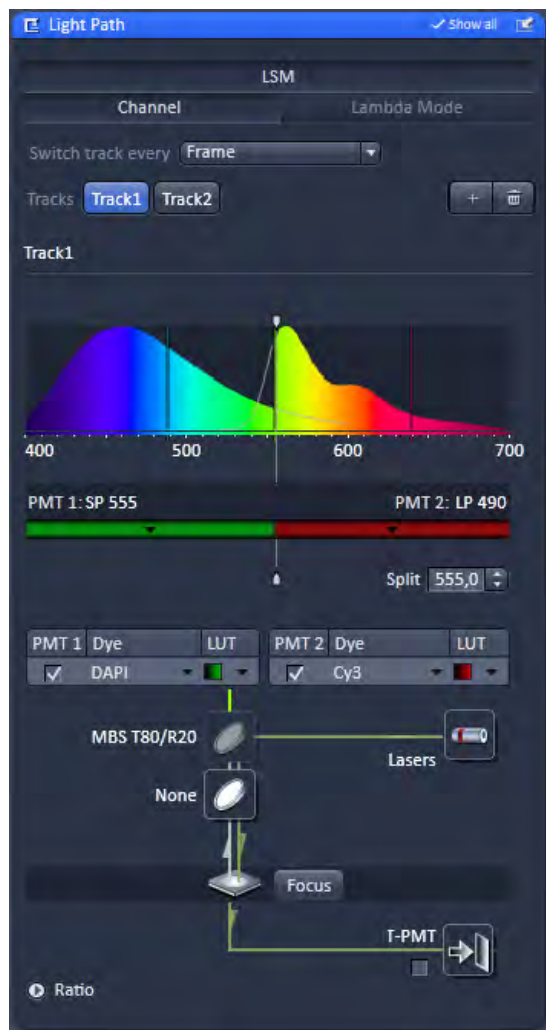


Fig. 50 Light Path tool: LSM tab

## 5.2.4 Setup Manager – Light Path

This tool displays the hardware settings of either the beam path in the scan head (**LSM** tab), or the beam path to image with the camera (**Camera** button). The settings can be changed in the corresponding beam path representation and used for a new imaging configuration.

### Channel Mode

The **Channel Mode** tab (Fig. 50) displays the current hardware settings within the LSM 700 scan head for an imaging track.

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** (see section **Acquisition Parameter – Channel**). 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 are displayed in addition. With the **Tracks** buttons the display can be switched between the different tracks.

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. 50 shows the **LSM** tab when channel mode is selected.

## Switch track every ...

The hardware settings for the different tracks can be switched every frame, every frame but fast, or every line (only available in **Show all** mode). 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.

## Tracks configuration

Within the **Tracks** line tracks can be added and subtracted by clicking  or .

Clicking on the track button allows switching between the tracks quickly.

## Lambda Mode

The **Lambda** mode is chosen for imaging heavily overlapping emission signals. When choosing this mode by activating the **Lambda** mode button 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 **Processing – Linear Unmixing**) using the acquired Lambda stack and allow to clearly separate even heavily overlapping emission signals.

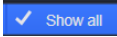
## (1) Light Path

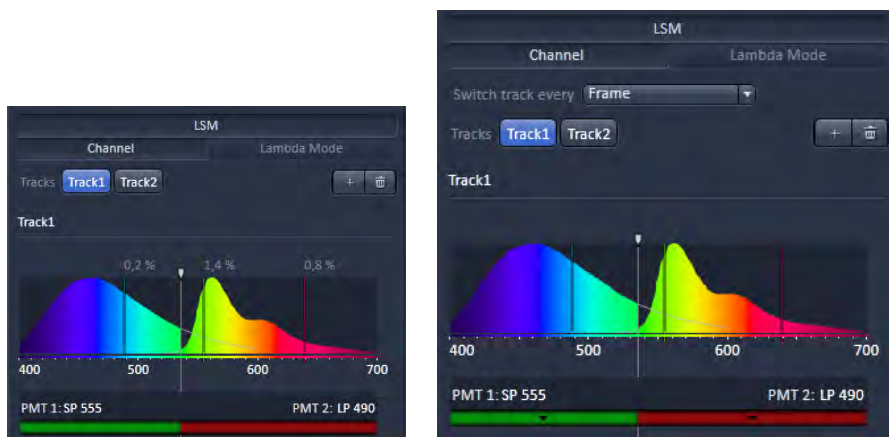
- Operation of the beam path tool with the **Show all** function deactivated  (Fig. 51, left):

The two channels can be activated by ticking the tick boxes under **PMT1** or **PMT2** . In the row named **Dye**, a dye from a list can be selected. The emission spectrum of the selected dye is then displayed in the main part of the beam path tool. In addition the so called splitter (or Variable secondary dichroic [**VSD**]) is then positioned automatically in the best position for the dye(s) selected. It can be positioned manually if desired. If available, suitable **emission filters** are selected also automatically by the system to minimize crosstalk between the channels. The part of the spectrum on the left side of the VSD position is detected by channel 1, the part of the spectrum on the right side is detected by channel 2. The parts of the spectra which are actually detected by the system are filled with color. In addition the laserlines in use are also displayed.

The beam path tool is giving a very good overview on the hardware settings for a given set of dyes.

The pull down **LUT**  hosts a number of different look up tables to display the data in pseudocolors.


- Operation of the beam path tool with the **Show all** function activated  (Fig. 51, right):  
All automatic functions of the beam path are deactivated in this mode.  
Emission filters and the position of the VSD are thus not automated in this mode. However, the emission spectra of the dyes are still displayed in the beam path tool.



**Fig. 51** Beam path tool with **Show all** function deactivated and activated

## (2) Emission Filter EF

- Below the spectra representation, the emission filters can be selected by simply clicking on the filter representation. The two pull downs each represent an emission filter wheel in front of the according channel 1 or 2. Once the filter is selected, the part of the spectrum which is actually detected is filled with color. Emission filters can be band pass filters (BP), passing the light in between the indicated wavelengths onto the detector, long pass filters (LP), passing the light above the indicated wavelength onto the detector, or short pass filters (KP), passing the light below the indicate wavelength onto the detector. For a more intuitive way to select the right filter, the part of the spectrum transmitted by the filter is displayed in bright color. Together with the position of the VSD, the emission light can be flexibly chosen for both PMT's.
- To select an emission filter, click on the colored bar and choose it from the drop down menu.




 It is possible to change the filters directly on the LSM 700 scan module (see printed manual CHAPTER 6: ANNEX).

The use of the monitor diode function is described in detail in the annex of this manual.

## (3) Main Dichroic Beam Splitters MBS and Variable Secondary Dichroic Beam Splitter VSD

- MBS stands for main dichroic beam splitter. In the LSM 700 a fixed "Fixgate" dichroic beam splitter is used that is suitable for all laserlines on the system. It is stably inserted in the scanhead and allows high precision imaging with all 4 possible lasers on the system.
- VSD stands for Variable Secondary Dichroic. It can be continuously tuned and splits the light into the two channels. Its **variable** range is from 420 nm to 630 nm. The system detects light from app. 380nm to app. 750 nm
- To move the VSD, grab the line in the beam path and move it left and right. The part of the spectrum on the left of the split position is always detected by the PMT 1 and the part on the right side of the spectrum is always detected by PMT 2.

### 5.2.4.1 Activation/Deactivation of Channels

- To activate a channel for imaging, tick the check box under the PMT1 or PMT 2 part of the list in the beam path . 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 triangle and select a color from the drop down menu (Fig. 52).
- To set the dye click onto the  dye triangle and select it from the drop down menu.



**Fig. 52** Drop down menu for selection of the channel color

### 5.2.4.2 Reflector Turret

The filter wheel FW1 is the reflector turret of the microscope that holds push and click filter cubes and fixed beam combiner cubes. When using the LSM 700 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.

### 5.2.4.3 Stage / Focus

This icons provide access to the control for the focus (position of the specimen in Z) and the stage (position of the specimen in X and Y). But detailed description is given in section **Acquisition Parameter – Focus** and section **Acquisition Parameter – Stage**.

#### 5.2.4.4 Setting of Laser Line and Laser Attenuation

The **Laser** icon provides access to the control of the laser lines and their attenuation (Fig. 53). Activate a laser line by checking the according box. The attenuation slider is displayed and input box, which allows setting the desired laser power. Make sure the laser line is indicated as active with a warning triangle. Once the tick box is ticked, the lasers in the LSM 700 are switched on automatically.



- Use the **Transmission [%]** slider to set the utilizable laser intensity (recommendation: start at values between 2 and 5 %).

Fig. 53 Laser line selection and attenuation

#### 5.2.4.5 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 **Acquisition Parameter – Channel**).

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 **Channelstool** and **Light Path** tool.

In the **Light Path** tool choose **Channel Mode** as the mode for image acquisition and define the hardware parameters like Laser line, laser attenuation, position of the variable secondary dichroic beam splitter, 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 **Channels** 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 **Channels** 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 **Channels** 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 **Acquisition Parameter – Channel**), the scan parameters like **Frame Size**, **Zoom**, **Rotation & Offset**, and **Scan Direction** parameters (see section **Acquisition Mode Functions for LSM**) are stored.


### 5.2.4.6 Ratio Imaging

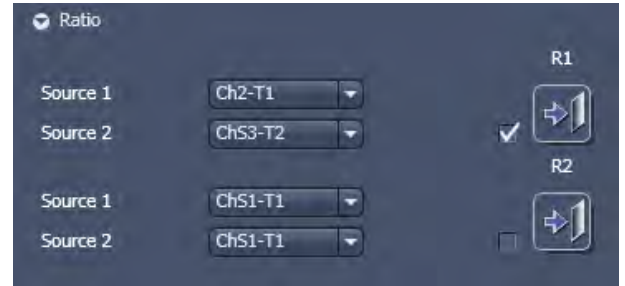
With the **Show all** function of the **Light Path** tool **activated** two additional ratio imaging channels **R1** and **R2** can be defined. These channels are displayed in addition to the maximum of 8 active imaging channels. They provide the possibility to display the ratio between two of the active channels.

A click on the arrow next to **Ratio** in the lower part of the **Light Path** tool window (Fig. 50) 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 **Acquisition Parameter – Channel**).

A suitable color is assigned to each of the two **Ratio Channels R1** and **R2**, in the same way as for the photomultiplier channels.

 The ratio to be formed between the selected channels can be defined more precisely using one of the four preset formulas in the **Channels** tool window after a click on the relevant ratio button (e.g.: R1) for online display of radiometric or single wavelength dyes. The Set by min/max function (in **Channels** tool window) allows the definition of the display scaling according to the expected minimal and maximal values.



**Fig. 54** Ratio channels in the Light Path tool

### 5.2.4.7 Imaging in Lambda Mode

(to be selected in the **Light Path** tool, if not automatically selected by using the **Smart Setup** routine).

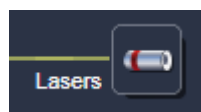
In the **Lambda Mode** the LSM 700 is recording the overall emission from the sample by moving the VSD (Variable Secondary Dichroic) **sequentially** to different positions over the selected spectral range. During this movement in steps the lower part and the higher part of the spectrum is detected by PMT 1 and PMT 2 respectively. Taking those signals together, represents a spectral signature of the dye (s) which can be used for spectral unmixing just as well as spectra acquired in a **parallel** way.

The variable range of the VSD is between 420 and 630 nm. In between those borders the number of steps the VSD performs to record the spectral information can be chosen freely.

By increasing the number of steps the step size can be reduced to a minimum of 1nm. In the **Show all** mode it is possible to enter non – linear step widths of the VSD in order to spectrally resolve heavily overlapping parts of spectra in a better way (see Fig. 55).

The new image format is called Lambda stack. The settings of the beam path for the **Lambda Mode** scanning procedure with regard to the positions of the VSD are done in the **Light Path** tool (see section **Setup Manager – Light Path**).

The **Light Path** tool displays the laser lines, the position of the VSD, and the selected spectral range. You can change the settings using the following function elements:



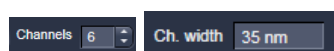
Activation / deactivation of the excitation wavelengths (check box) and setting of excitation intensities (slider).



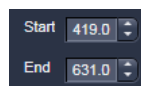
Definition of the spectral range and Display of emission spectra of used dyes.



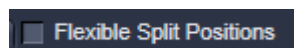
Access to the focus control (see section **Acquisition Parameter – Focus**).



Number of VSD steps and resulting step width.



Manual definition of the spectral range.



Activation or de-activation of non – linear step widths.

### Spectral range

Use the slider to define the spectral detection range.

**Resolution:** Step size can be variable down to 1nm. The higher the number of steps – the finer the resolution. Increasing the number of steps is also increasing the bleaching.

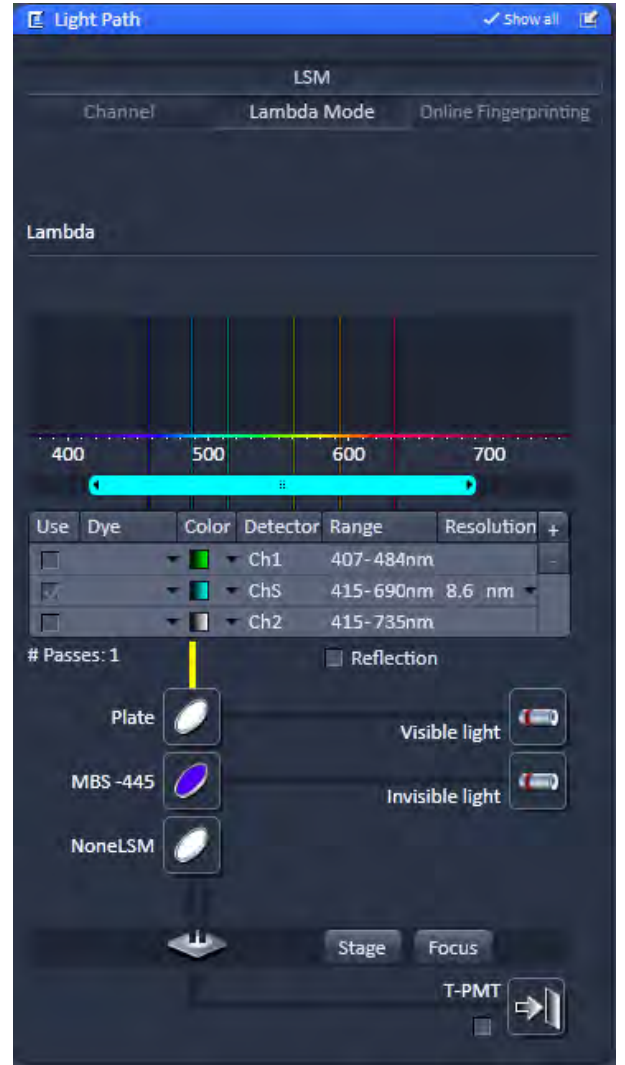
A good starting point for assessing the optimal number of steps is always the suggestion calculated by the smart setup algorithm for a given set of dyes. This suggestion is the mathematically optimal number of steps.



**(1) Light Path for imaging Lambda stacks**

(To be selected in the **Beam Path** tool, **Imaging Setup** tool or automatically set by **Smart Setup**).

Up to 11 split positions are displayed in the **Light Path** tool – above a number of 12 positions the individual lines are not displayed anymore. The split positions are nevertheless used for acquisition.



**Fig. 55 Light Path tool window; Lambda Mode**

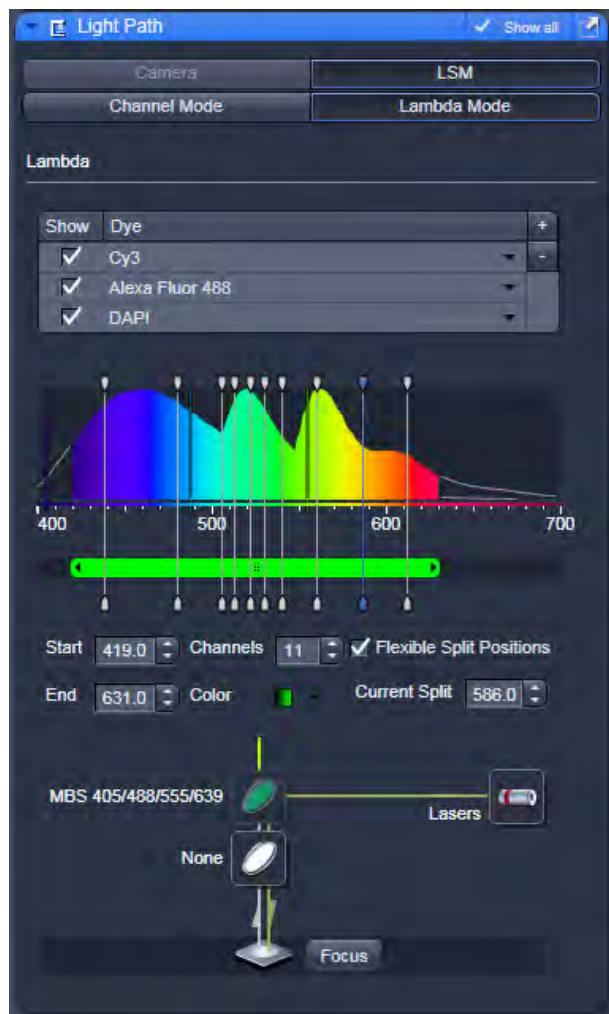


Fig. 56 Light path tool: non-linear split positions

## (2) Light Path for imaging Lambda stacks with variable split positions

In cases of heavily overlapping parts of 2 spectra the steps of the VSD can be setup non-linearly in order to resolve those overlapping parts better. Tick the **Flexible Split Positions** tick box and move the split positions with the mouse to the desired positions.

### 5.2.4.8 Camera Mode

The **Camera** mode of the light path tool 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 **Light Path** tool.


The Camera mode provides access to the conventional illumination sources, the halogen lamp or HBO / X-Cite 120. It also provides access to the condenser aperture and filters and the tube lens.

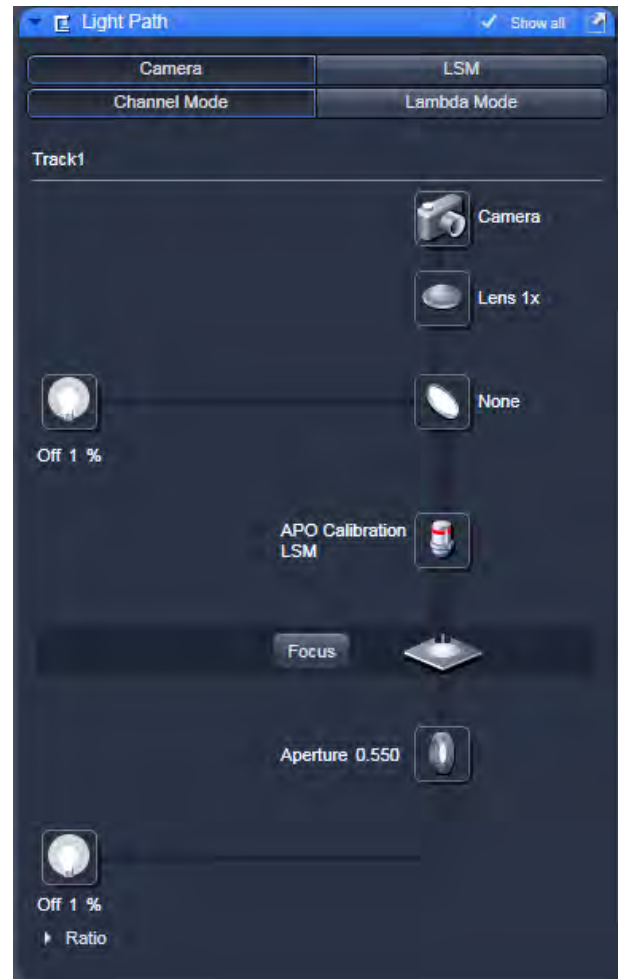
The use of this function permits the use of a Zeiss AxioCam camera (various models, see ANNEX for description) as an alternative external detector.

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 X-Cite 120 or HBO 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.

 If TV and LSM tracks are mixed, the active detection port of the microscope has to be set according to the first track.



**Fig. 57** Light Path tool: Camera tab

### 5.2.5 Acquisition Mode Functions for LSM

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


### Frame Mode for LSM

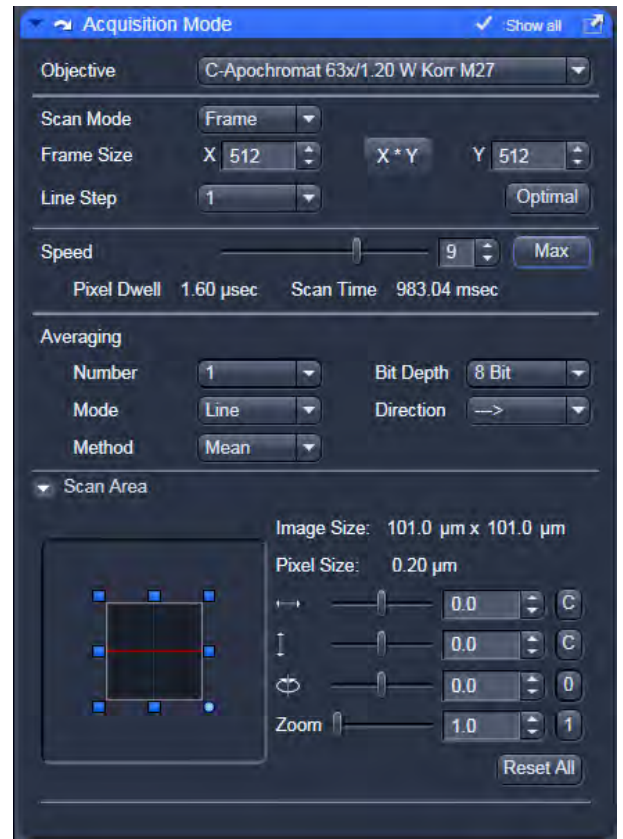
When the scan mode **Frame** is active, a frame of variable size is scanned pixel by pixel and line by line. The laser beam is moved over the specimen line by line.

The following additional parameters can 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  or type in the required values next to X and Y. Recommended setting to start with: 512 x 512 pixels.
  - It is also possible to enter different values for X and Y. The value for **Y** is freely selectable between 1 and 2048 pixels (integers). The value for **X** must always be an integral multiple of 4. The maximum value for **X** is also 2048 pixels.
  - The **Optimal** button sets the image resolution to an optimal value corresponding to the optical magnification (objective) the zoom and the emission range detected. This provides an image where no information is lost as optimal sampling is achieved.
- Select the **Line Step** size between 1 and 10. Only every n-th line is scanned. The lines in between are interpolated. This fast scan mode is called Step Scan.
- Select the **Scan Speed** from the 13 preset steps via slider or input box. Recommended: 7 for the first scan. A click on the **Max** button sets the maximum speed for the current zoom.
  - The **Scan Speed** determines the **Pixel Dwell** time. In the case of different image formats, the **Pixel Dwell** time is constant for the same Scan Speed, but the **Scan Time** is different.
  - **Pixel Dwell** time of the laser beam on the pixel
  - The **Scan Time** duration of the acquisition for the entire frame



**Fig. 58 Acquisition Mode - Frame with LSM tab selected in the Light Path tool**

<b>Speed:</b>	1 ... 8	9	10	11	12	13
<b>Zoom:</b>	0.5	0.8	1.1	1.8	2.6	4.1

- 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 via 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. For automatic scan correction, click on the **Auto** button.
- For **Averaging**, select the Line or Frame mode.
- 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.

**ROI HDR**

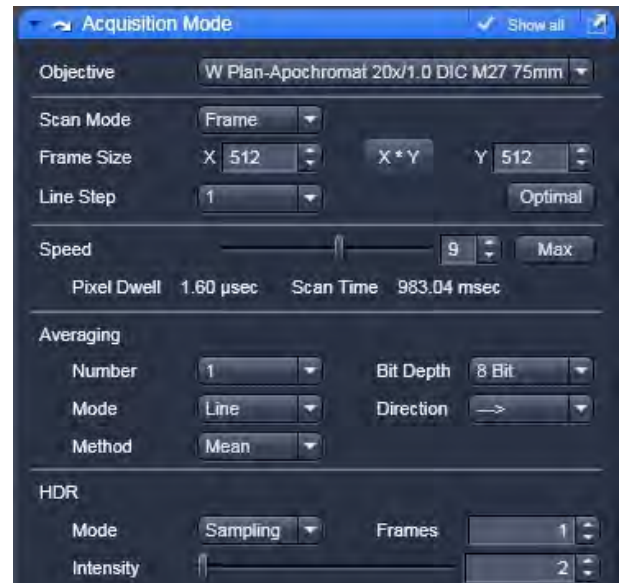
The **HDR** function is located under the field **Averaging** of the **Acquisition Mode** tool (Fig. 59). HDR is active when the checkbox is ticked.

Two modes are available (Fig. 60):

- **Sampling** (HDR) and
- **Illumination** (ROI HDR).

Both modes create the typical HDR effect of boosting weak structures without saturating bright areas in the image.

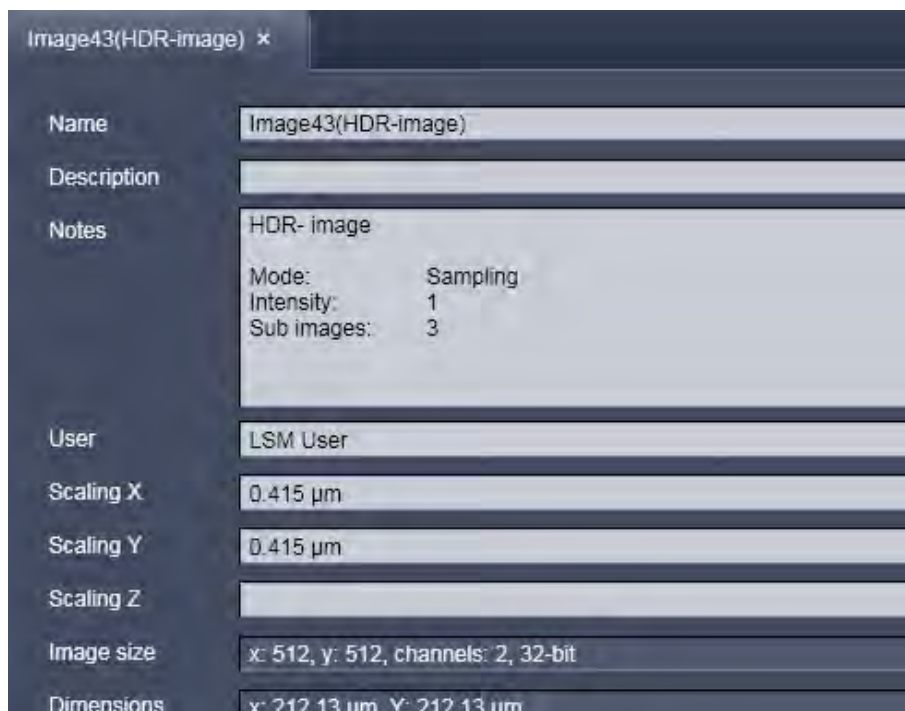
Both modes create a HDR image with a 32-bit grey dynamics, which covers a much wider dynamic range than conventional acquisition. The used HDR method is also shown in the image info (Fig. 61).



**Fig. 59 Acquisition Mode tool, HDR panel**



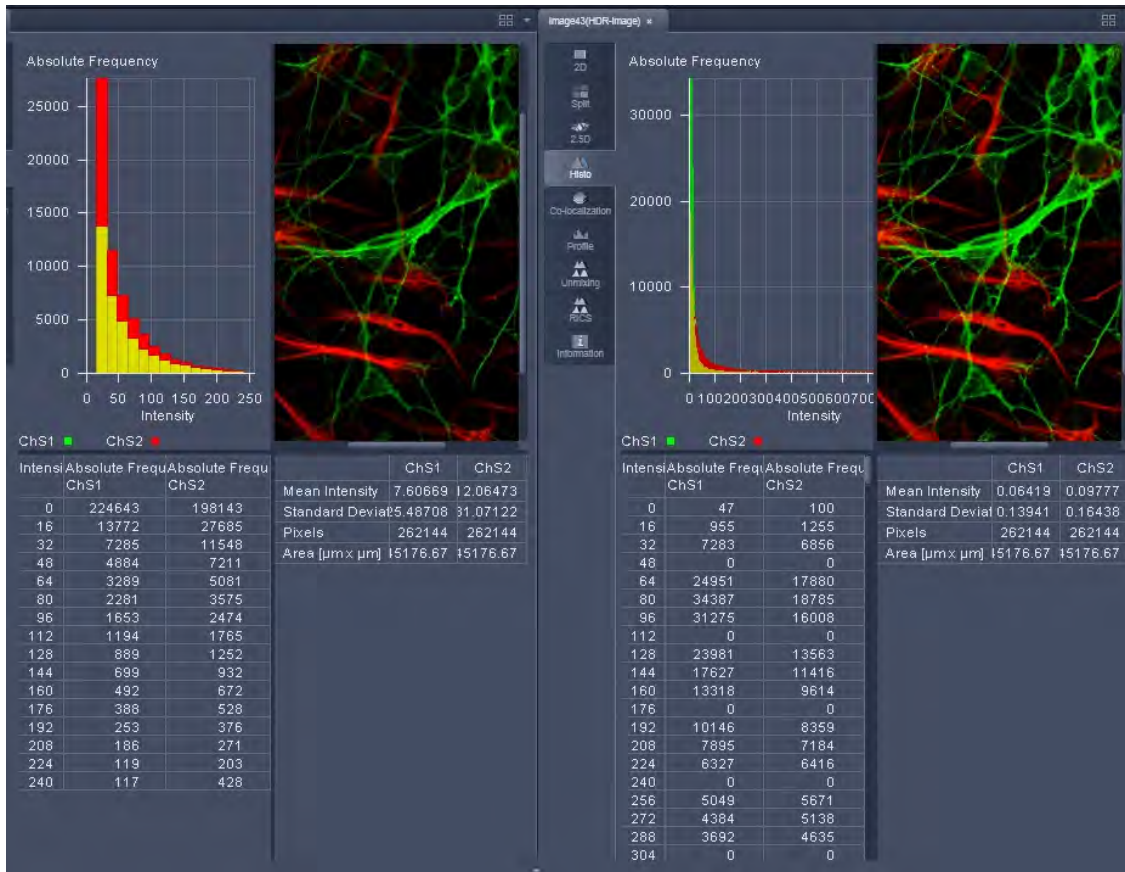
**Fig. 60 HDR modes**



**Fig. 61 Image info of a HDR image**

As a result of the HDR process, the grey scale is independent from the initial bit depth, but covers a range from 0-1.

Also, the grey stepping is much finer, with much better visibility of weak structures (Fig. 62).



**Fig. 62 HDR image display (left side without, right with HDR)**

The **Sampling** mode is a means to improve the image display as it evens out the differences between the very high and the very low intensities in the image. However, it is not suitable for quantitative analysis as the grey level steps become non-linear.

The image re-calculation works already with a single image (**Frame** = 1) and is done in the following way taking **Intensity** 3.

1. Image x 1; cut off of grey levels above the dynamic range; Result divided by 1
2. Image x 2; cut off of grey levels above the dynamic range; Result divided by 2
3. Image x 3; cut off of grey levels above the dynamic range; Result divided by 3

The three resulting images are summed up and again divided by 3.

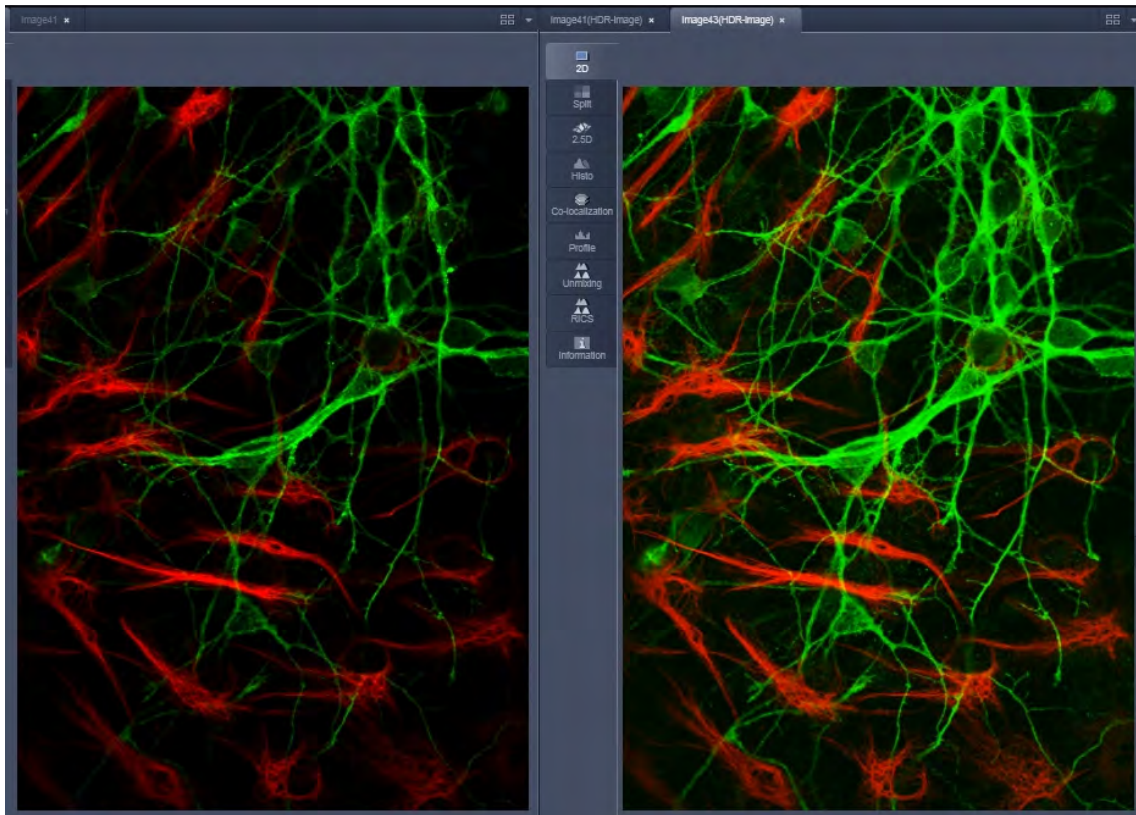
This procedure is adapted accordingly if a higher **Intensity** number is set.

If two images are **acquired** (**Frame** = 2) and Intensity is still at 3, the second image is rated lower and is multiplied and divided in three steps with the value 4,5 and 6; The then 6 resulting images are summed up and divided by 3.

The image acquisition parameters are not changed for the acquisition of the two (or more) **Frames**.



After acquisition, the grey dynamics of the image are automatically compressed (see description above) to match the range of the output display. This step visibly intensifies weak signals (Fig. 63).



**Fig. 63** HDR image display using Sampling mode (left side without, right with HDR)

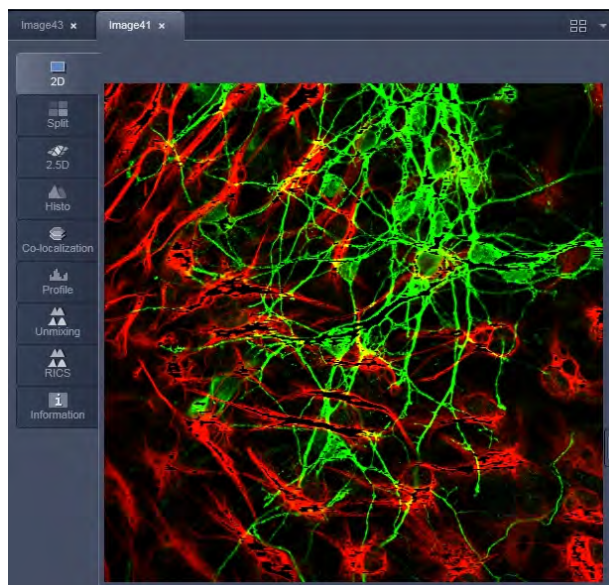
The **Illumination** mode still provides a linear grey stepping which allows quantitative measurements.



**Fig. 64 HDR Illumination mode**



**Fig. 65 Laser Power setting for Illumination mode**



**Fig. 66 HDR image display after using Illumination mode**

To use the HDR **Illumination** mode, select **Illumination**. The number of **Frames** is automatically set to **3** (Fig. 64).

The **Illumination** mode changes the laser power (Fig. 65) to acquire a series of varying exposures. The degree of variation is fixed to allow better reproducibility of experiments. The second image is acquired with 3 times more laser power and the third image is acquired with 10 x more laser power.

The default laser power set in the channel menu should be kept to a minimum.

For the acquisition step with increased laser power, those regions of the image are excluded from exposures where no reasonable data can be obtained with any higher laser power. This mask reduces unwanted bleaching of the sample. The respective ROIs are displayed in an additional image (Fig. 66).

The calculation of the resulting image follows the same procedures as for the sampling mode. However, the regions, which are excluded from a second and/or third illumination step are divided by 2 instead of 3.

After acquisition, the grey dynamics of the resulting image are not automatically compressed to match the range of the output display.

Use the **Gamma** and **Contrast** sliders of the image display (Fig. 67) to adjust the image.

For manual adjustment, a result can be achieved which fits the individual requirements, without affecting the actual raw data stored with the image.

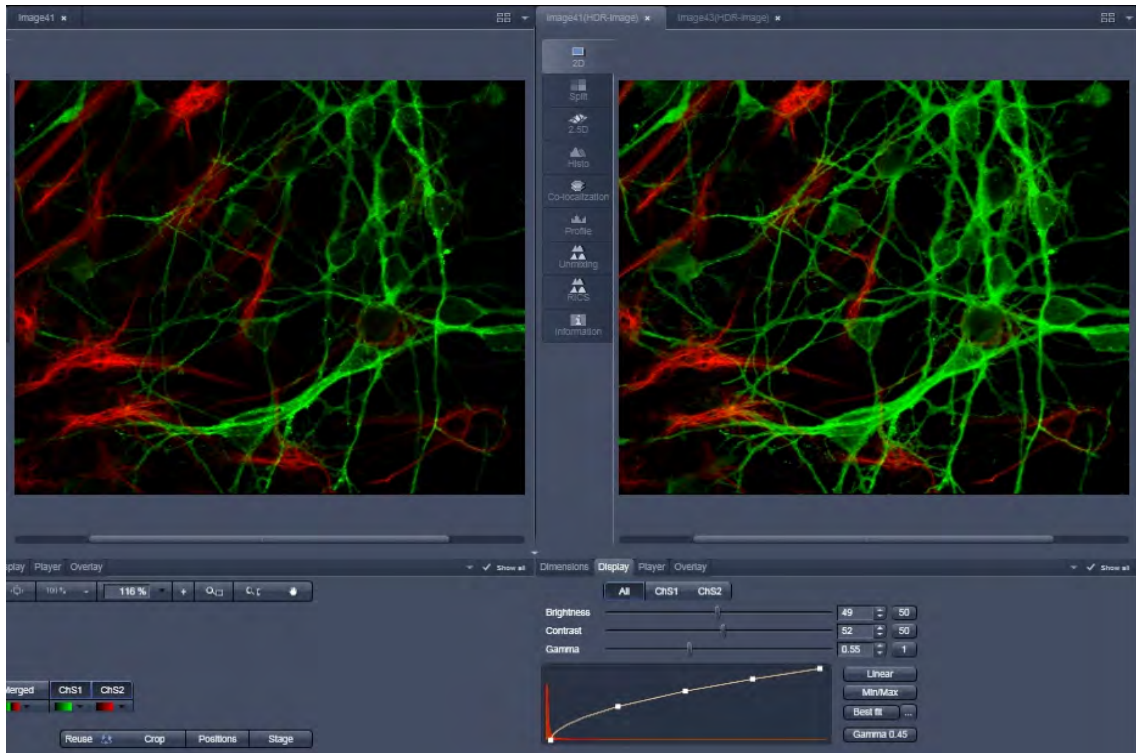
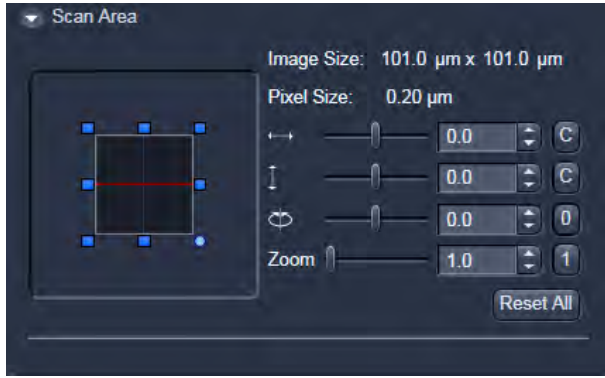


Fig. 67 Image adjustment of the HDR image (left side without, right with HDR)





### 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.

**Fig. 68** Scan Area panel

- 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 0.5 to the maximum of 30, and is displayed in the relevant input box. The value 0.5 corresponds to factor 1, and value 30 to factor 60, related to the field of view.
- 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  $\mu\text{m}$  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°.

### Frame Mode for Camera


When images are acquired with the camera only **Frame** mode is available.

The following additional parameters can 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**, **12 or 16 Bit** Data Depth, i.e. 256, 4096 or 65536 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 **Continuous**.

 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.

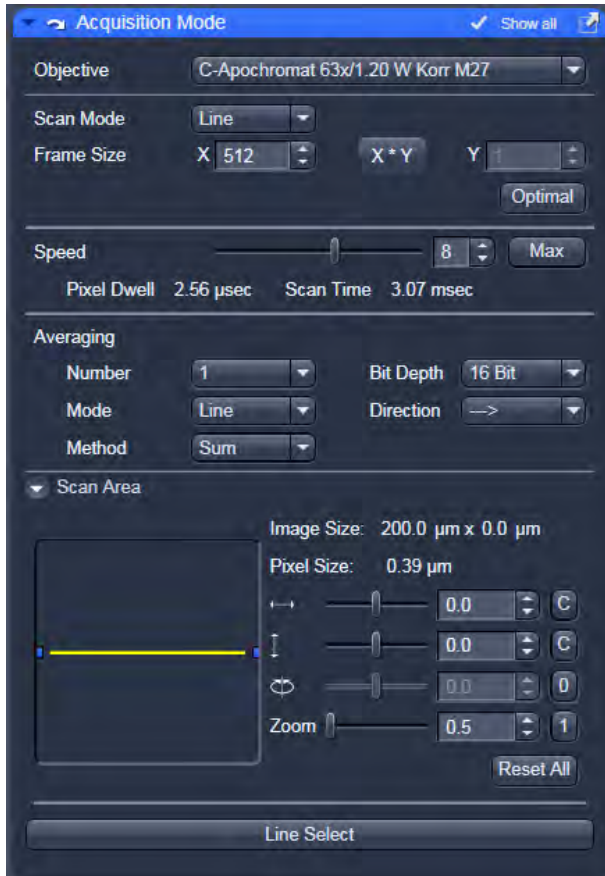
Averaging can be performed in different ways, depending on whether the **Mean** or **Sum** method has been activated.

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. 69** Acquisition Mode - Frame with Camera tab selected in the Light Path tool



**Fig. 70 Acquisition Mode – Line with LSM tab selected in the Light Path tool**

## 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**. Frame size in this context sets the length of the line in x direction. The width of the line is one pixel.

☞ 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 (see printed manual CHAPTER 5 "Maintenance Tool").

### Spot Mode

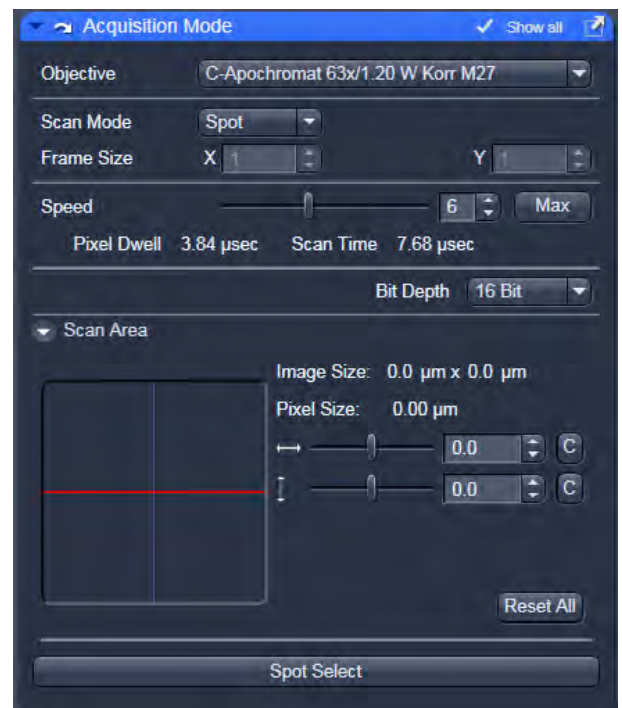
In the **Spot** mode fluorescent or reflected light occurring from a single voxel xyz is detected. In this mode a spot can be defined by two perpendicular lines in the **Image** using the Spot Select button which is located in the lower part of the **Acquisition Mode** tool window (Fig. 71).

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**.

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**.



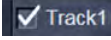
**Fig. 71** Acquisition Mode tool - Spot Mode

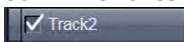


**Fig. 72** Channels tool using channel mode (in this case multiple tracks) for imaging

### 5.2.6 Acquisition Parameter – Channel

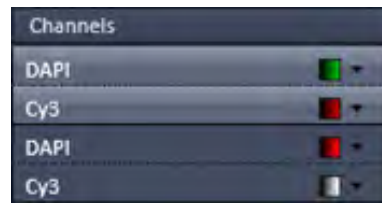
The **Channels** tool provides the control of the parameters of the individual detection channels.

It also allows to activate and de-activate tracks by ticking and un-ticking the track check box .

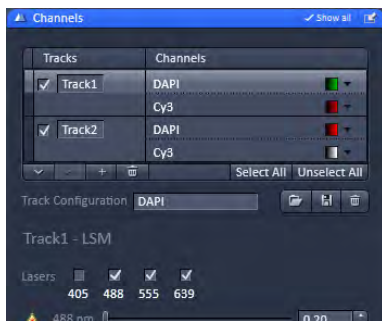
The channels and tracks defined in the **Light Path** tool or by **Smart Setup** are listed channel by channel as selectable list entries. Clicking on the list entry **Track**  displays controls (laser, pinhole, gain, ...) for all the channels in the respective track.


Clicking on the **Channels**  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** or the **Light Path** tool the names of the dyes used or the names of the tracks are displayed in the **Channels** tool.



Using the  buttons you can modify the order of the tracks in tracks table display.

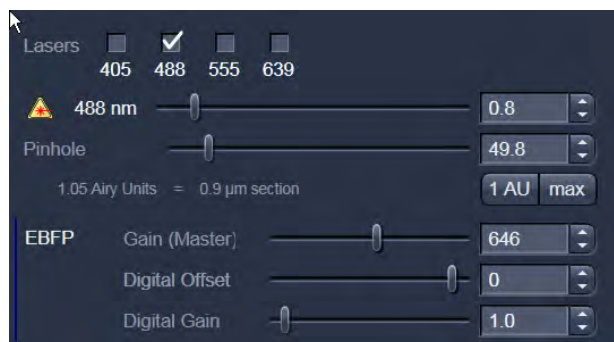
The buttons  allow to add new or remove the selected tracks.

With the  buttons all tracks can be selected or unselected.

In the Track configuration line, you can load, save or delete track configurations.



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)

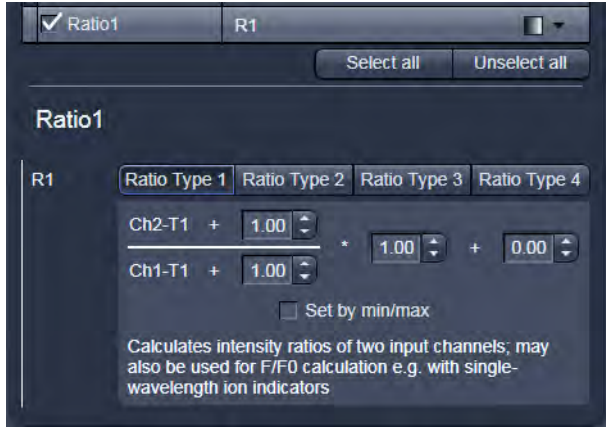
When using 12 bit grey resolution, gaps can occur in the histogram of the acquired image, in case the gain slider is moved beyond a gain level of 25.

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.

If the **Auto copy from last acquired** check box is activated, the display settings of the last scanned image are automatically used for the new image acquisition procedure.

The **Range indicator** check box activate / deactivate the range indicator function.



**Fig. 73 Channel Settings panel of a Ratio Channel**

### 5.2.7 Ratio Channels

The parameters for ratio metric imaging can be set when a ratio channel is selected.

- Click on the button of a ratio channel (e.g. **R1**).
- Clicking on the required tabs enables you to choose from four formulas (**Type 1** to **4**) for ratio calculation. The relevant decimal values can be entered in the input boxes. The entered values remain unchanged even after switchover to another formula and can be reactivated any time.

The formula type activated last is always used for ratio formation during the scan procedure. If the input box does not contain any value at all or no suitable value, the useful value last used will be activated.


The ratio channels are displayed as additional images.

Select the required formula and enter the relevant values.

Letters can be entered into the formula fields which will be valued as 1; it is also possible to make no entry, which will also be valued as 1, but will not be displayed.

Set by min/max allows the definition of the display scaling according to the expected minimal and maximal values.

- Laser lines and laser power are displayed according to each track. It is possible to vary laser intensities (in the same way as in the **Laser** tool or **Light Path** tool).

 All parameters can be varied while imaging.

### 5.2.8 Acquisition Parameter – Focus

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  $\mu\text{m}$ .

The Current Z-Position can be **Set** as **Zero**

**Position** by clicking the  **Manually** button.

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  $\mu\text{m}$  and 10  $\mu\text{m}$ .

As soon as the focus position of the microscope is changed (via hand wheel or software), the piezo objective focus is automatically leveled.

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



Fig. 74 Focus tool window



**Fig. 75** Stage tool window


### 5.2.9 Acquisition Parameter – Stage

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



If an LSM 700 scan head is attached to the side port of an inverted microscope (Axio Observer, Axiovert 200M) 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**.

### 5.2.10 Acquisition Parameter – Regions

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. 76).

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.

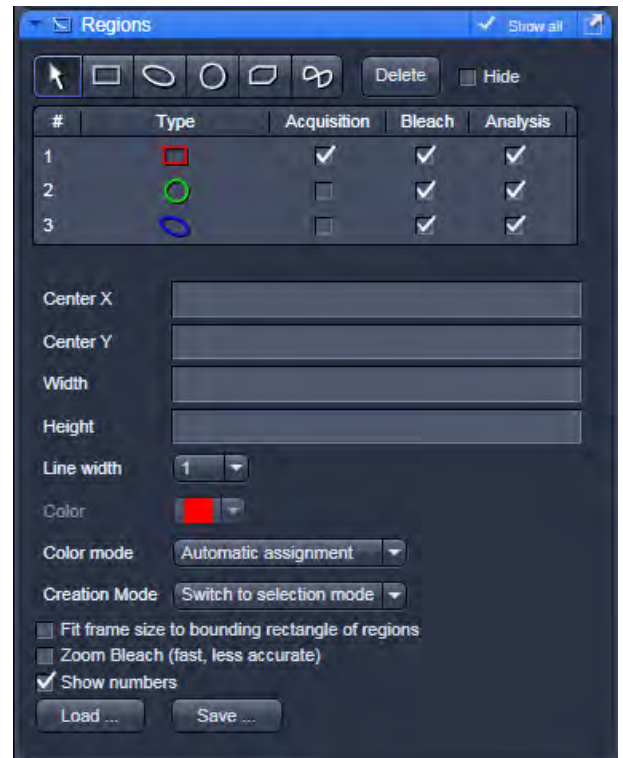


Fig. 76 Regions tool

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.

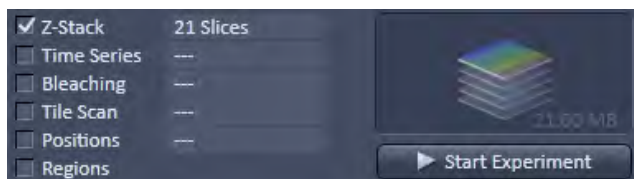
If a region is used to define a position for bleaching within the sample, the bleach process can be accelerated by checking the box next to **Zoom Bleach (fast, less accurate)**. In this case the scanner movement will be restricted to the bleach region zooming in onto this region. This may result in 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.

### 5.2.11 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.



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. 77) or to use **Center** (Fig. 78). In case a **Piezo** focusing device is attached to the system, the Piezo device can be chosen for the acquisition of the stack.

#### 5.2.11.1 Performing a Z-Stack Using First/Last Mode

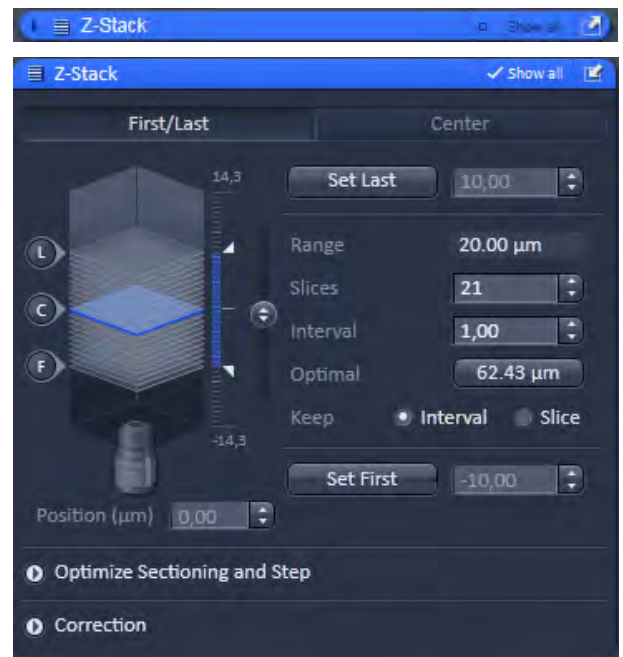
Focus onto the specimen and start a **Continuous** scan. While scanning change the focus using the **Focus** tool (see section **Acquisition Parameter – Focus**), or the **Z-Stack Navigation** (see below), or use the focus drive of the microscope (see manual of the microscope). When you reach the position 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.



**Fig. 77** Z-Stack tool Start/End mode with Z-Stack Navigation displayed

**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. In case of a MAT configuration (database) the **Optimal Interval** is even half this value in order to ensure precise height measurements.

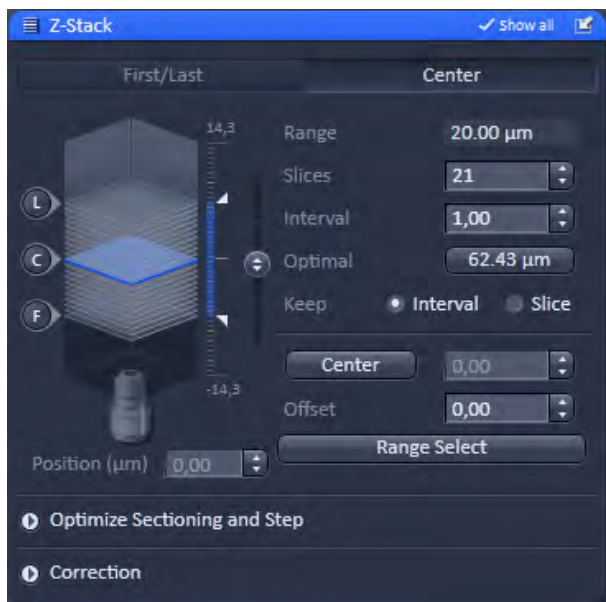
**Fast Z Line** performs a fast XZ scan for overviews using a continuous movement of the microscope focus (only in **Line** scan mode and not with the Piezo). The stack size is retained; the interval is adapted depending on the scan speed. Not available in **Frame** mode.

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.

☞ To obtain best image quality with Z-Stacks and scanning stages which are critical regarding the focus impulse, a waitstate can be activated in the **Maintain Tab** -> **Options** – System Options -> **Hardware** menu. Such a waitstate is only beneficial at certain acquisition speeds and not needed by default.




**Fig. 78** Z-Stack tool Center mode with Z-Stack Navigation displayed




### 5.2.11.2 Performing a Z-Stack Using Center Mode

The **Center** mode (Fig. 78) 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.



**Fig. 79** Use Z-Piezo stage for Z-Stack acquisition – appearance for the LSM imaging mode

### 5.2.11.3 Performing a Z-Stack Using the Z-Piezo Stage Insert

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 **First/Last** mode. Activate **Piezo** mode (Fig. 79). The total Range of the Z-Stack is limited to 250  $\mu\text{m}$  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.


When changes are made to **Interval** or **Number of slices** in **First/Last** 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.


### 5.2.11.4 Optimize Sectioning and Step

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.

 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 choose 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.



**Fig. 80 Optimize Sectioning and Step: Optimal Interval is set starting with one Airy unit for all channels**



**Fig. 81 Optimize Sectioning and Step: Match Pinhole to Step resulting in equal optical sections for all channels**

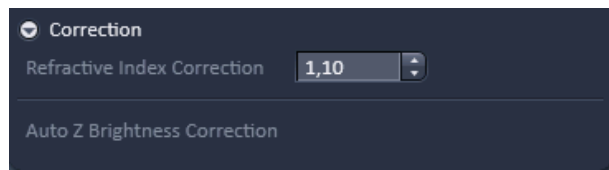


Fig. 82 Correction field

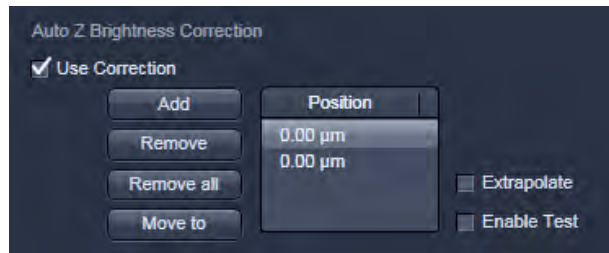


Fig. 83 Auto Z Brightness Correction

### 5.2.11.5 Correction

Expand the **Correction** field by pressing the triangle (Fig. 82). The **Refractive Index Correction input box** allows you to consider 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 by typing or using the arrow keys. The number should correspond to the ratio  $r=n/n'$ .

**Auto Z Brightness Correction** (Fig. 83) allows setting of different values for the scan parameters **Gain (Master)**, **Digital Offset**, **Digital Gain** and **Laser Attenuation** of several freely selectable slices within a Z-Stack. During the scan procedure, the interim values of these three parameters are automatically linearly interpolated between the initial and end values. This function can be activated and deactivated with the check box **Use Correction**. **Add** takes the above mentioned parameters and the actual **Focus Position** and correlates them with a given position (for example the first or the last slice of a stack).

**Move to** moves the actual focus to the position highlighted in the list without scanning. Starting a **Continuous** scan then allows setting or correcting the parameters and storing them again hitting **Add**.

When **Enable Test** is active, scanning at single positions of the stack or while focusing through the stack is done using the settings for a given z-position or the interpolated values calculated for the respective focus position. This allows controlling the settings before starting the actual acquisition of the stack.

## 5.2.12 Multidimensional Acquisition – Bleaching

The use of this function permits setting the bleach parameters. The bleaching of the sample can be observed sequentially with the LSM 700.

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 **Acquisition Parameter – Regions** ) 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 6: ANNEX).

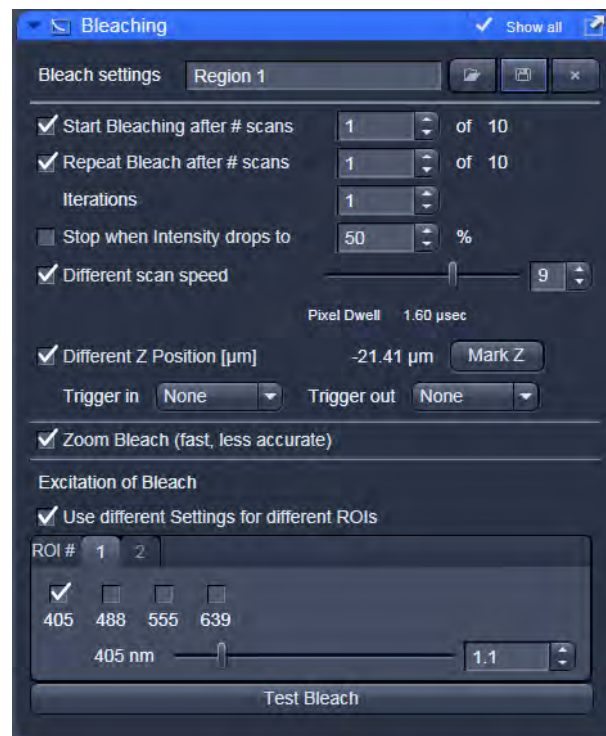


Fig. 84 Bleach Control window

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.



**Fig. 85** Excitation of Bleach area for setting laser line and intensity for bleaching

Define the region(s) for bleaching in the Regions tool (see section **Acquisition Parameter – Regions**). Only a region activated under Bleach is considered for bleaching. In the **Excitation of Bleach** area (Fig. 85) choose 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.

**Test Bleach** performs one bleach cycle ignoring the time series settings. This can be used to test the bleaching before using the protocol for the actual experiment.

### 5.2.13 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 Gigabytes.

**Interval** defines a potential time interval, set in milliseconds (msec), 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.

An **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.

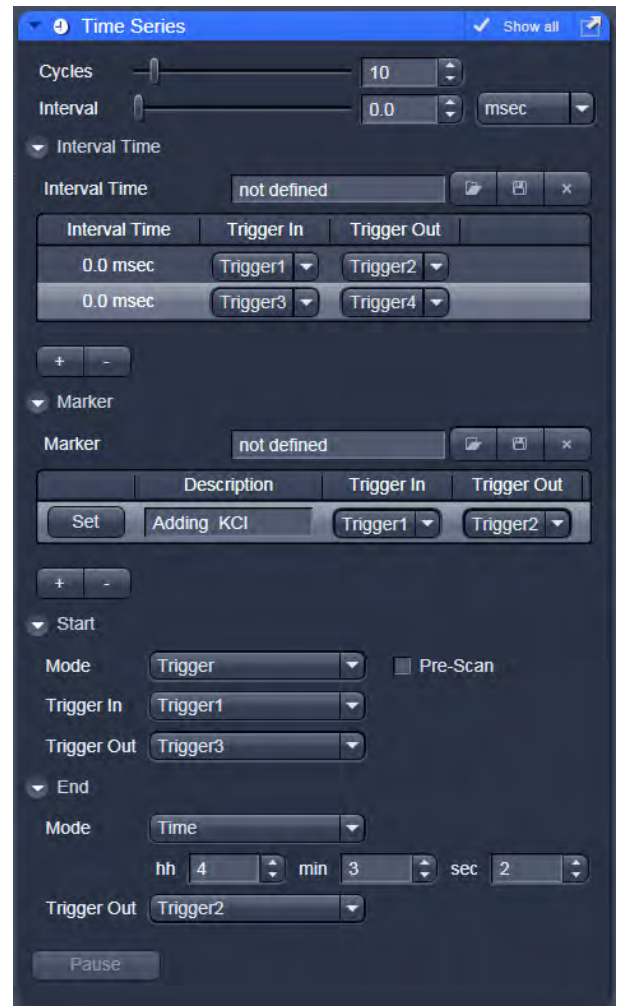


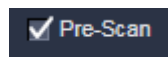
Fig. 86 Time Series tool


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 labeling 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 timepoints is below 11s – the focus stabilizer will not be activated between the two timepoints.



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.



### 5.2.14 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 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.

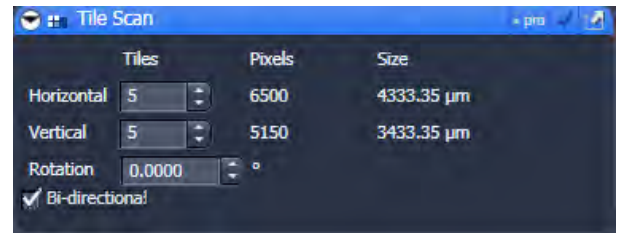


Fig. 87 Tile Scan tool

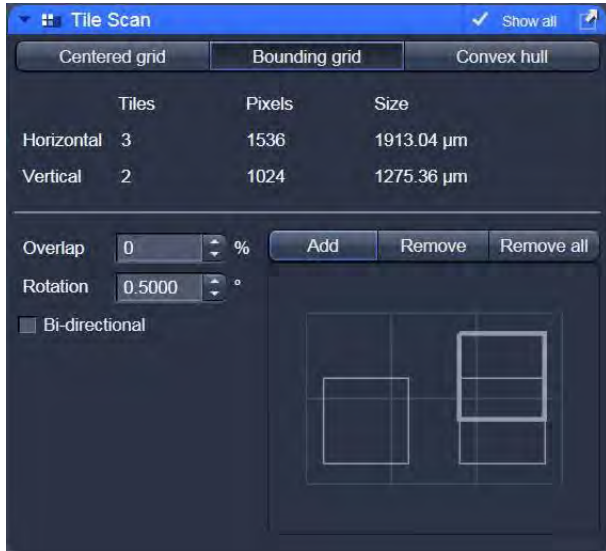
#### Tile Scan with StitchArt plus

The StitchArt plus license allows to create 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.

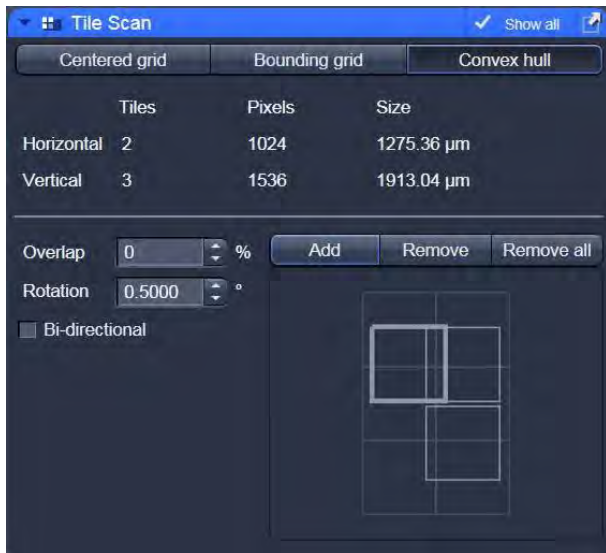


Fig. 88 Tile Scan tool with StitchArt plus



**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.

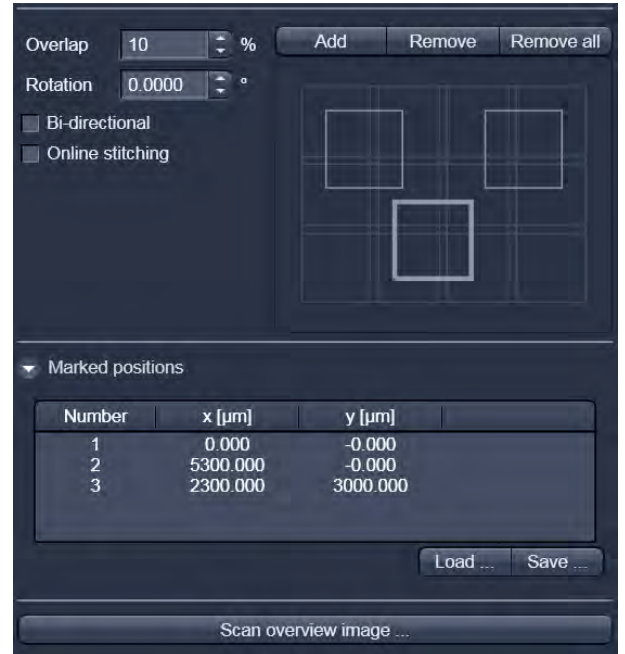
Fig. 89 Tile Scan: Bounding grid



**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. 90 Tile Scan: Convex hull

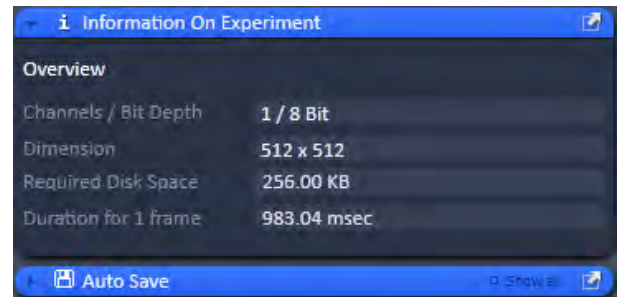
**Marked positions** are listed up. This list can be saved and loaded.



**Fig. 91 Tile Scan: Marked positions**

### 5.2.15 Multidimensional Acquisition – Information On Experiment

This tool displays the information on 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.



**Fig. 92 Information on Experiment**

## 5.2.16 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.

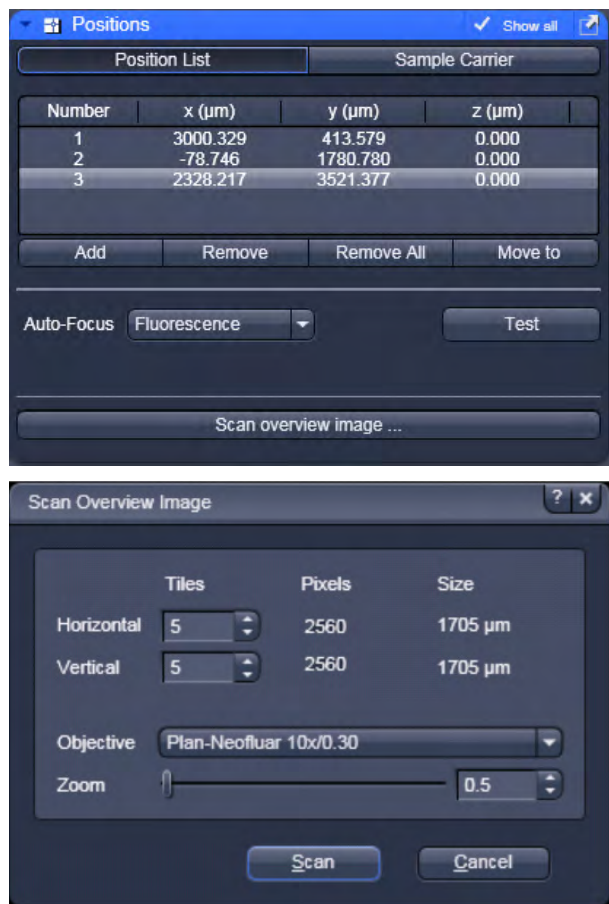



Fig. 93 Positions Tool, Position List tab; sub-tab Scan Overview Image

Using the first tab, **Position List** (Fig. 93), 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 **Acquisition Parameter – Stage**).

 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. 93) to define the number of **Tiles**, the **Objective** (choose 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.

Using the second tab **Sample Carrier** (Fig. 94) 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. 95).

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.

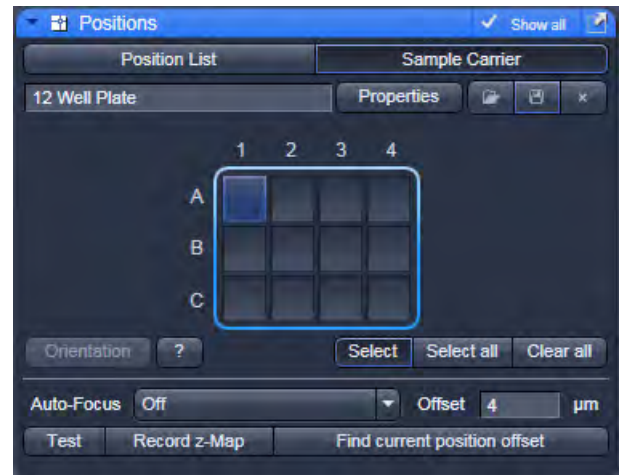


Fig. 94 Positions Tool, Sample Carrier tab

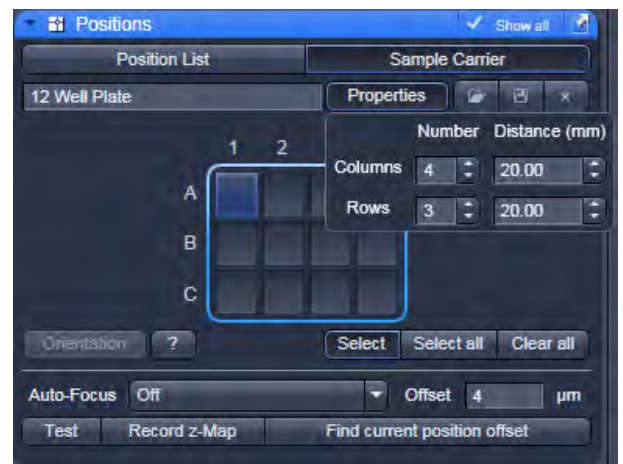
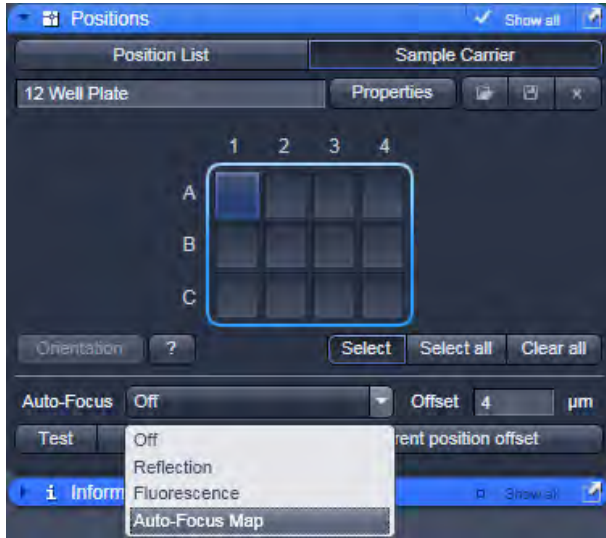
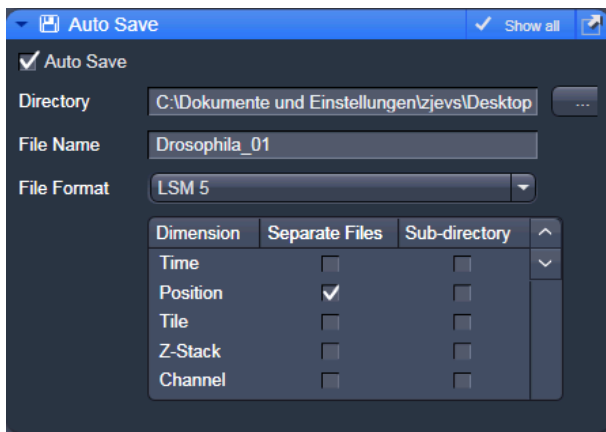


Fig. 95 Positions Tool, Sample Carrier tab, Properties interface



**Fig. 96** 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. 96). 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.



**Fig. 97** Auto Save dialog

### 5.2.17 Multidimensional Acquisition – Auto Save

Images which are acquired clicking the **Start Experiment** button can be automatically saved by activating the **Auto Save** tool (Fig. 97).

The image series can be saved in a user defined directory using an arbitrary file name. The file name (or name of the sub-directory) is extended by the exact date and time of the image acquisition.

In addition the letters t, z and p and a number is used to indicate the image position in a Z-Stack or time series for example. There is a list of file formats which can be chosen alternatively to the lsm format to store the data.

Note, for each individual image a separate file will be generated.

### 5.2.18 Image Optimization

This section describes an example how to acquire an image, using an excitation wavelength of 488nm and a fluorescence emission range above 510 nm. We presume that no predefined configuration is available for this imaging task and that Smart Setup is not used to do this task.


The description applies to the use of any microscope.

#### 5.2.18.1 Requirements

- Click on the **Locate** tab on the **Left Tool Area** and set the microscope according to your needs.
- Position the specimen under the objective and focus onto the desired imaging plane.
- Click **Acquisition** tab in the **Left Tool Area** toolbar.

Click on **Show all tools** to display the tools in the tool group **Setup Manager**.

- Open the **Light Path** tool. Choose **Channel** mode for the image acquisition (Fig. 98).
- Click on the laser icon, select the 488 line, and set the transmission slider to about 2 %. Make sure other lasers are not checked (Fig. 99).

 Optimize the laser intensity subsequently via the transmission slider when imaging.

- Set the **VSD** to the position 500 nm and activate PMT 2 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 LUT part of the list to open the color table and select the desired color (Fig. 100).
- Click on the **Dye** pull down list and select the desired dye (e.g. FITC). This will display the emission spectrum of the desired dye in the upper part of the light path.
- If required, deactivate all the other channels (Ch1, transmission, R1-2) via the check boxes.

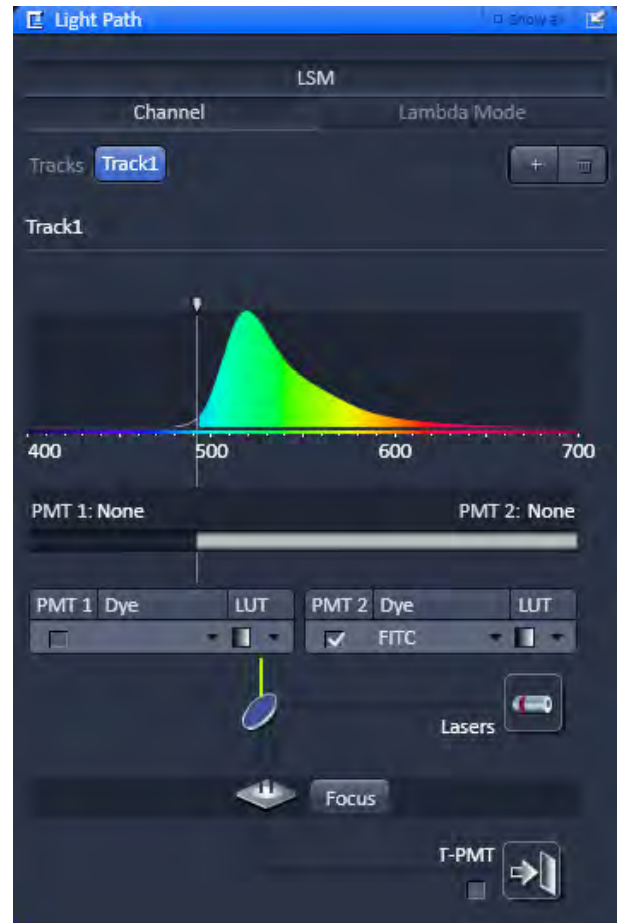


Fig. 98 Light Path tool

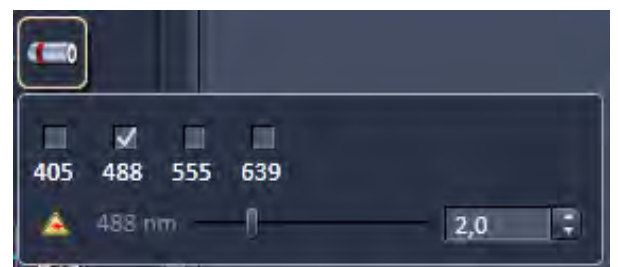


Fig. 99 Laser line and laser power selection

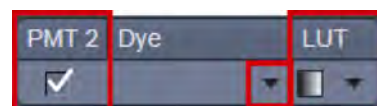
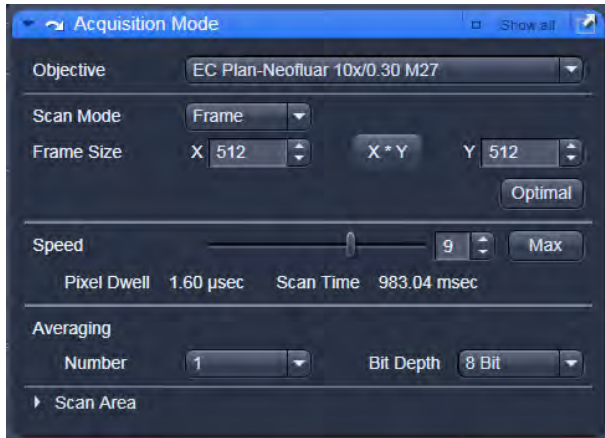
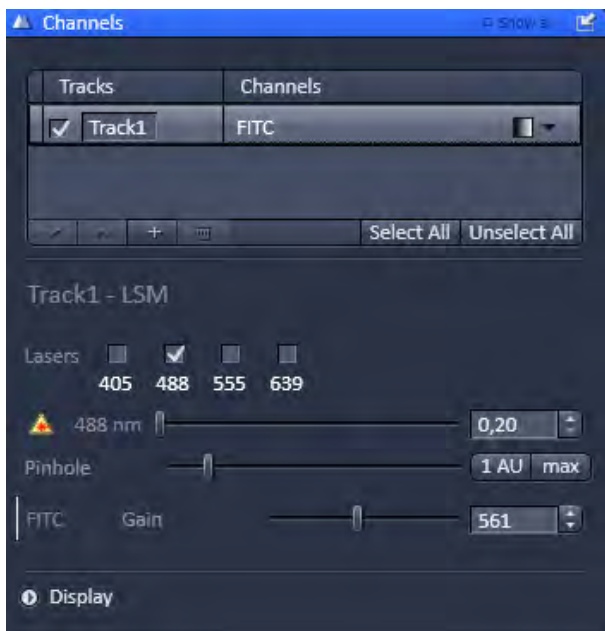


Fig. 100 Channel activation and color selection



**Fig. 101 Acquisition Mode tool**

- Start with the following settings on the **Pixel Depth, Scan Direction & Scan Average** panel:  
Data depth: 8 bits  
Average: Number: 1 (no averaging)
- ☞ The default settings for Acquisition Mode are suitable for good image quality and do not need to be changed for initial scanning.



**Fig. 102 Channels tool**

### 5.2.18.2 Defining the Scan Parameters

- Open the **Acquisition Mode** tool (Fig. 101).
- 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.

### 5.2.18.3 Defining the Detection Parameters

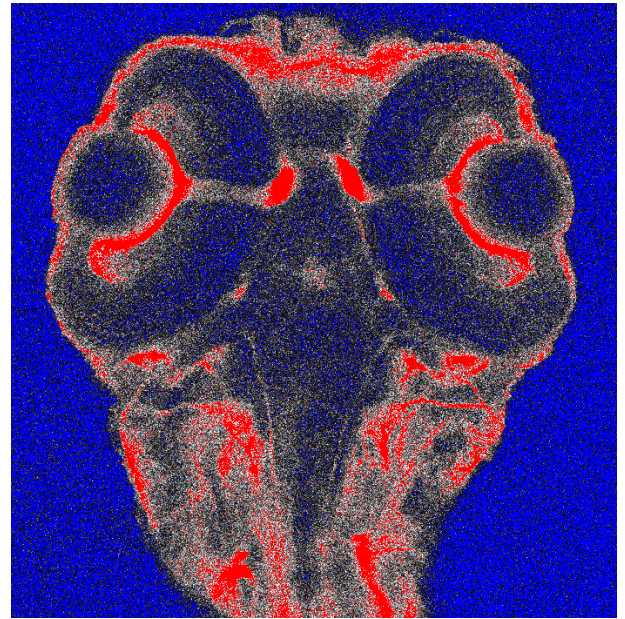
- Activate the **Channels** tool (Fig. 102).
- Set the **Pinhole** to two Airy Units using the slider. The value for the pinhole is displayed in the tooltip.
  - The pinhole diameter should be so small that there is still enough variation for the setting of the **Gain (Master)** and that sufficient image 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 0.3 Airy Units).
- Use the **Set 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 **Digital 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. 103** Image displaying bad adjustment of gain and offset (blue regions are underexposed, red regions are overexposed)

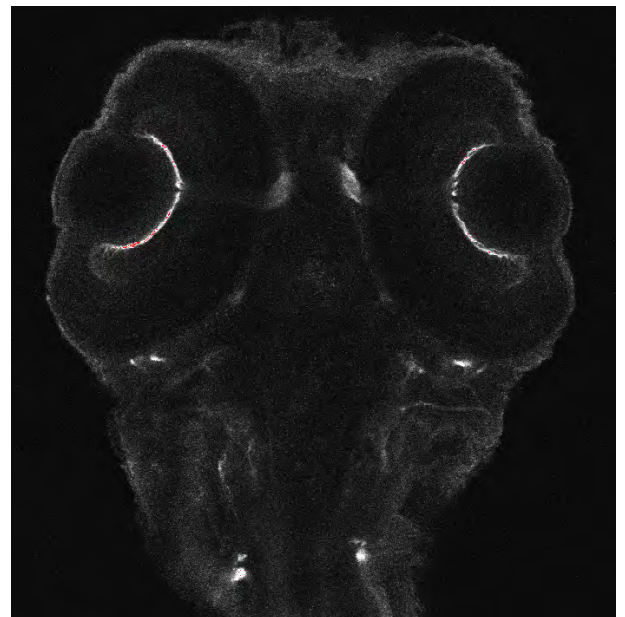
- To adjust the black level (background), use the **Digital Offset** slider so that areas without picture content just show a trace of blue.
- If necessary, re-amplify brightness with the **Digital Gain** slider.

 Do not change the **Digital Gain** setting unless the settings made so far are insufficient for image optimization.


- Stop scanning.
- Save the imaging configuration again as this time it will also save the correct settings for the detector, the laser, and the pinhole.

The signal-to-noise ratio can be substantially improved by reducing the scan speed to an acceptable level and averaging over several scans (i.e. with an average **Number** greater than 1 for the **Mean** average **Method** in the **Scan Control** window).

- Use the **Speed** slider in the **Acquisition Mode** tool set the slowest acceptable scan speed.
- In the **Number** text box of the **Averaging** panel enter the number of images to be averaged.



**Fig. 104** Image displaying good adjustment of gain and offset

-  Image optimization can be achieved faster if you select a smaller frame, since less data have to be processed.  
The greater the number of averages selected using **Mean** as average **Method**, the better the image quality will be; the scan time is prolonged accordingly.

A similar procedure applies for image optimization if more than one channel is used and simultaneous image acquisition is performed.

Make sure the suitable lasers are switched on and the channels are activated.

Pay attention to address the right channel when **Pinhole** size, **Gain (Master)**, and **Digital 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

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.

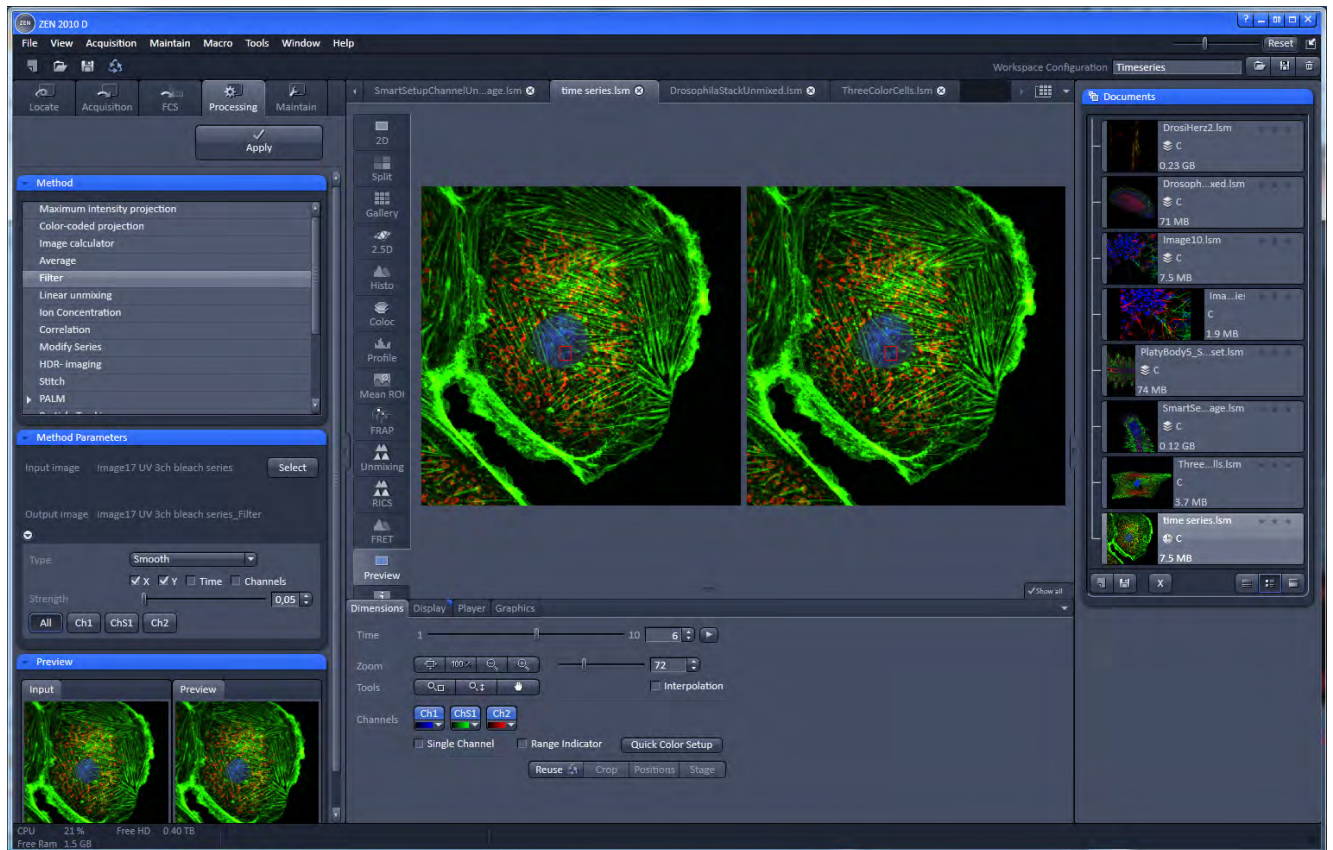


Fig. 105 The Main Application window with the Process main tab activated

By clicking on the third of the four **Main** tool tabs in the top left corner of the **Main Application** window (see Fig. 105), the list of available processing tools becomes available.



- 1 Main tool tabs
- 2 Apply button
- 3 Method panel with the available processing methods
- 4 Method Parameters panel with the input image and output image selection field
- 5 Method Parameters panel with with the method-specific settings
- 6 Preview panel with input and preview thumbnails

**Fig. 106 The Process tab structure**

The **Processing** tool tab is structured as follows (see Fig. 106): On the top, under the **Main** tool

tab, is the Processing tab header with the **Apply** button. To process a loaded image, first select the required methods from the List of available processing methods (Fig. 106); select the active image as the input image with the **Select** button(s). The thumbnail panel at the bottom of the Processing tab now shows thumbnails of the selected input images and a preview of the processing result. In the method-specific settings, all the parameters for the active methods can be set, edited or viewed.

When an input image in a processing tool is selected, in addition to the Preview panel at the bottom of the Processing tab, a new **View** tab – the **Preview** tab - in the Center 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 Center Screen Area – is immediately updated.

When everything is set, click **Apply** on the top of the **Processing** tab and the set processing action will be performed.

All actions in the Processing tab automatically generate a new image document if the Processing Output is not explicitly directed to an already existing channel or image.

### 5.3.1 Processing – 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. 107).

- To open the **Maximum Intensity Projection** Processing tool, select it from the list in the **Processing Main** tab.
- Click on the triangle buttons to show or hide the **Method Parameters** and **Preview** panels of the function.
- Select the **Input Image** with the  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  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.

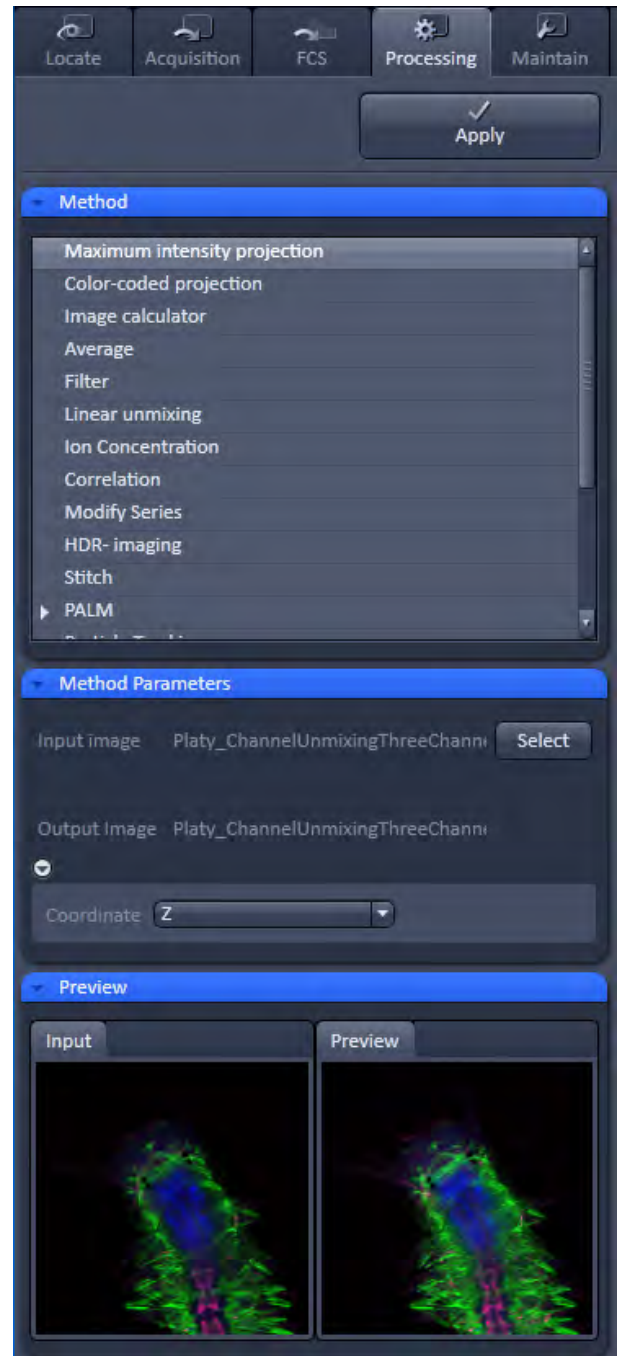


Fig. 107 Maximum Intensity Projection tool

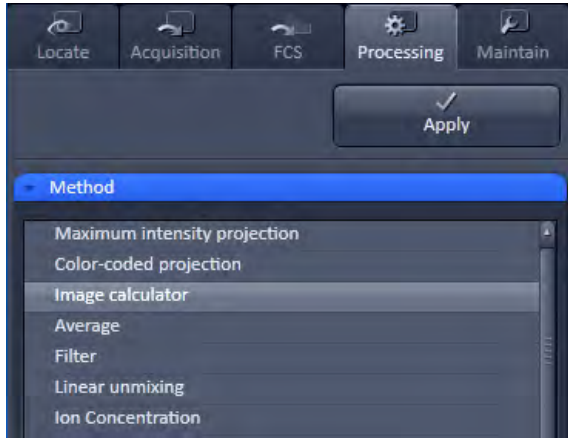


Fig. 108 Activating the Image Calculator

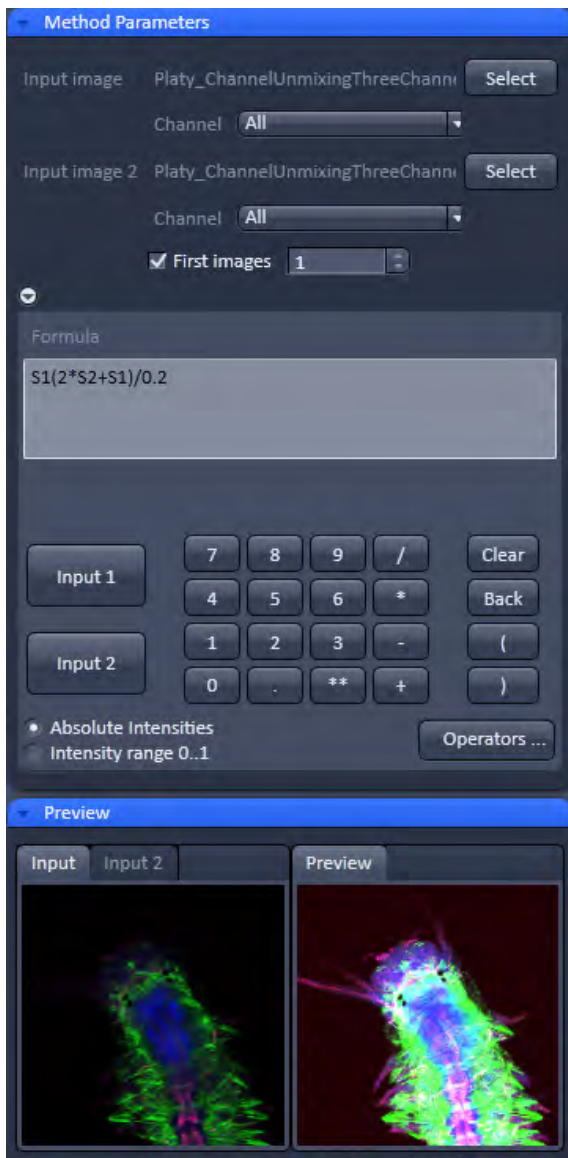


Fig. 109 The Image Calculator window

### 5.3.2 Processing – 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. 108), click on the **Image calculator** in the list of the **Method** panel in the **Processing** tool tab.
- Click on the triangle buttons to show or hide the **Method Parameters** and **Preview** panels (Fig. 109) of the function.

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. 109).

By highlighting the selected operator a description of the function of this operator is displayed in the lower part of the operators list (Fig. 110).

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.

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 created.

A good example for an application of the Image Calculator is offsetting/normalizing two images or an image series with a reference image to enhance contrast for small changes in intensity:

- Select a time series as **Input 1** and the first frames of this series (or another offset image) as **Input 2**.
- Then type/edit an operator as shown in figure Fig. 111 that enhances the relative changes in the image with respect to the relatively bright signal level, for example:  

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

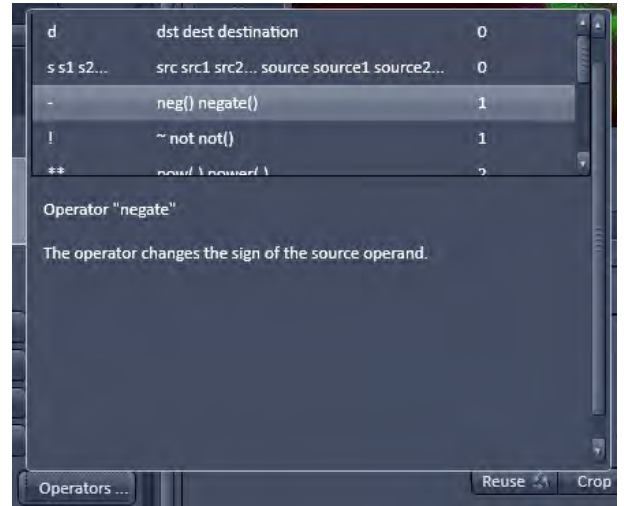


Fig. 110 Operators window



Fig. 111 Image calculation window

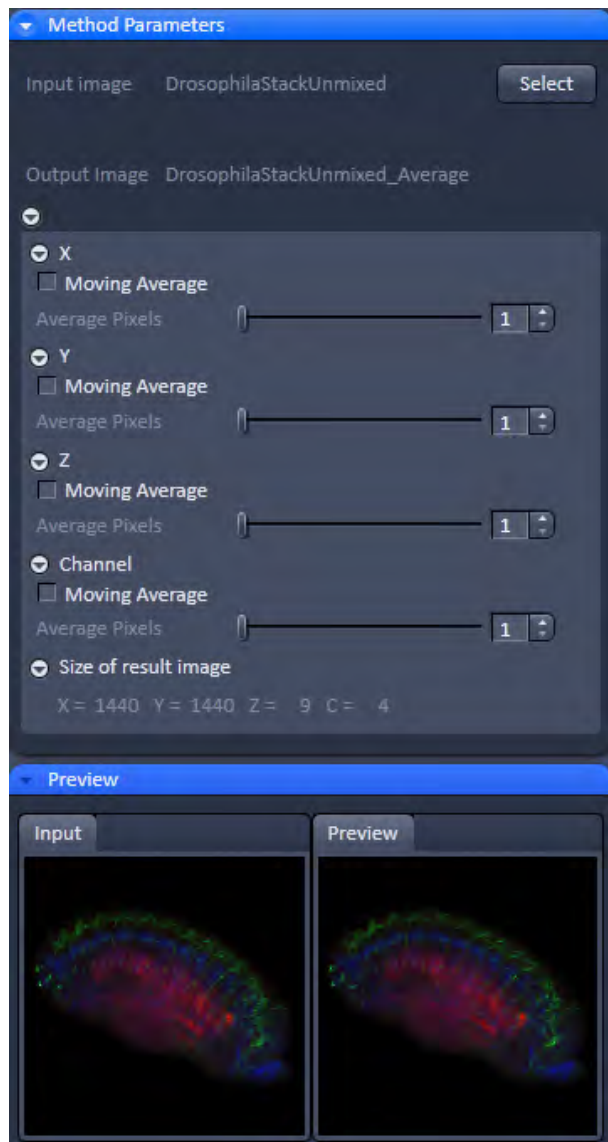


Fig. 112 Average window


### 5.3.3 Processing – 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. The **Average** processing function will calculate an average image.

- Click on the triangle buttons to show or hide the **Method Parameters** and **Preview** panels (Fig. 112) of the function.
- 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.



### 5.3.4 Processing – Filter

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

- To open the **Filter Processing** tool, select it from the list in the **Processing** main tab (Fig. 113).
- 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. 114). A larger kernel, however, also increases the processing time. Other settings of this filter cannot be modified.



Fig. 113 Filter window

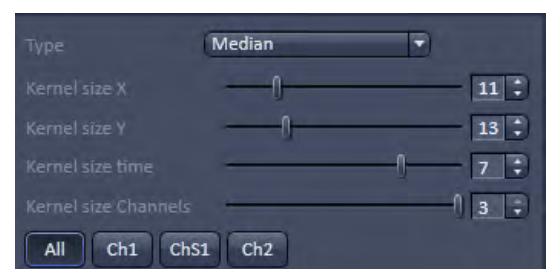


Fig. 114 Median filter settings

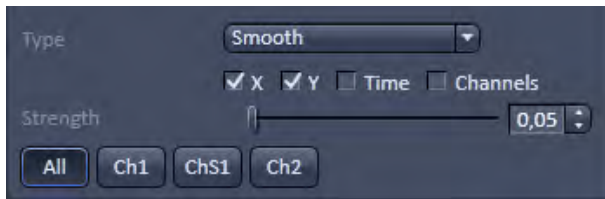


Fig. 115 Smooth filter settings

### 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. 115). 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.

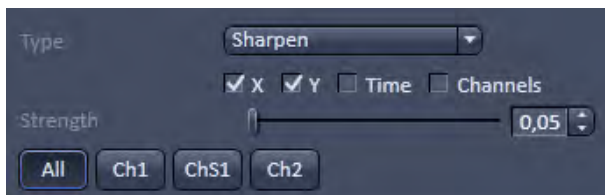


Fig. 116 Sharpen filter settings

### 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. 116) are the same as in the **Smooth Filter**.

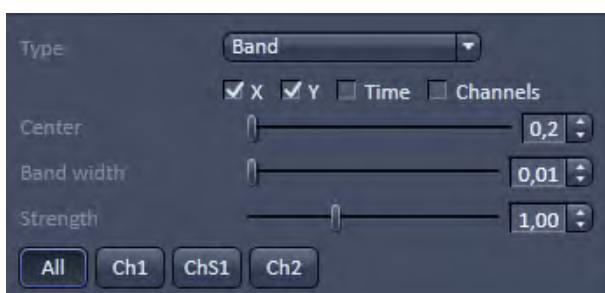


Fig. 117 Band filter settings

### 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.

### HDR offline mode

Two images with different exposures can be processed with the offline HDR function (see section **Frame Mode for LSM** for operation) to increase the grey dynamics and to improve the visibility of weak structures.

### 5.3.5 Processing – Correlation

- Activate **Correlation** in the **Processing** tool. The **Correlation** menu will appear (Fig. 118).

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.

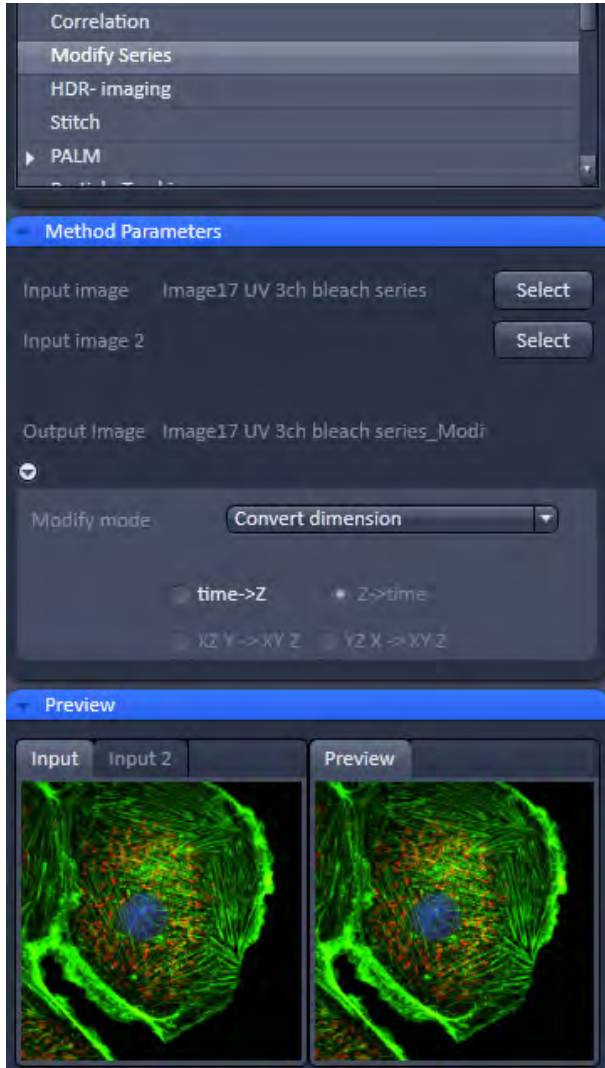
- You can select which kind of correlation you want to perform by activating the **X**, **Y**, **Z** and **Time** check boxes to perform spatial correlations in x, y and z-direction or a temporal correlation.

 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.



Fig. 118 Correlation window



### 5.3.6 Processing – Modify Series

This function allows to modify dimensions of images or image series (Fig. 119). Depending on the input image (for example **Z-Stack** or **Time Series**) the available modifications vary (Fig. 120). It allows modifying the acquisition date, rotate or mirror the image(s), convert or reverse dimensions or make time series from Z-stacks, tiles scans or images from various positions.

Fig. 119 Modify Series tool

#### Z-Stack options



#### Time Series options



Fig. 120 Modification options depending on the selected input image

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### 5.3.7 Processing – 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 analysis 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. 121** Linear Unmixing in the Processing tab



**Fig. 122** Linear Unmixing panel

- To open the **Linear unmixing** tool, click on **Linear unmixing** in the **Processing** tool list in the Processing tab (Fig. 121).
- 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. 122), 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. 123).
- Assign appropriate colors to the fluorescence channels by clicking on the color flag and choose from the available lookup tables (LUTs) (Fig. 123).
- 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. 124 and Fig. 125).

The following additional settings and functions are available for linear unmixing (see Fig. 122):

**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. 123** 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.

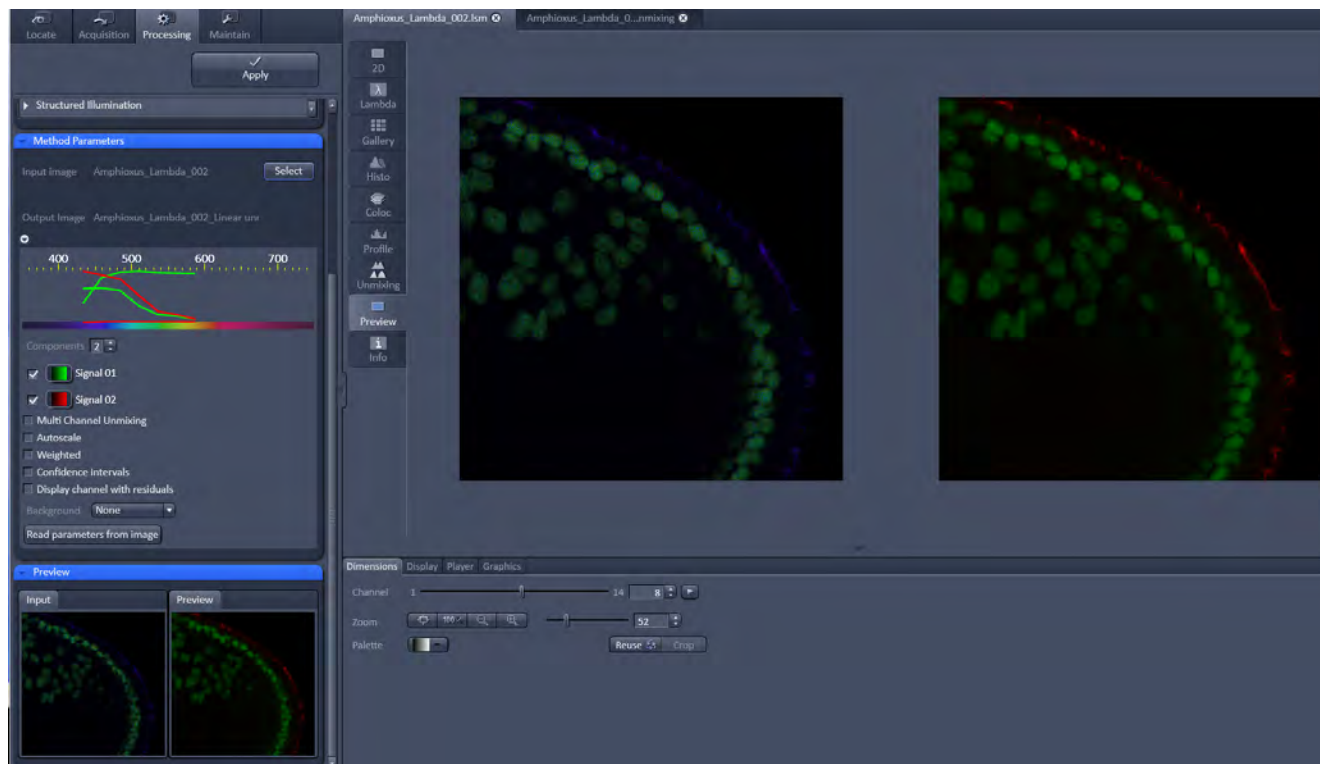


Fig. 124 Image Display window before unmixing in Preview



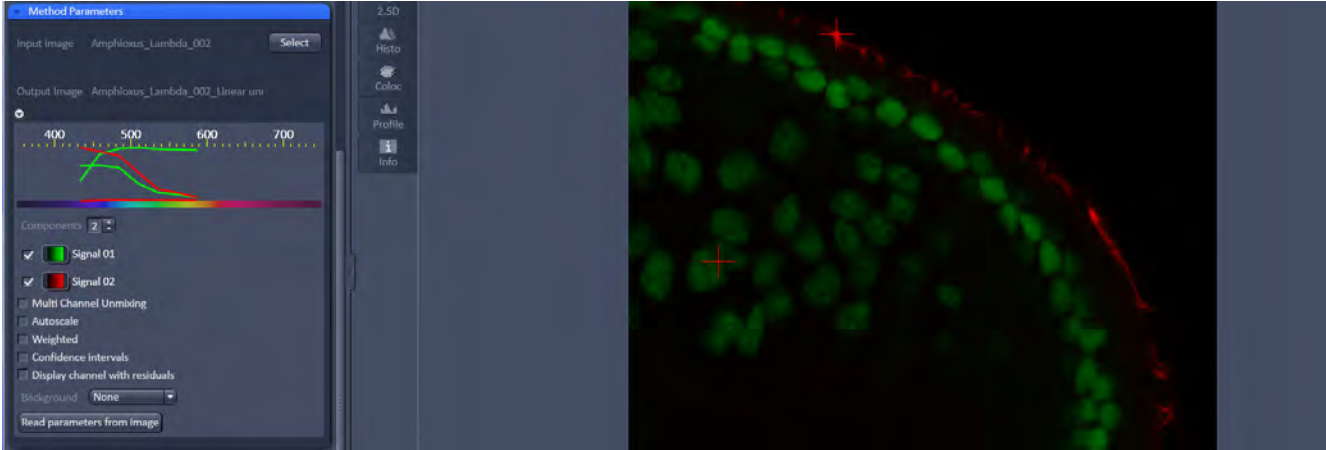


Fig. 125 Image Display window after unmixing



Fig. 126 Processing tab - Ion Concentration

### 5.3.8 Processing – Ion Concentration

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

To open the **Ion Concentration** tools click **Ion concentration** (Fig. 126).

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.

In the **Preview** panel 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 (Fig. 127).

After opening the **Method Parameters** panel by clicking the little arrow, the full tool window opens and the following functions are available (Fig. 128).

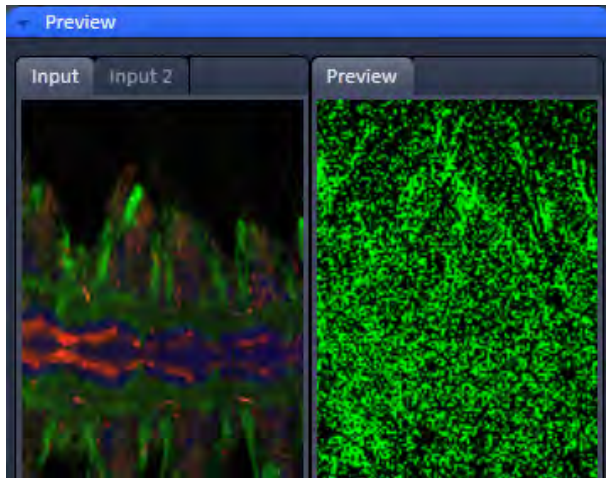
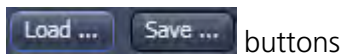


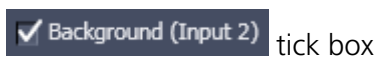
Fig. 127 Ion Concentration tool window



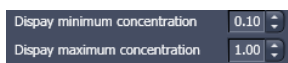
Opens the display for the resulting calibration curve.



To load & save calibration curves from / to files.



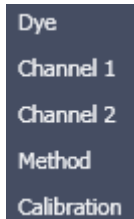
To select Input Image 2 as background image.



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

Image scaling

Pull down menus for



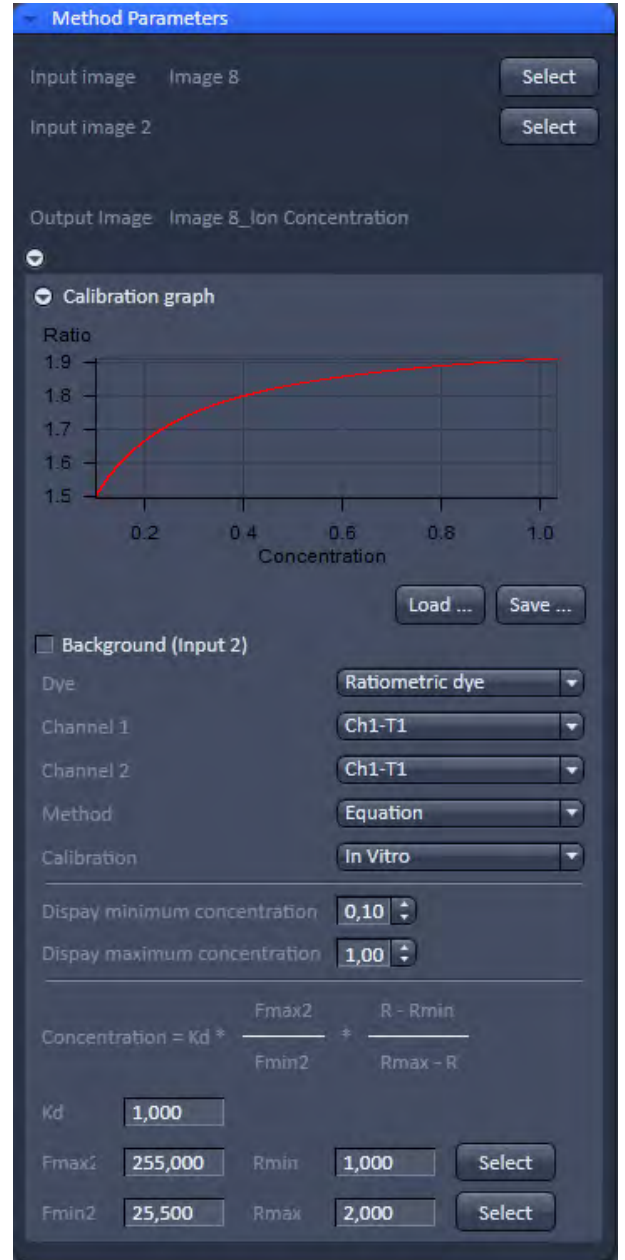
To set three different calibration options, according to the dyes (single wavelength, vs ratiometric), the calibration method and if the calibration is carried out in vitro/situ. Ch 1 and 2 select the channels from Input Image 1.

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. 128** Fully opened Ion Concentration tool window - Ratiometric dye in equation mode

### 5.3.8.1 Single Wavelength Dyes – Offline Calibration

- Subtract background/autofluorescence image from raw images to obtain better raw data to start with (Fig. 129).
- Perform equation or titration calibration (compare F with a calibration curve → titration calibration or put F values in calibration formula).

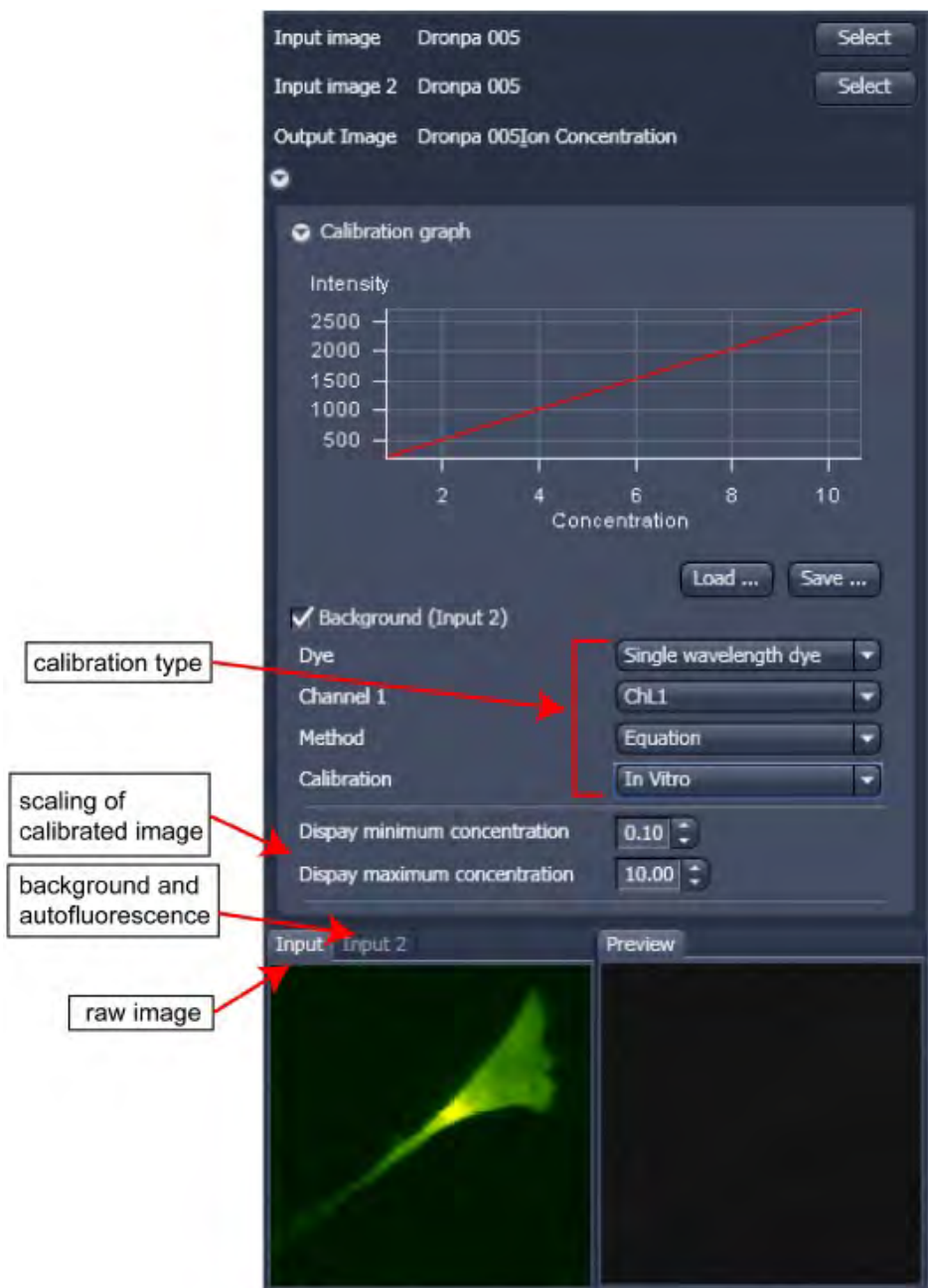


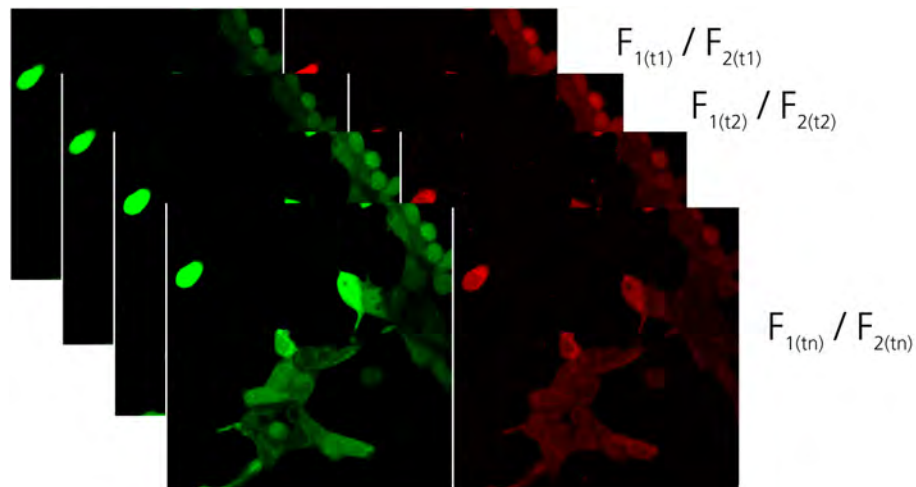
Fig. 129 Ion Concentration panel

### 5.3.8.2 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. 130)

$$R(t_1) = F_1(t_1) / F_2(t_1), R(t_2) = F_1(t_2) / F_2(t_2) \dots$$



**Fig. 130 Ratiometric Dyes – Online ratio**

#### Ratiometric Dyes - Calibration (Fig. 131)

- Subtract background/autofluorescence images from raw images to obtain

$$R_{\text{korr}} [= (F_1 - F_{1\text{Background}}) / (F_2 - F_{2\text{Background}})]$$

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 parameters: Input image 'Dronpa 005', Input image 2 'Dronpa 005', and Output Image 'Dronpa 005Ion Concentration'. The 'Background (Input 2)' is checked, and the 'Dye' is set to 'Ratiometric dye'. Channel 1 and Channel 2 are both set to 'ChL1'. The 'Method' and 'Calibration' are set to 'In Vitro'. The 'Display minimum concentration' is 0.10 and the 'Display maximum concentration' is 10.00.

**Left Screenshot (Titration Method):**  
 The 'Method' is set to 'Titration'. The 'Fit' is set to 'Hill function fit'. The 'Calibration table rows' is set to 4. The table below shows the calibration data:

Used	Concentration	Ratio	Select
<input checked="" type="checkbox"/>	5.000	300.000	Select
<input checked="" type="checkbox"/>	3.000	250.000	Select
<input checked="" type="checkbox"/>	2.000	180.000	Select
<input checked="" type="checkbox"/>	1.000	0.000	Select

**Right Screenshot (Equation Method):**  
 The 'Method' is set to 'Equation'. The 'Fit' is set to 'Equation'. The calibration formula is displayed as:

$$\text{Concentration} = K_d * \frac{F_{\text{max}2}}{F_{\text{min}2}} * \frac{R - R_{\text{min}}}{R_{\text{max}} - R}$$

The parameters are set as follows:

- Kd: 1.000
- Fmax2: 255.000
- Fmin2: 25.500
- Rmin: 1.000
- Rmax: 2.000

Both screenshots show a 'Calibration graph' with 'Ratio' on the y-axis and 'Concentration' on the x-axis. The left graph shows a red curve fitting the data points. The right graph shows a red curve fitting the data points. Both screenshots show 'Input' and 'Input 2' images (green fluorescence) and a 'Preview' image (grayscale). A red arrow points from the 'calibration type' label to the 'Equation' method selection in the right screenshot.

Fig. 131 Ratiometric Dyes - Calibration

**Ratiometric Dyes - Equation Calibration (Grynkiewicz)** (Fig. 132)

Fura-2, Indo-1, ..

$K_D$  (dissociation constant) taken from literature

$R_{min}$ : derived from ion-free state of the dye (e.g. 0  $Ca^{2+}$ )

$R_{max}$ : derived from ion-bound state of the dye (e.g. saturated with  $Ca^{2+}$ )

$F_{min2}$  and  $F_{max2}$  are the minimum and maximum fluorescence intensities at wavelength 2

$R_{min}$ ,  $R_{max}$ ,  $F_{min2}$  and  $F_{max2}$  may be determined in the cells under investigation (in situ) or in solutions (in vitro)

Calibration parameters may be saved and reloaded (\*.cal)

Dye: Ratiometric dye  
Channel 1: ChL1  
Channel 2: ChL1  
Method: Equation  
Calibration: In Vitro

Display minimum concentration: 0.10  
Display maximum concentration: 10.00

$$\text{Concentration} = K_d * \frac{F_{max2}}{F_{min2}} * \frac{R - R_{min}}{R_{max} - R}$$

Kd: 1.000  
Fmax2: 255.000  
Fmin2: 25.500  
Rmin: 1.000  
Rmax: 2.000

**Fig. 132 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)

### 5.3.9 Processing – Stitch

With this function tiled images acquired with Versions of ZEN 2011 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.

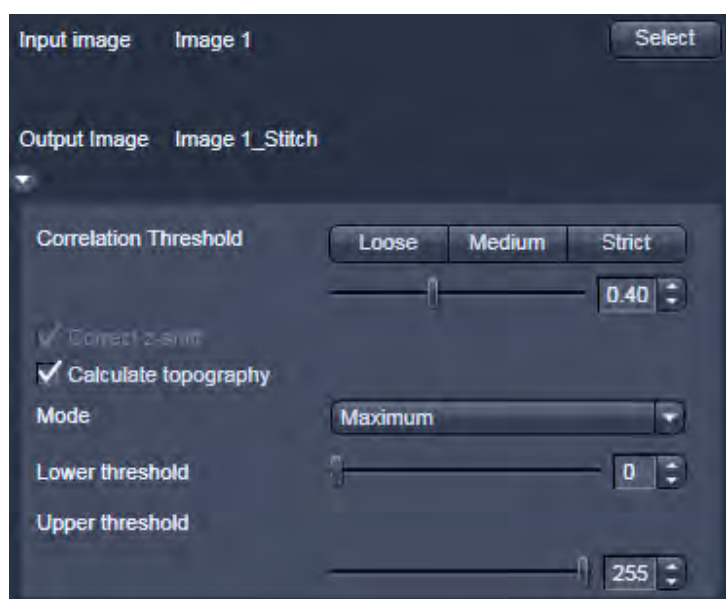


Fig. 133 Stitch function

**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.



### 5.3.10 Processing – Copy

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

**Copy Channel** (Fig. 134), produces a copy of the selected image.

The first **Select** button in the selection panel selects the active image in the **Image Display** as Input Image. The **Channel All** selection pull down specifies which channel to copy.

The second **Select** button is used to choose a destination image if the copied channel is to be added to an existing image document. In the second **Channel New image 8-bit** pull down menu, the destination channel is specified – or a new image document can be chosen as destination.

**Apply** starts the copy process.

☞ For Z-Stacks or Time Series, the entire series of the selected channel is copied.

#### Duplication

**Duplication** creates a new image document which is a duplicate of the selected input image.

#### Delete Image

Single images or series of images can be deleted from the selected image series (input image) acquired as time series or Z-Stacks or combinations of those.

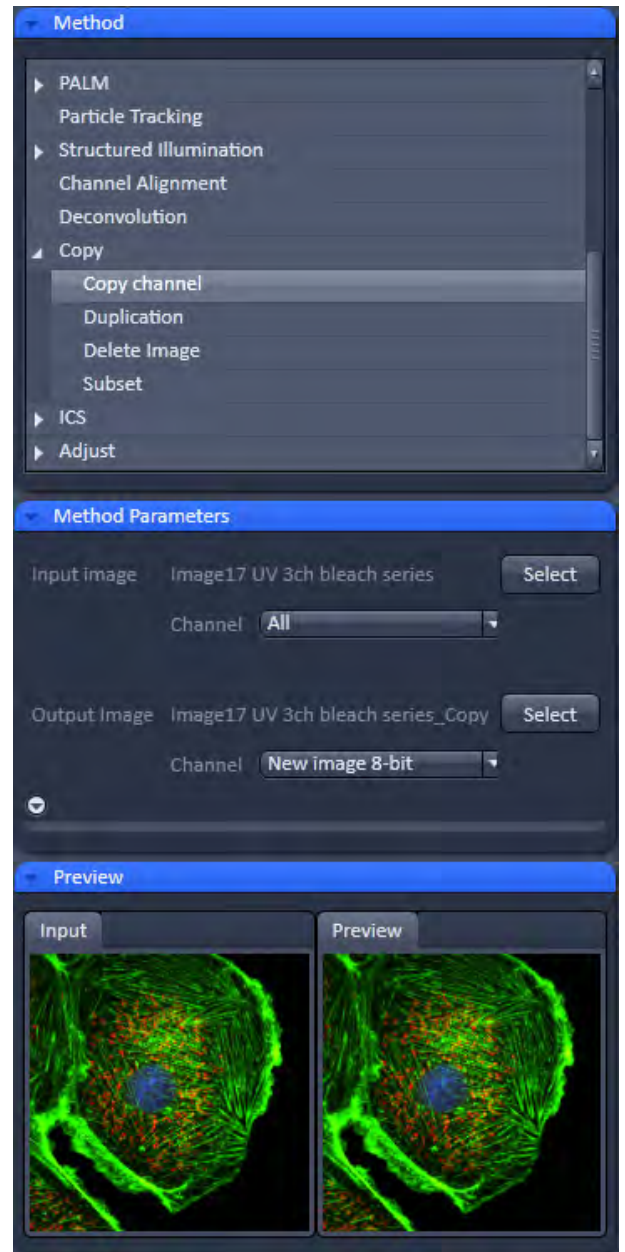
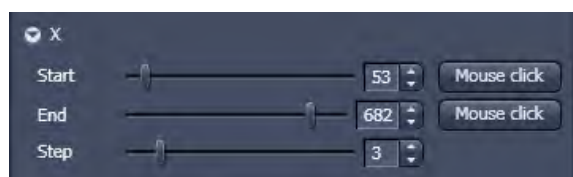


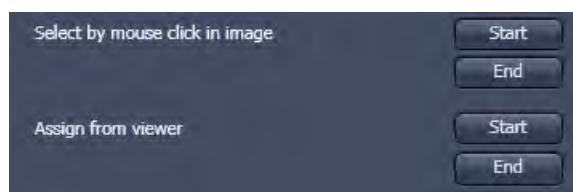
Fig. 134 Copy window



**Fig. 135** Subset tool with loaded Multiposition Tile-Scan



**Fig. 136** Subset panel for the x coordinate



**Fig. 137** 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. 135).

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. 136) 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 (Center Screen Area). The **Step** coordinate determines how many data points in the respective dimension are dropped out.

For example: setting **Step** to 3 takes 1 and drops 2 pixels along the respective dimension, reducing the data to 1/3. The scaling is calculated to stay correct in the resulting image.

This subset-panels are available for all coordinates of the selected image (x, y, z, t, channel, position and tile). The panel **Selection for all visible coordinates** is shown in Fig. 137. It always applies only to those coordinates with the subset-panel (above) open.

The **Select by mouse click in image** option allows to click in the Image Display and the respective coordinates are set (if displayed in the image: x, y, tile in the 2D view, z and position in the gallery view, channel in the split view, etc).

The assign from viewer selects the coordinate from the slider in the **Dimensions View Options** control block.

### 5.3.11 Processing – Adjust

#### 5.3.11.1 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. 138).

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.

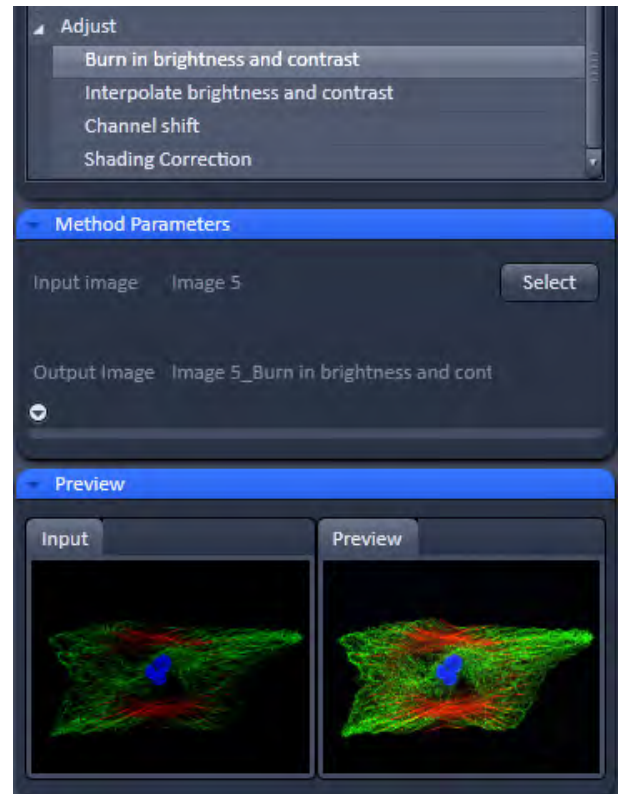


Fig. 138 Burn in Brightness and contrast tool

#### 5.3.11.2 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. 139).

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

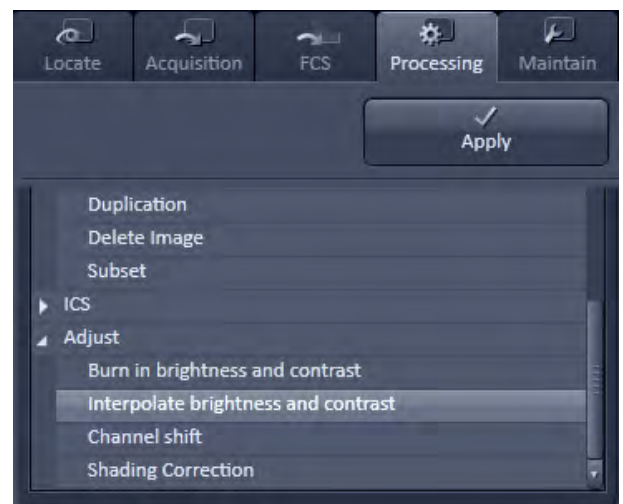


Fig. 139 Interpolate brightness and contrast tool



**Fig. 140** Interpolation brightness and contrast tool with loaded Z-Stack

In the **Interpolation** panel (Fig. 140) 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.

### 5.3.11.3 Channel Shift

**Channel Shift** is used to produce a congruent image with relation to the pixels of the various channels (Fig. 141).

This pixel correction function is particularly important in UV applications.

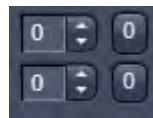
 This is a manipulation of your data that has to be considered very carefully when co-localization analysis is part of your experiment.

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

The image has to be a multi-channel image.

- Select the channels you want shift by ticking the **Ch1** or **Ch2** check box:  Ch1-T1  Ch2-T2

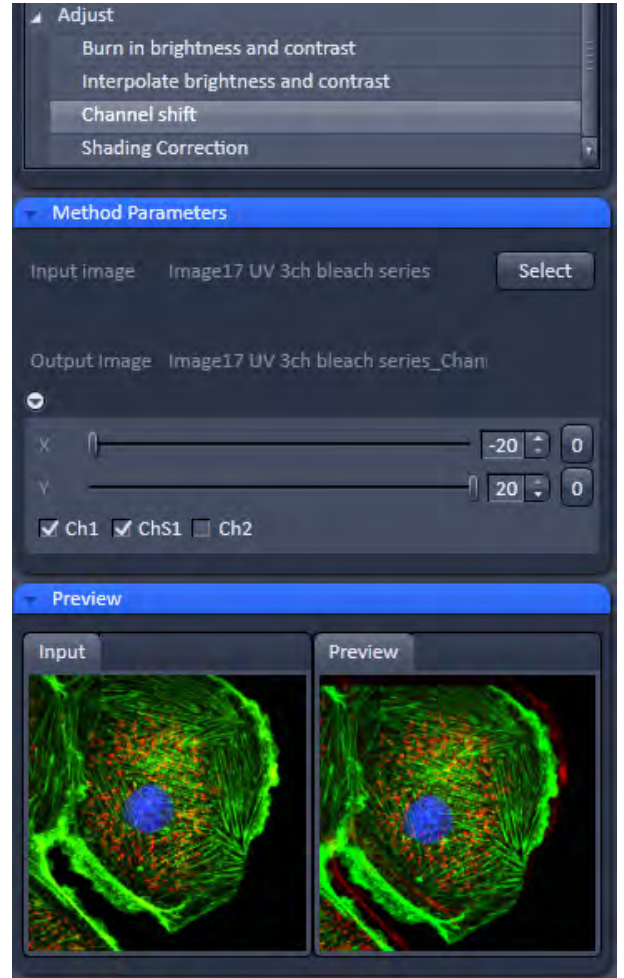
- Use the sliders



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 are and at the same time. A large preview is shown in the Preview view tab in the Image display in the Center Screen Area.

- Click on the **Apply** button to generate a new image with the set pixel shift.



**Fig. 141 Channel Shift window**

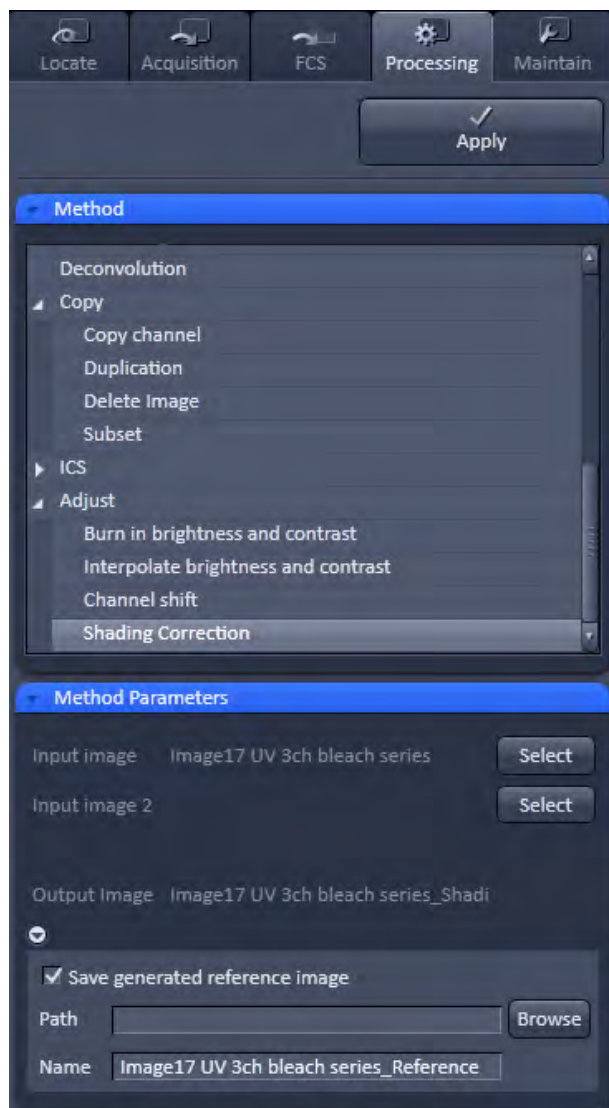


Fig. 142 Shading Correction interface

#### 5.3.11.4 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 Tab

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

### 5.4.1 Maintain – Adjust Pinhole

This function permits pinholes 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** tool of the **Maintain** tool group (see Fig. 143).

The position of the pinhole (X-Y 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.

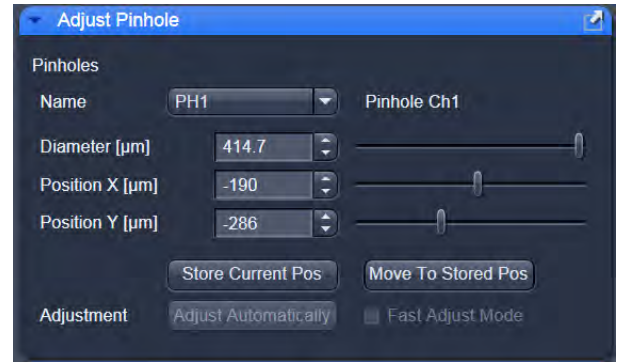


Fig. 143 Adjust Pinhole tool

### 5.4.1.1 Pinholes Section Panel

The **Pinholes** section panel has the following options (see Fig. 144):

- Name display box                      Selection of Pinholes (PH1 to 4) to be adjusted via the drop down menu.
  
- Description** field:                      Display of the relevant active channel.
  
- Diameter [µm]** slider                      Setting of diameter, X-, Y- position of the pinhole in relation to the beam path using the slider or arrow buttons
- Position X[µm]** slider
- Position Y[µm]** slider
- Store Current Position** button:                      Storage of the current pinhole setting.
  
- Move to Stored Pos** button:                      Pinhole setting is reset to the position last stored.
  
- Adjustment: Adjust Automatically** button:                      Automatic pinhole adjustment.
  
- Fast Adjust Mode** check box:                      If this check box is activated, the pinhole adjustment is only performed in a limited area. Used for readjustment.

No further software function can be activated and executed while automatic pinhole adjustment is running.

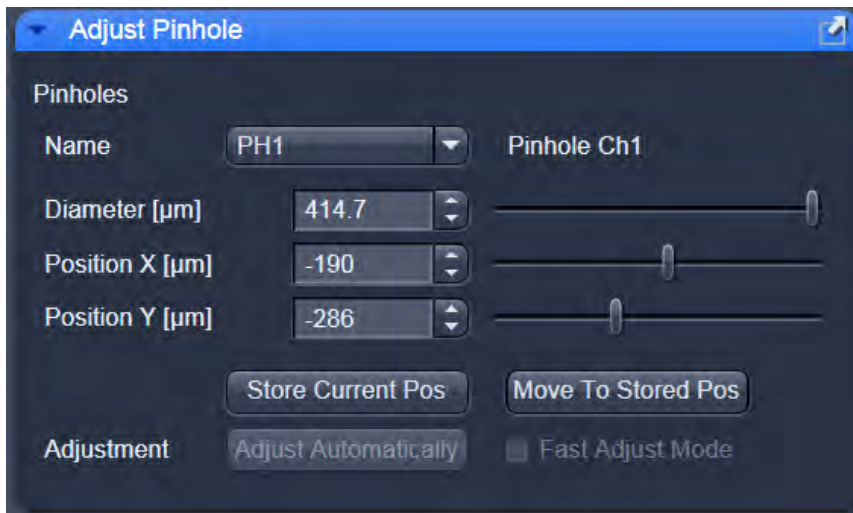


Fig. 144 Pinholes section panel



### 5.4.1.2 How to Adjust the Pinhole

Adjustment of pinholes can be performed manually or automatically.

If several channels are used to produce the image, all the used pinholes must be adjusted separately.

#### Manual adjustment

The position of the pinhole relative to the detector in terms of X-Y 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. 145).
- Open the **Adjust Pinhole** tool in the **Maintain** tool group in the **Maintain** panel.



Fig. 145 Channels tool

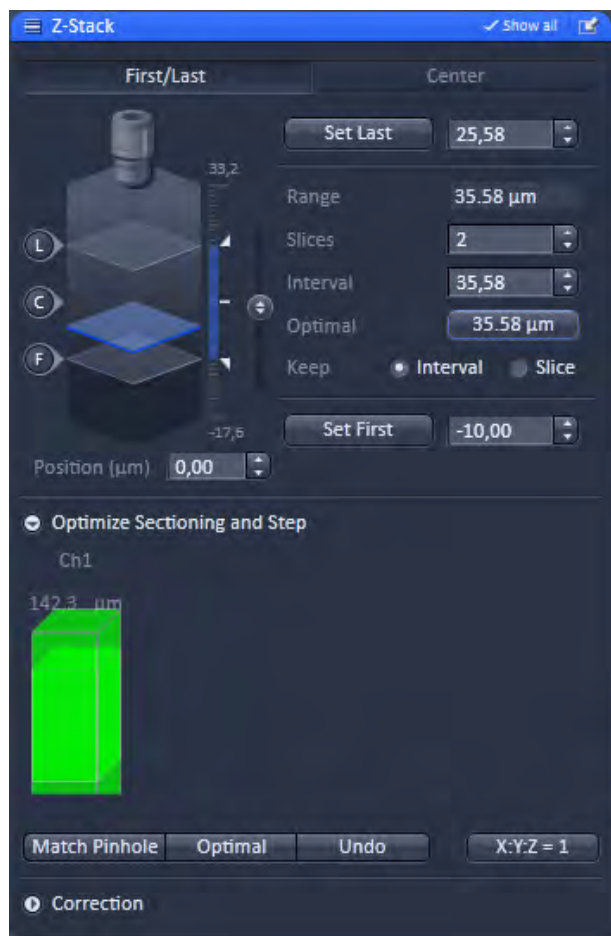


Fig. 146 Z-Stack tool

- 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** button of the **Z-Stack** tool in simultaneously displays the depth resolution corresponding to the pinhole diameter (see Fig. 146).
- ☞ Image optimization can be done with the **Range Indicator** or in the **Line-Scan** mode.



Fig. 147 Manual pinhole adjustment

- Click the LSM **Continuous** action button to start a continuous scan. Optimize the pinhole position in X, Y relative to the PMT using the **Position X [µm]** and **Position Y [µm]** sliders to maximize image brightness (see Fig. 147).
- Click on the **Current Pos** button to save the pinhole adjustment.
- Click on the LSM **Stop** action button to stop the continuous scan.

### 5.4.2 Maintain – 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. 148).

To start the image field curvature compensation click **Topography Calibration**. This only affects the topography calculations based on Z-Stacks.

#### 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. 149).
- 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. 150).
- 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.

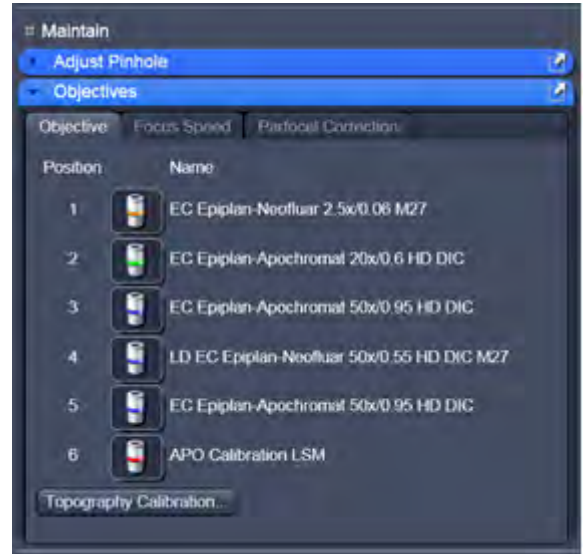


Fig. 148 Maintain and Options tool groups

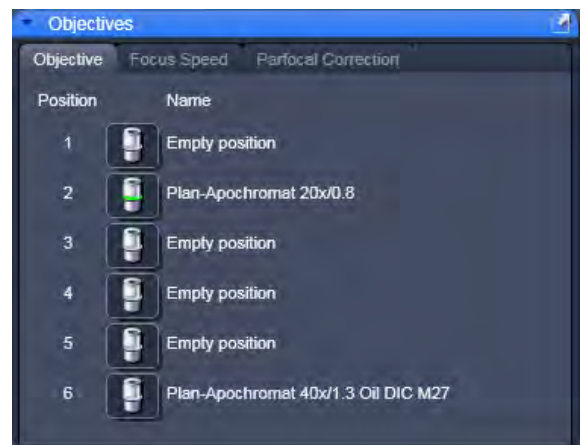


Fig. 149 Objectives tool

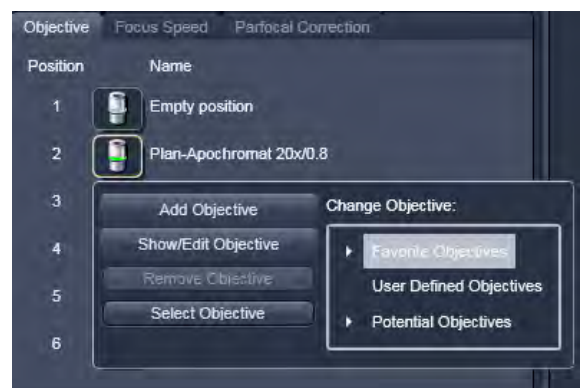
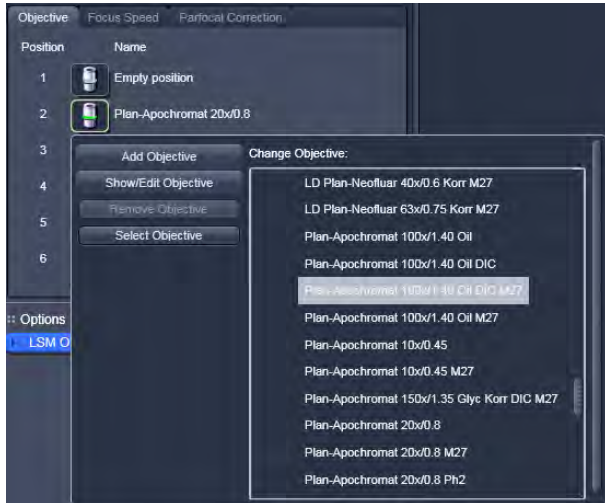


Fig. 150 Change objective window



**Fig. 151 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. 151).
  - An expansion (**Create new Objective**) window is opened (Fig. 152).
- 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**.

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



**Fig. 152 Editing a new objective**

### 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. 153).

- 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. 153 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.

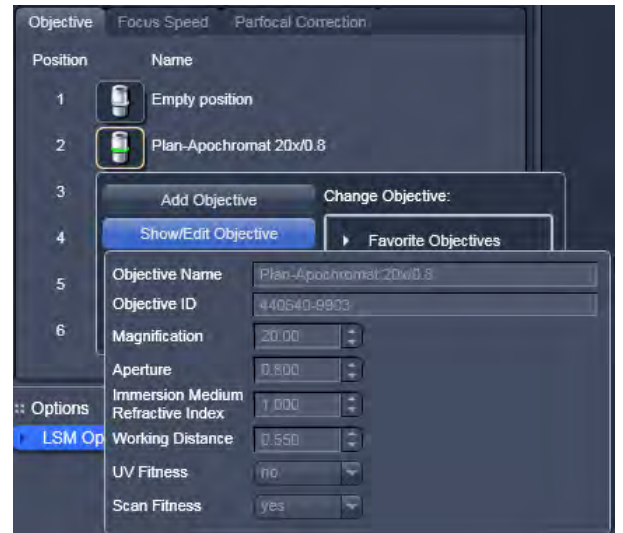


Fig. 154 Edit an objective

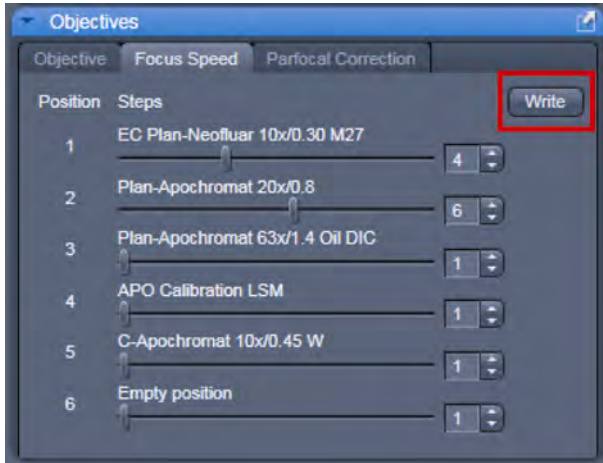


Fig. 155 Focus speed setting

### 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.

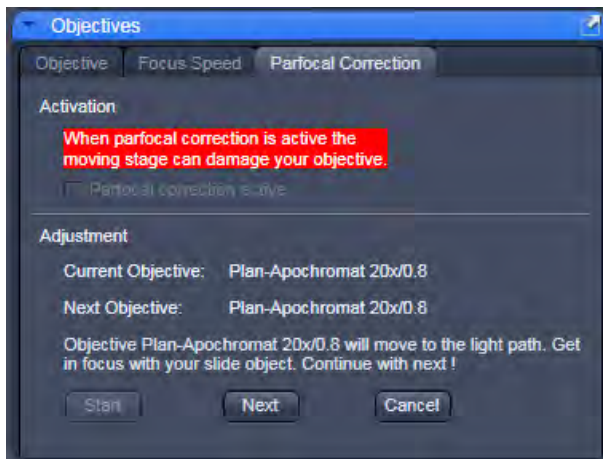


Fig. 156 Parfocal correction

### 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 Options – System Options

In the **System Options** tool the following user-accessible program **Settings** of the ZEN software are available (see Fig. 157):

- **Load configuration**
- **Re-use**
- **Hardware**
- **Image display**

- Click on the respective register to display the panel.

#### (1) Load configuration

**Load configuration** (see Fig. 157) lists parameters to be taken into consideration (if checked active) for loading an imaging configuration.

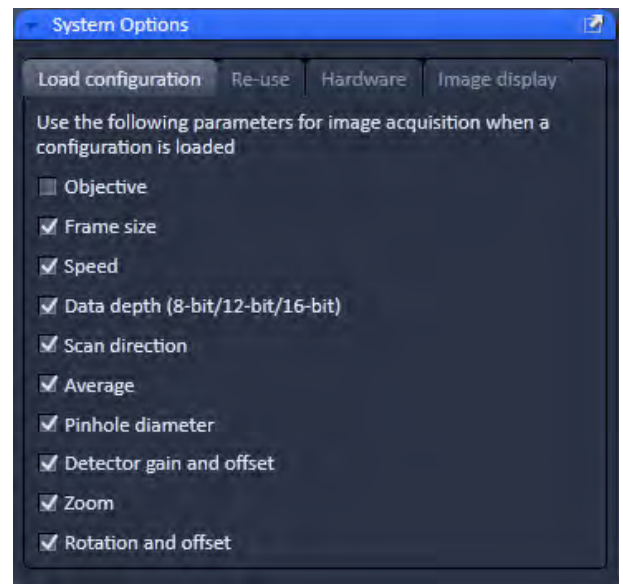


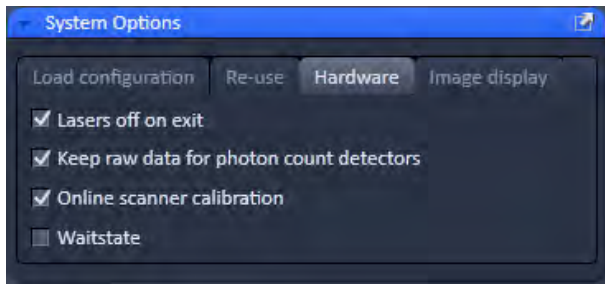
Fig. 157 Options tool with load configurations panel opened

#### (2) Re-use

Within the **Re-use** panel (see Fig. 158) 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. 158 Options tool with re-use panel opened



**Fig. 159** Options tool with hardware panel opened

### (3) Hardware

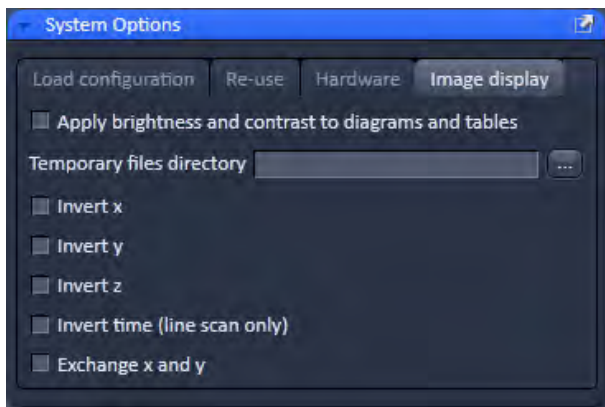
Press the **Load configuration** register to open the panel

The **Hardware** tab (see Fig. 159) allows you to set several hardware defaults at the shutdown of the system.

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.

The Z-Stack **Waitstate** function is recommended for acquisition with scanning stages, high power

objectives and short acquisition times on Axio Imager stands. **Waitstate** is on as default setting. This function delays the image acquisition during time series or Z-Stacks in case vibrations of the stage occur and are registered at the beginning of individual images. The delay is set to 350 ms. This can occur for specific image acquisition settings and is dependent on the speed, the stage and the objective in use.



**Fig. 160** 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. 160) 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.

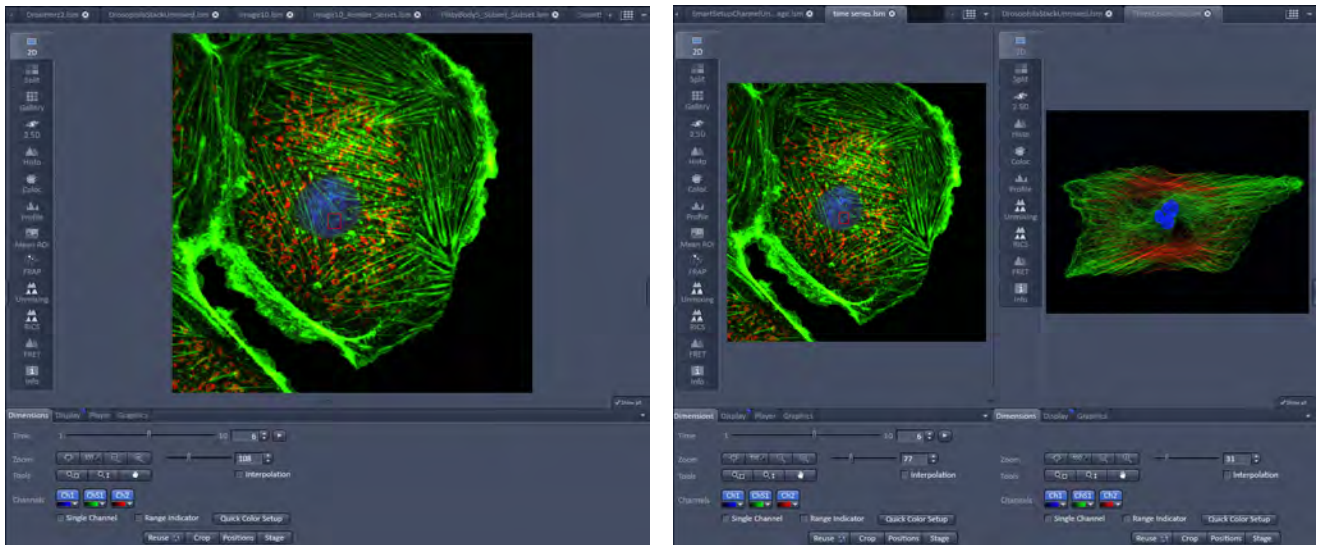


## 6 Center Screen Area / Image Containers - Display and Image Analysis

### 6.1 Structure and Functional Concept of the Center 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. 161 shows the layout with one or two Image Containers.



**Fig. 161** The Center Screen Area of the ZEN Main Application window – Left: 1 Image Container, Right: 2 Image Containers

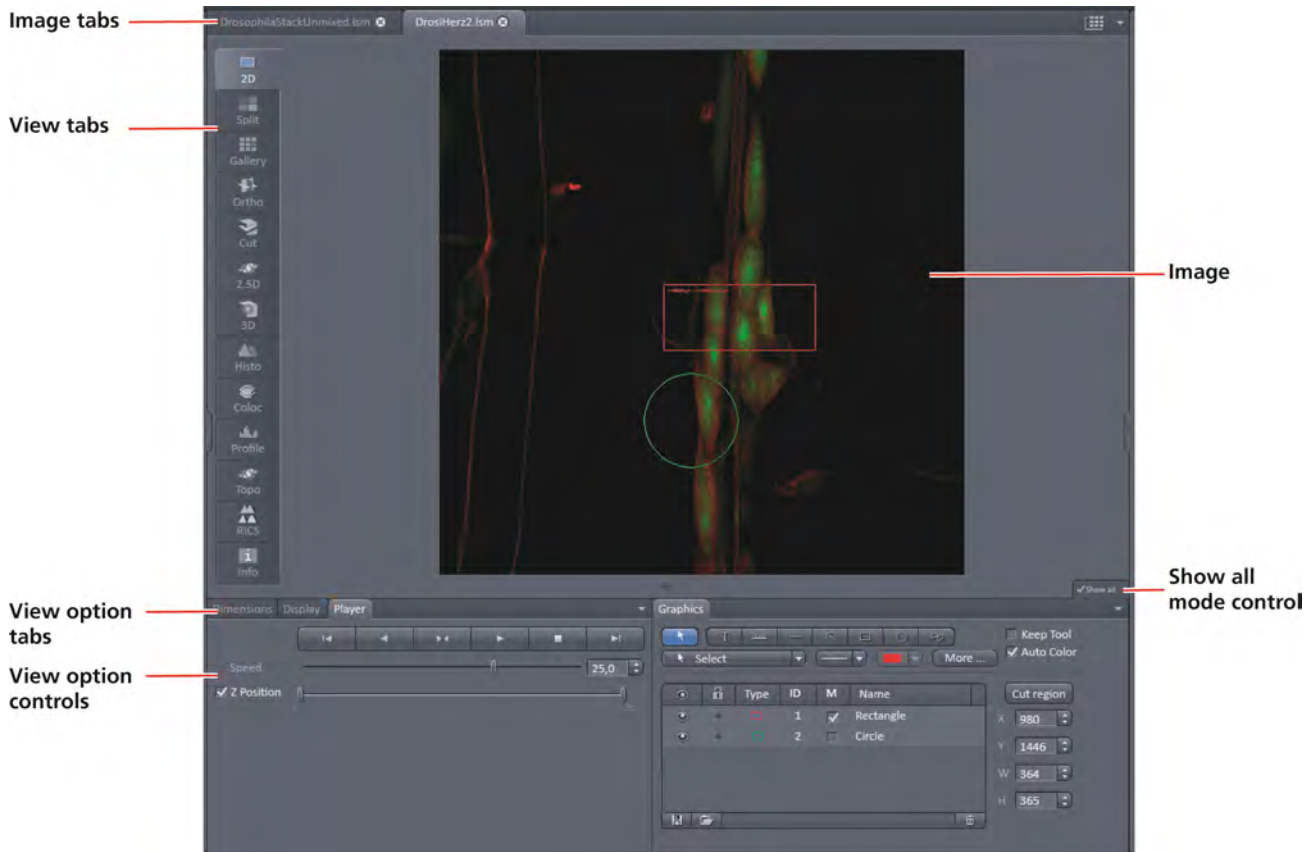


Fig. 162 Image Display window – image tabs show options

**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. 163/a), the name and a small image thumbnail (Fig. 163/b) or the file name and a larger image thumbnail (Fig. 163/c). This can be configured in the Container Context menu (see Fig. 166).



Fig. 163 Image Display window; image tabs show options

**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 **Graphics**. 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:

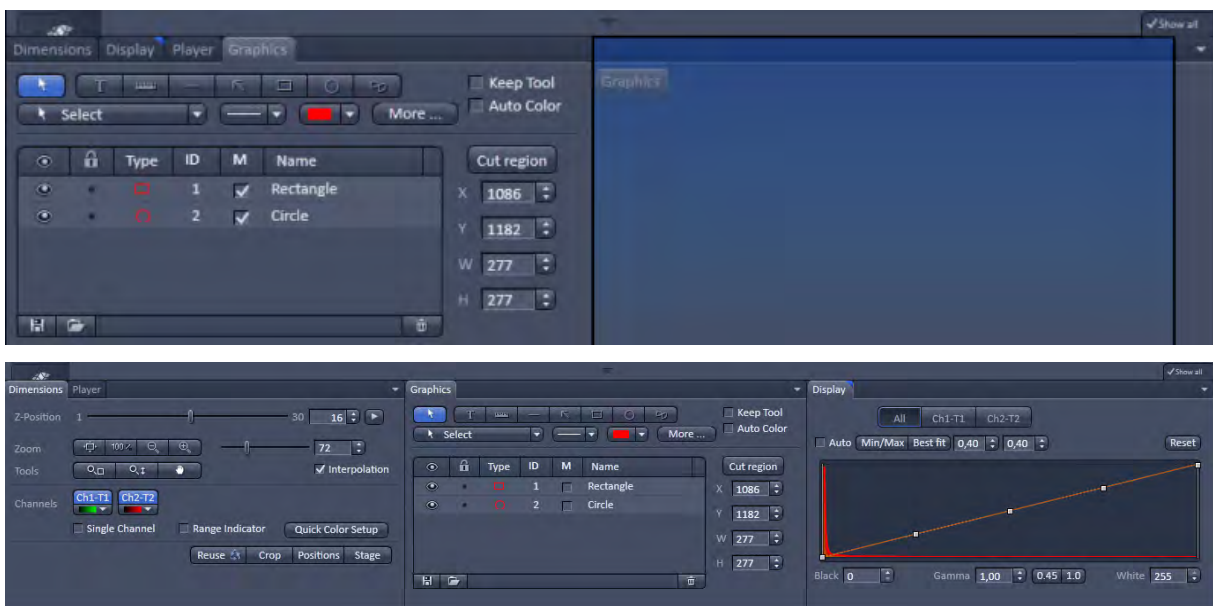

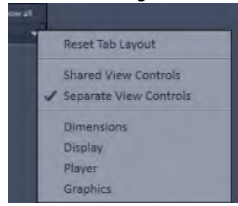


Fig. 164 General View Options control tabs

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.

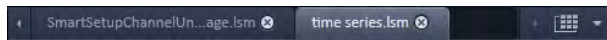


**Show all mode of the View Options Area:**

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 **Options** – System Options.



**Fig. 165** Control area of the top of the image container

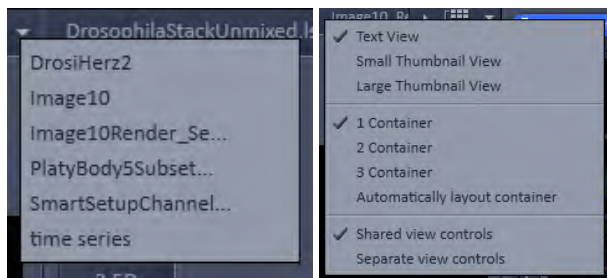
**Image Handling and Container Layout Configuration:**

The control area (Fig. 165) on the top right corner of the Image Display is for scrolling (click on the left or right triangle button) and listing the Image tabs which cannot be displayed if too many images are opened (left click on the down triangle).

This is just an alternative way of handling the open images (Fig. 166, left).

The main tool for this purpose is the **Open Images** panel (see section **Right Tool Area, Data Management and Storage**)

A right click on the image tab row opens the context menu (Fig. 166, right) for the layout of the Center Screen Area.




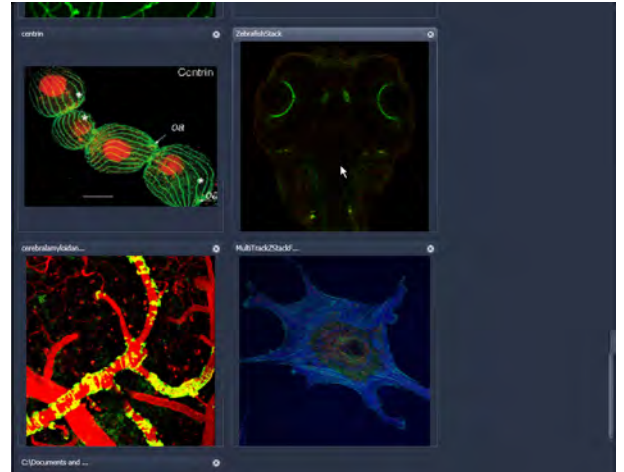
**Fig. 166** Open Image pull down menu (left) and context menu of the Center Screen Area (right)

**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. 167).



**Fig. 167 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:

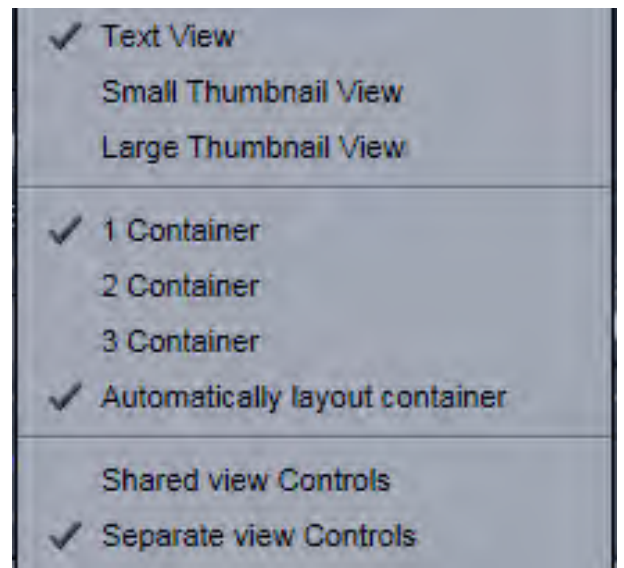
- setting the view for the Image tabs (see above),
- choosing to split the Center Screen Area in 1, 2 or 3 containers and setting the automatic container layout
- choosing between separate or shared view controls between the containers.

The same menu is available in the menu bar in the **View** part of the menu.

Splitting the Center 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. 168 Container Context menu**

## 6.2 2D View

This function allows to:

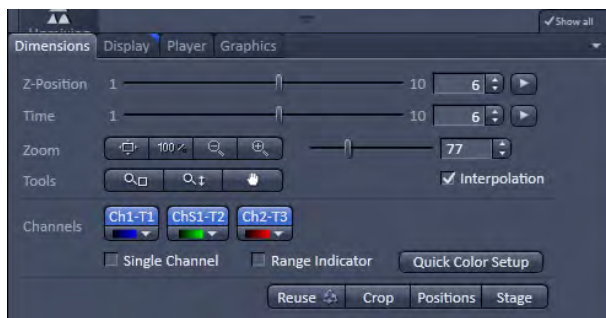
- display a single image in frame mode,
- display multiple channel images in superimposed mode.

The following **View Option** control blocks apply to this View Type:

**Dimensions, Display, Player** and **Graphics**.

In the 2D view, only the general **View Option** control blocks are 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. 169 View Options control block - Dimensions**

### 6.2.1 Dimensions

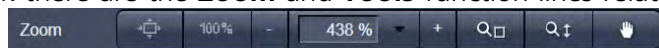
The **Dimensions View Options** control block holds the tools to modify the image display with respect to the (multiple) dimensions of the image data set.

The sections (slices) in the dimensions can be scrolled with sliders (**Z-Position** and **Time** in Fig. 169) 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 (**Z-Position** and **Time**) starts and stops an animation in the respective dimension. This basic animation cannot be customized. For all other player-functionality refer to the **Player View Block**.





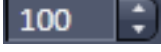



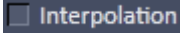
#### (1) Dimensions – Zoom

In the **Dimensions View Options** control block there are the **Zoom** and **Tools** function lines related to changing the zoom factor of a displayed image:



Any changes on these elements affect the displayed image immediately. The zoom function can be performed online.

The functions of the **Zoom** and **Tools** lines are:


- 
**Zoom Normal:** The image is fitted automatically to size of the **Image Display** window which is always the originally displayed size.
- 
**Zoom 100 %:** displays one pixel of the image also as one pixel on the screen.
- 
**Zoom -** reduces the image.
- 
**Zoom +** enlarges the image.
- 
**Spin-box and slider:** type in your magnification value in percent directly. The zoom factor can be set alternately by moving the slider. The spin-box displays the current zoom factor in %. 100% corresponds to the original size.
- 
**Zoom selection via mouse:** draw the area to zoom in directly on the image.
- 
**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.
- 
**Navigation hand:** allows you to scroll the position of a zoomed image inside of the image window.
- 
**Interpolation check box:** activates / deactivates the zoom interpolation function.

## (2) Dimensions – Channels

The **Channel(s)** function 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 **Ch1-T1**, **ChS1-T2**, **Ch2-T3** buttons switches the respective channels on/off in the display.

Clicking on the  buttons or white arrow opens the channel color lock-up-table to select the color, LUT or no color for this channel (see Fig. 170).

The **Single Channel** check box activates / deactivates single mode operation.

The **Range Indicator** check box activates / deactivates the **Range Indicator** function.


Clicking on the **Quick Color Setup** button opens a selection menu. Here you can change the image view between **BGR**, **GRB**, **RGB**, **Grey** and **Range** indicator mode quickly.

Any changes on these functions affect the displayed image immediately.



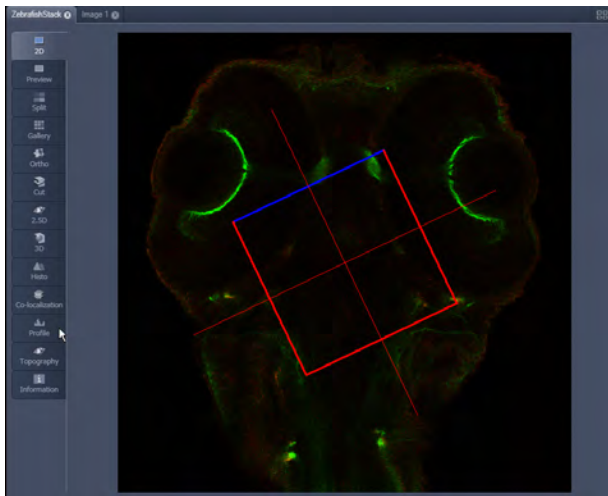
**Fig. 170** Image Display - Dimensions - Channels

**(3) Dimensions – 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 **Options – System Options**) (**System Options - Re-use** 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.





**Fig. 171 Image Display - Dimensions - Crop**

**(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 **Acquisition Mode**).

Click on  will display the **Crop Rectangle** in the **Image Display** (Fig. 171). 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.

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.



### 6.2.2 Display

In the **Display** View Option control block, the intensity minimum can be set to the Black value and the intensity maximum to the White value of the image using the **Min/Max** button.

Clicking on the **Best fit** button sets the intensity minimum to Black and percentile value and the intensity maximum to White and percentile value.

Activating the  **Auto** check box sets the intensity minimum and maximum values automatically.

With the **0,40** spin-boxes you can set the lower and upper percentile values.

The **Black**, the **Gamma** and the **White** values of the displayed image can be adjusted (Fig. 172) individually.

With the **All** **Ch1-T1** **ChS1-T2** **Ch2-T3** buttons, the effect of the settings can be restricted to an individual channel or be applied to all channels. By default, the settings apply to all channels simultaneously.

The parameters can be changed using the spin-boxes – or directly by typing in numbers in the number-field. With the **Reset** button, the original settings are easily re-set.

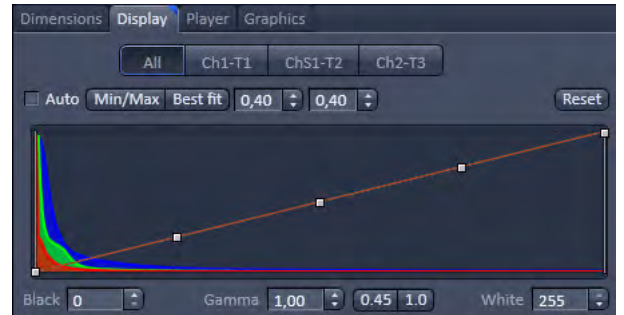



Fig. 172 View Options control tab - Display

 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 on the **Processing** tab (section **Processing Tab**).

### 6.2.3 Player

This function allows to:

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

When the image displayed in the **Image Display** window is neither a Z-Stack nor a time series this View Option control block is empty.

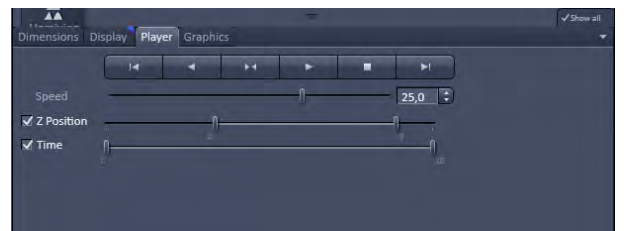



Fig. 173 View Options control tab - Player

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.



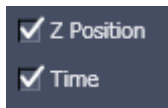
Move to the first slice.



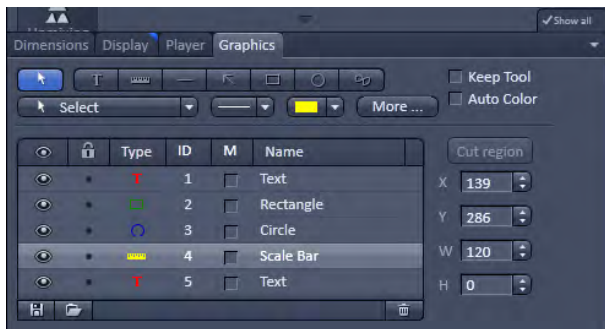
Move to the last slice.



**Speed** can be set through a slider or spin-box (selection box).



**Multidimensional** animations can be selected by including individual dimensions by checking tick-boxes.



**Fig. 174** View Options control tab – Graphics

### 6.2.4 Graphics

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).

### Functional Description

The graphics function uses a plane separate from the image plane (the graphics plane) and therefore does not change the content of the image(s).

The **Graphics** 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 Graphics View Control Block

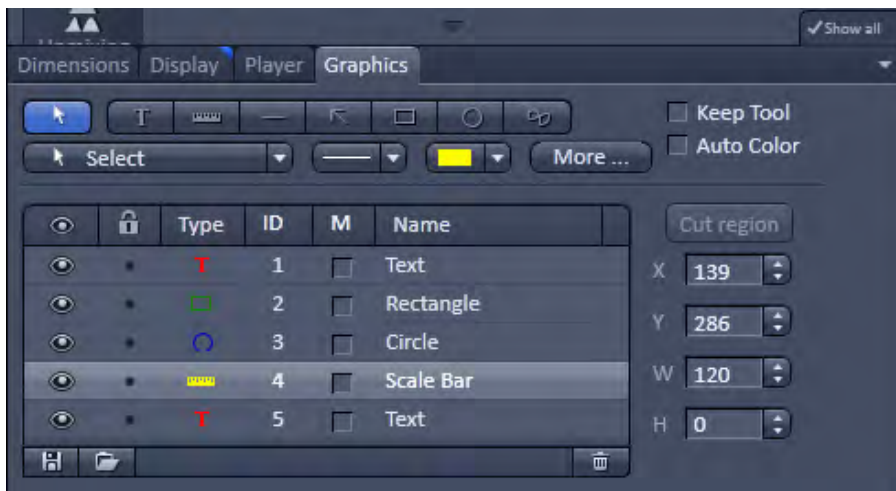

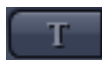


Fig. 175 Graphics view control block

The following list describes the most common functions in the **Graphics** 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 graphic 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.



**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.



**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.



**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.



**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.



**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.



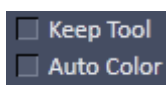
**Circle tool:** Creation of a circle in the Image Display.

Click and hold down the mouse button, draw a circle in any required direction, release the mouse button to end the procedure.



**Closed Bezier tool:** Creation of a closed 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 and closes the figure.



**Keep Tool check box:** keeps the selected tool for creation of further objects, if activated.

**Auto Color check box:** sets the color of the object automatically, if activated.



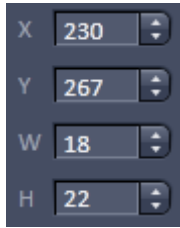
**Line thickness pull down:** sets the line thickness of the selected element.



**Color selection pull down:** In this pull down, different colors can be assigned to the graphic elements with a click of the mouse. A selected color is automatically assigned to the currently selected graphic element and to all elements created afterwards.



**Cut Region tool:** The region of a Z-Stack or 4D-image surrounded by an graphic element is extracted and displayed separately in a new Image Display. This function is only active if the active graphic element defines a closed contour or volume.



**Coordinate and dimension display:** shows the XY coordinates of the object center and the width and height of the graphic element.

**Function elements of the graphics list box:**



Activate/deactivate the display of the graphic element in the image.



Locks / unlocks the graphic element in the image



Shows the type of the graphic elements as an icon.



Shows the number of the graphic elements.



Activate/deactivate the display of the parameters of the graphic elements, e.g. area, etc.



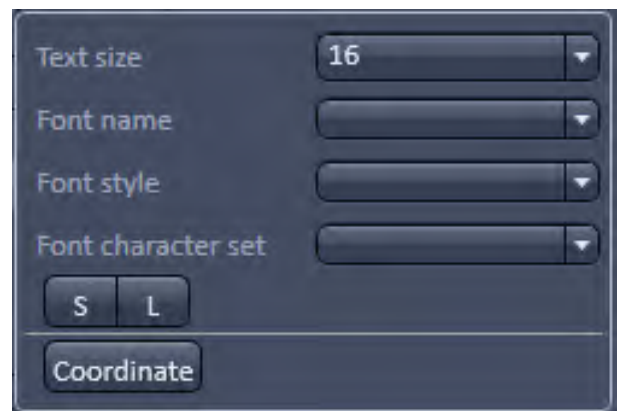
Shows the name of the graphic elements.



**The More** options (Fig. 176) allow the selection of the following text parameters in the appropriate list box:

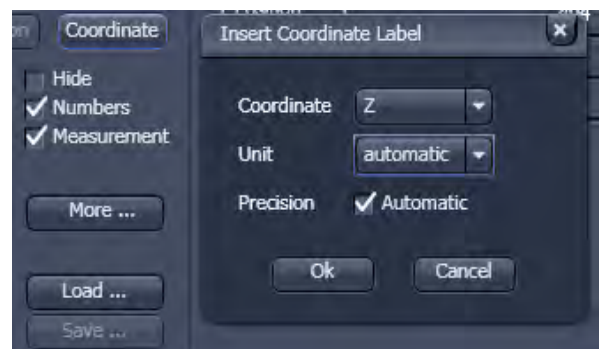
- Text size
- Font name
- Font style
- Font character set

Clicking on the **S** or **L** buttons toggles between small or large font size.






**Fig. 176 View Options Graphics - More**

To insert a **Coordinate Label** in the image display, click the **Coordinate** button in the **More ...** dialog. The coordinate settings dialog (Fig. 177) 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



**Fig. 177 View Options Graphics - Coordinate**

presentations (see Fig. 175).

To load / save graphics from / to a file use the **Load**  / **Save**  buttons in this View Options control block. To delete a graphic element from the image select this element and click on the **Delete**  button.

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**.

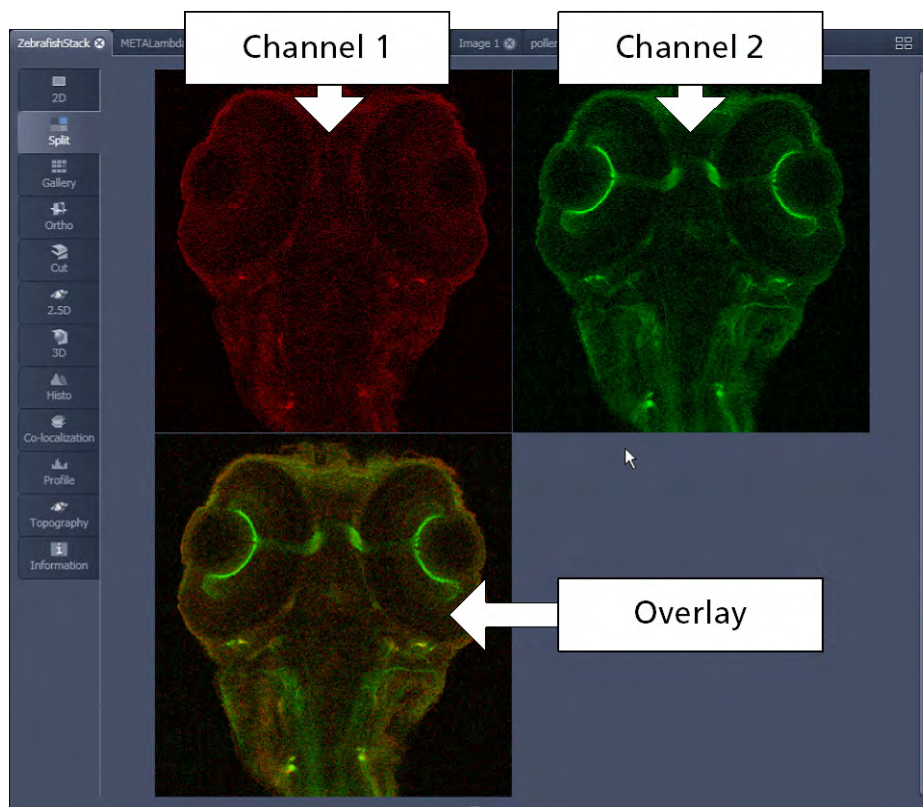
### 6.3 Split View

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 **Graphics** 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.
- Graphics overlay elements are always displayed in all channel displays.



**Fig. 178** 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 **Graphics** 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. 179).

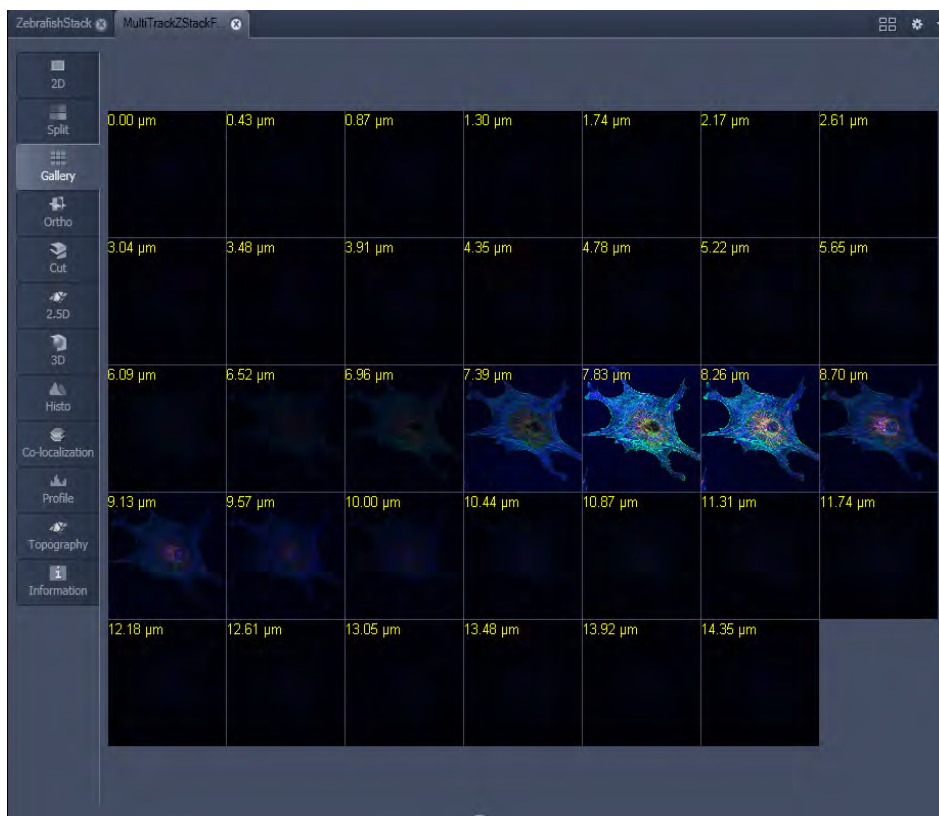


Fig. 179 Image Display window - Gallery display



### 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 **Graphics** 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. 180).

In the **Ortho** View, section sliders appear in the **Ortho View Option** control block together with orthogonal projections in the image (Fig. 181).

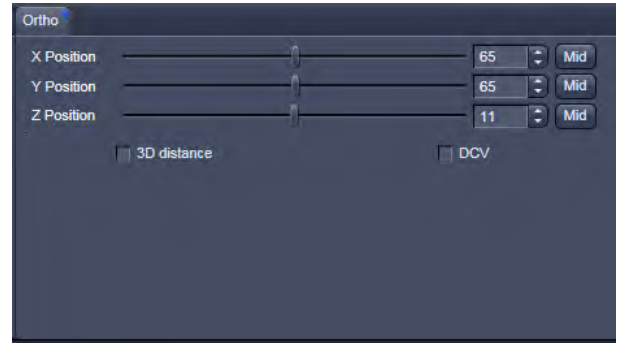


Fig. 180 View Options control block - Ortho

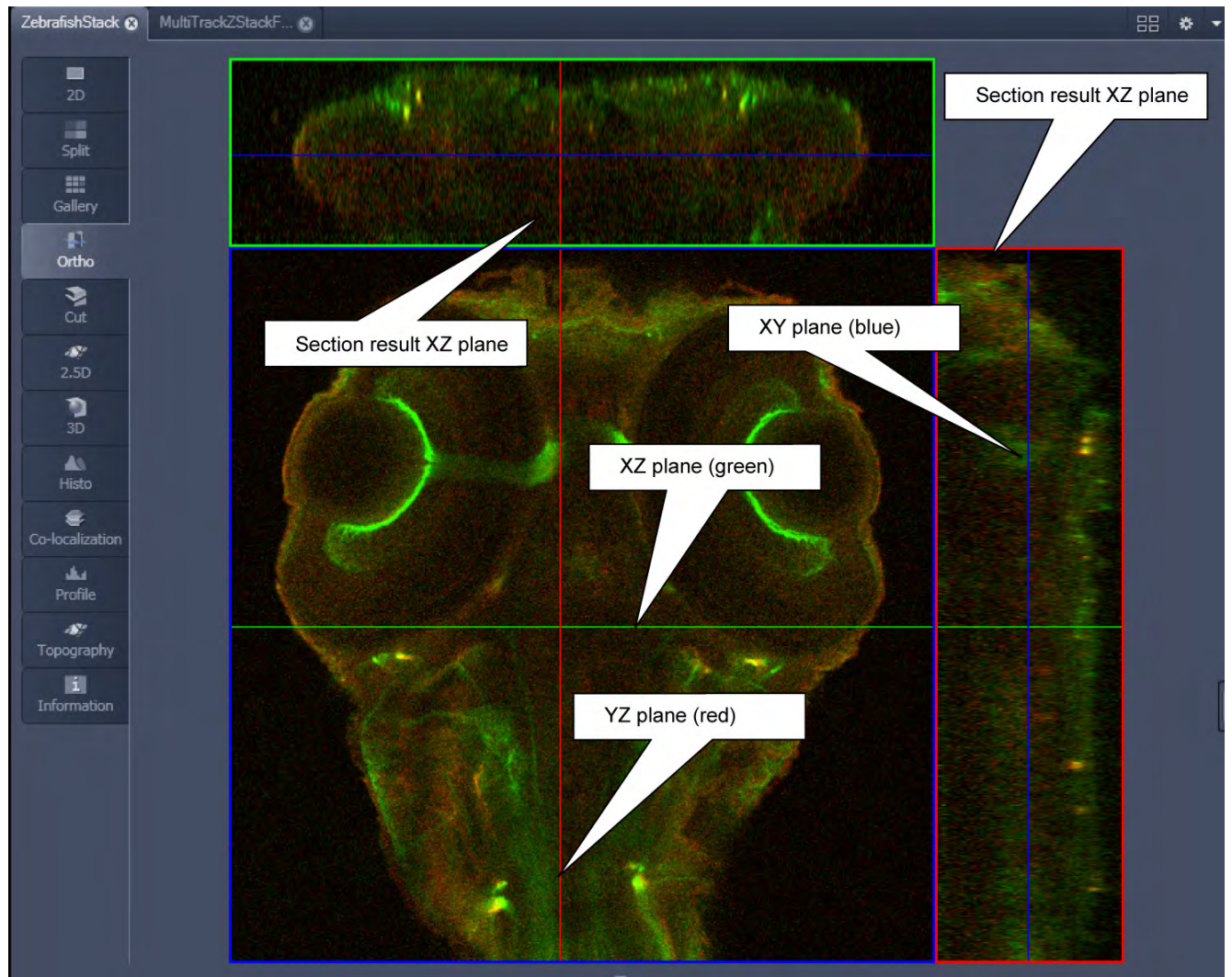








Fig. 181 Image Display - Ortho View

### 6.5.1 Ortho - Select Function

- By changing the parameters X, Y and Z in the **Ortho** View Option control tab, 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:

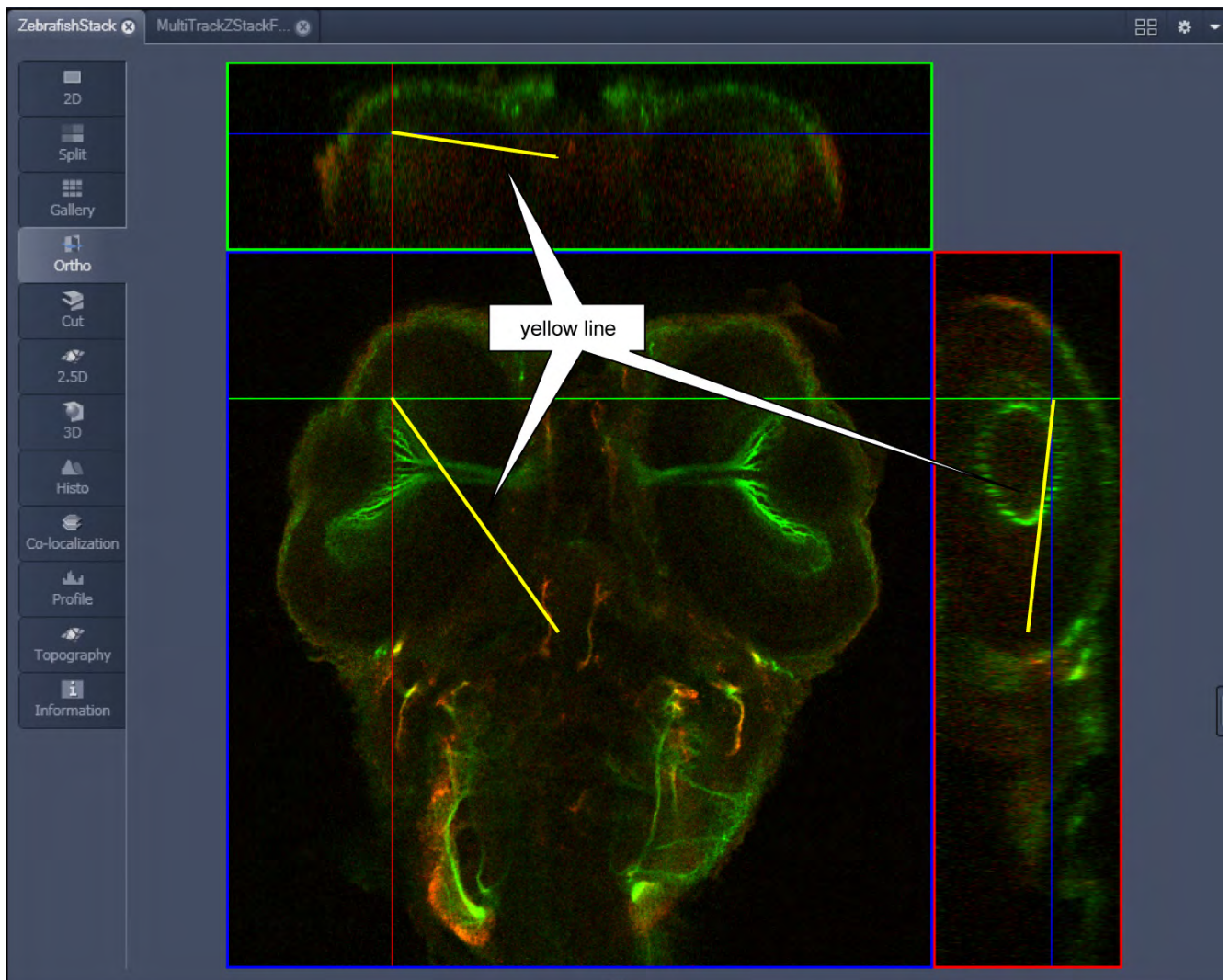
- By moving the sliders on the **Ortho** View Option control tab.
  - X and Y settings may range from 1 up to the maximum number of pixels scanned (in the example shown: 512).
  - Z settings may range from 1 to a maximum of n, with n standing for the number of slices produced in the stack.
- By directly 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 . 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  onto the intersection of the red and green section planes, it changes into the  symbol. If you now press the left mouse button and keep it pressed you can reposition both section planes **simultaneously**.
- If you move the crossline symbol  onto the green section plane, it changes into the  symbol. If you now press the left mouse button and keep it pressed, you can reposition the (green) XZ section plane.
- You can reposition the (red) YZ plane in the same way using the  symbol.

The result of an orthogonal section is visible at the image margin.

- Section of the XZ plane (green line) through the stack: above the XY image.
- Section of the YZ plane (red line) through the stack: right of the XY image.
- Section of the XY plane (blue, slice plane of the stack): center image.

### 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. 182). The actual spatial distance is calculated and shown in  $\mu\text{m}$  next to the **Mark** button.



**Fig. 182** Image Display – Ortho View - 3D Distance Measurement display



**Fig. 183** Cut View – control block

## 6.6 Cut View

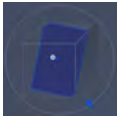
This function allows to display a user defined section plane (= cut plane) of a Z-Stack. It automatically improves the image of the section plane by trilinear interpolation.

The settings of the **Dimensions**, **Display**, **Player** and **Graphics** 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.

## 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 **Graphics** View Options control blocks do not apply.

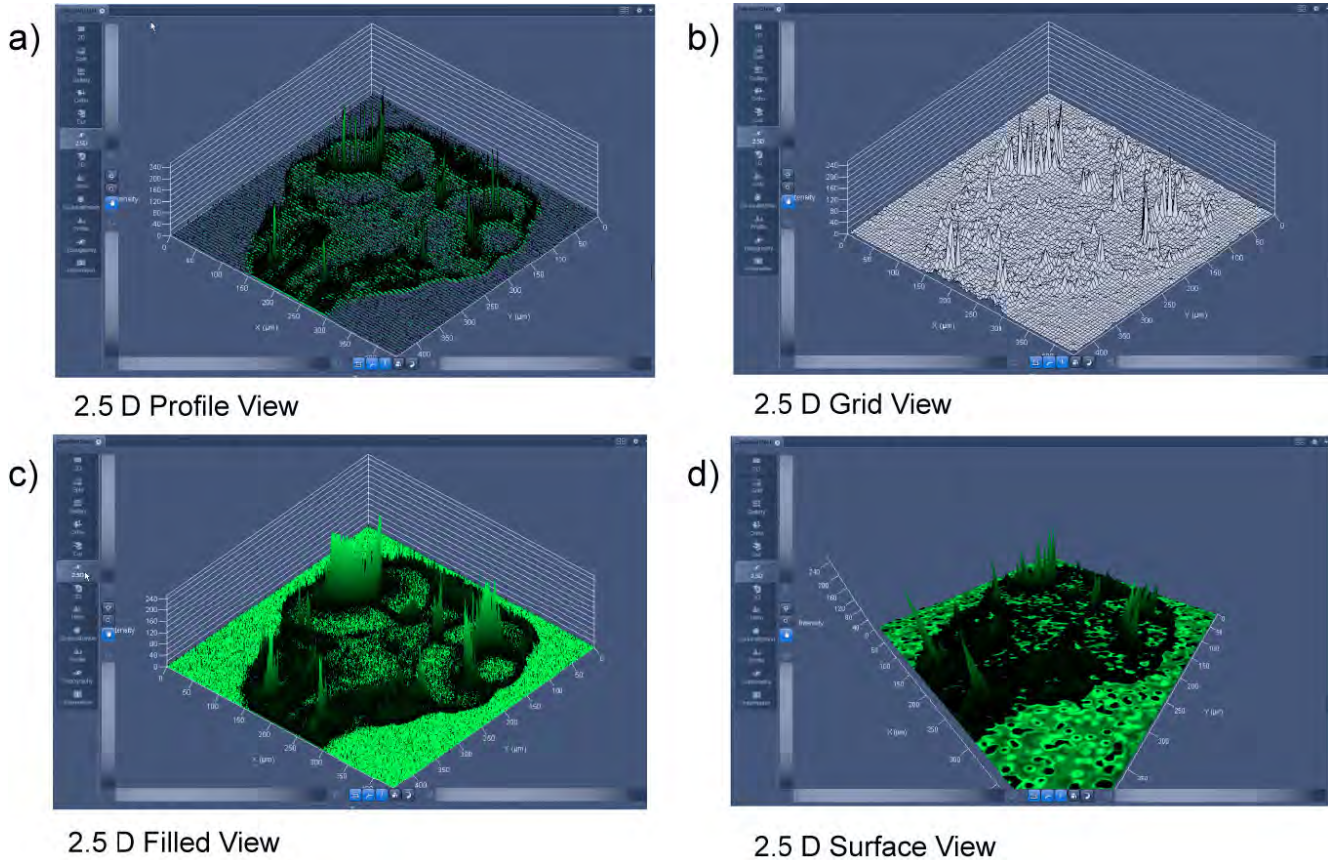
Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily hidden while the toolbar is displayed.

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. 184** 2.5 D View Option control tabs



**Fig. 185 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 **Graphics View Option** control tabs are not applied.

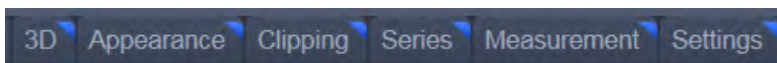
A click on the **3D View** tab will display the **3D View Controls**. Any changes done within these controls are effective immediately.

 The **3D View** can also be used online during scanning.



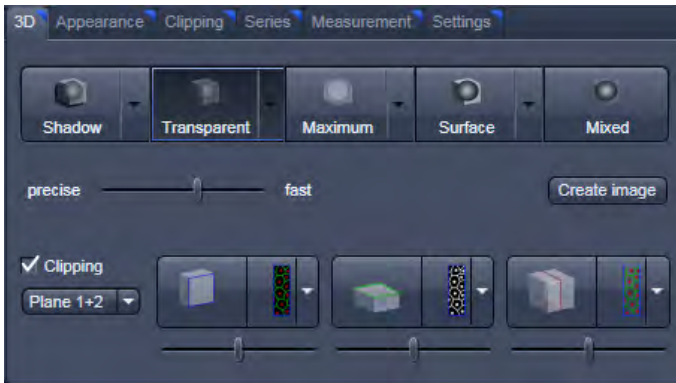
**Fig. 186** Image Display window - 3D display

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.



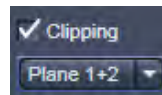
On the Clipping Planes different surfaces can be chosen from a pull down list:



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.

**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. 188).

- **Front** button: Shadow rendering front view
- **Back** button: Shadow rendering back view
- **Any View** button: Shadow rendering with user defined view

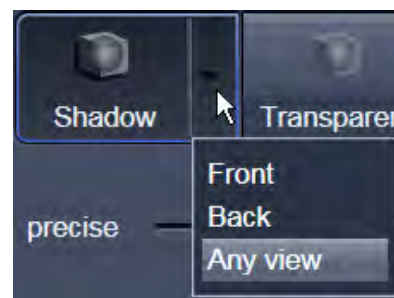
With a click on **Front**, the 3D reconstructed image is displayed in a shadow projection where it is illuminated at a 45° angle from the front left.

A click on the **Back** button creates the same projection with illumination from back left.

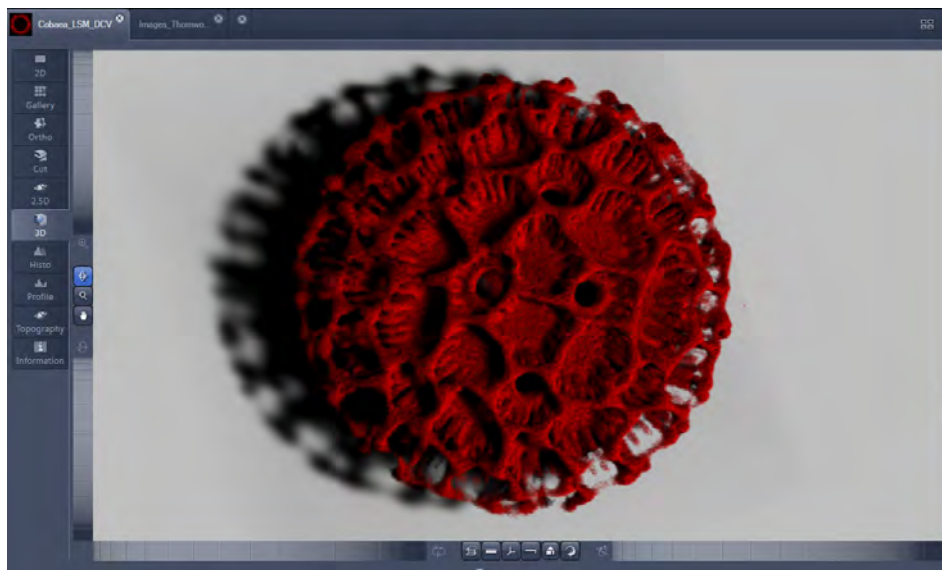
Detailed settings for shadow projection can be accessed via the **Appearance** tab.



**Fig. 187 3D panel - Shadow button**



**Fig. 188 Shadow pull down menu**

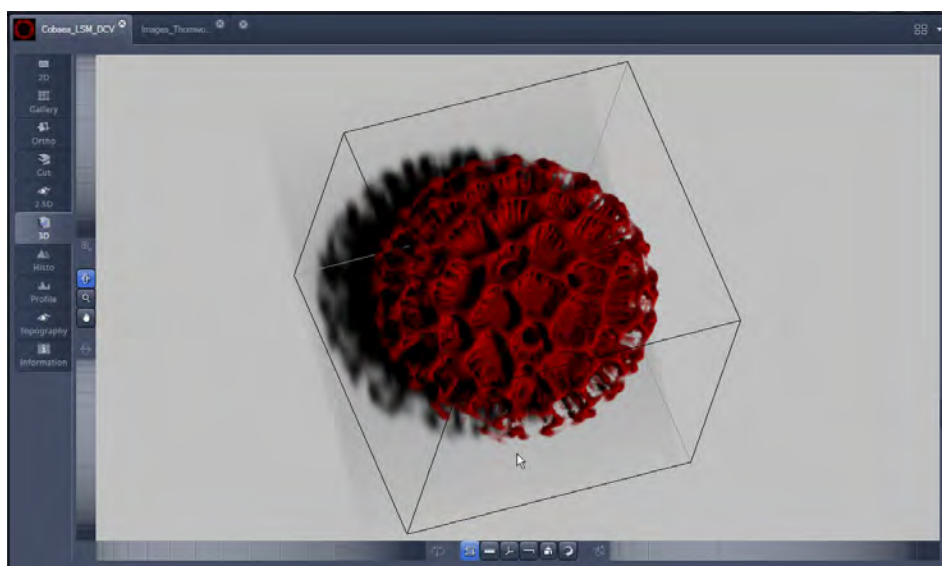


**Fig. 189** Image Display window, 3D display, Shadow projection, Front view

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. 190** 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.

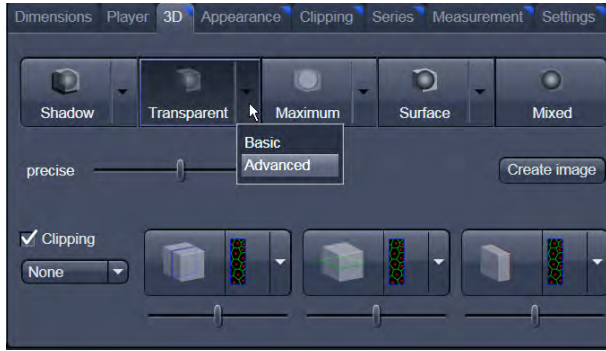


Fig. 191 3D View Control Panel, Transparent

## 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").

**Basic** Transparency rendering (voxel based) (CPU)

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

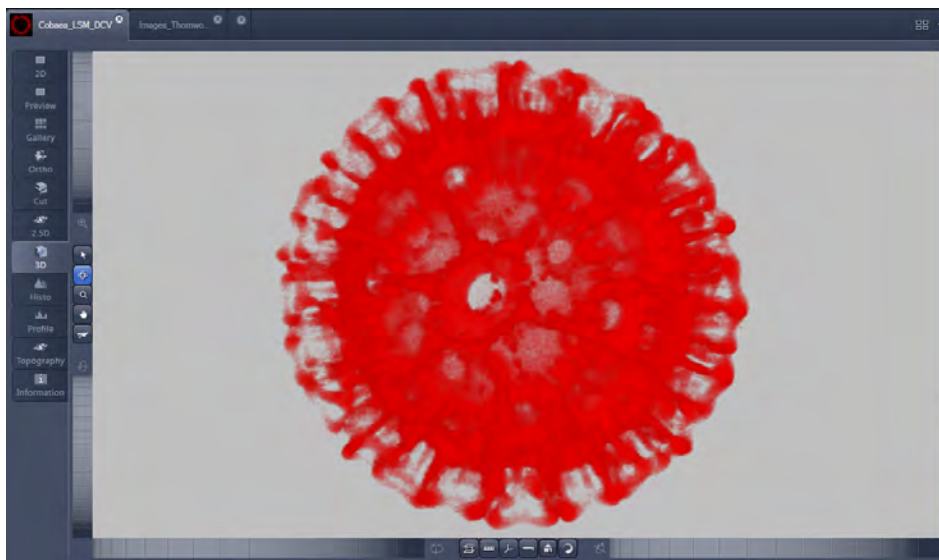

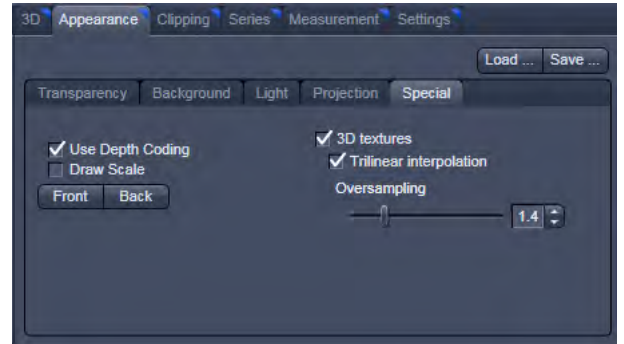


Fig. 192 Image Display window, 3D display, Transparency projection, Advanced

By means of the **Depth Coding** function (Fig. 193), 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.

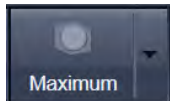
The **3D Textures** option can be used for faster and better rendering of transparency renderings.

 Note that this function is only available in Transparency and Maximum Intensity Render Modes.




**Fig. 193 3D toolbar – Transparent – Depth Coding**

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

**Maximum Basic**                      Maximum Intensity CPU based

**Maximum Advanced**            Maximum Intensity GPU based

### 6.8.4 Surface Render Mode

In the **Surface** mode (Fig. 194) 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.

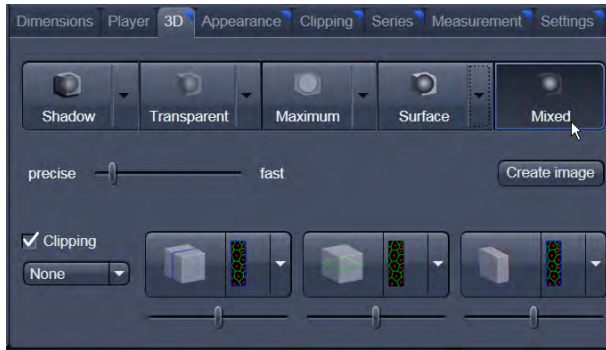
In this mode you also can select between Voxel (CPU) [basic] and OpenGL (graphics card) [advanced] display.



**Fig. 194 3D toolbar – Surface**

The [full resolution] projection is based on a high precision calculation method for 3D information on the basis of triangles with maximum resolution.

- Basic** button                      Surface rendering (voxel based)
- Advanced** button                Surface rendering (triangle based)
- Full Resolution** button        High accuracy surface rendering (triangle based)



**Fig. 195**      3D toolbar – Mixed


### 6.8.5      Mixed Render Mode

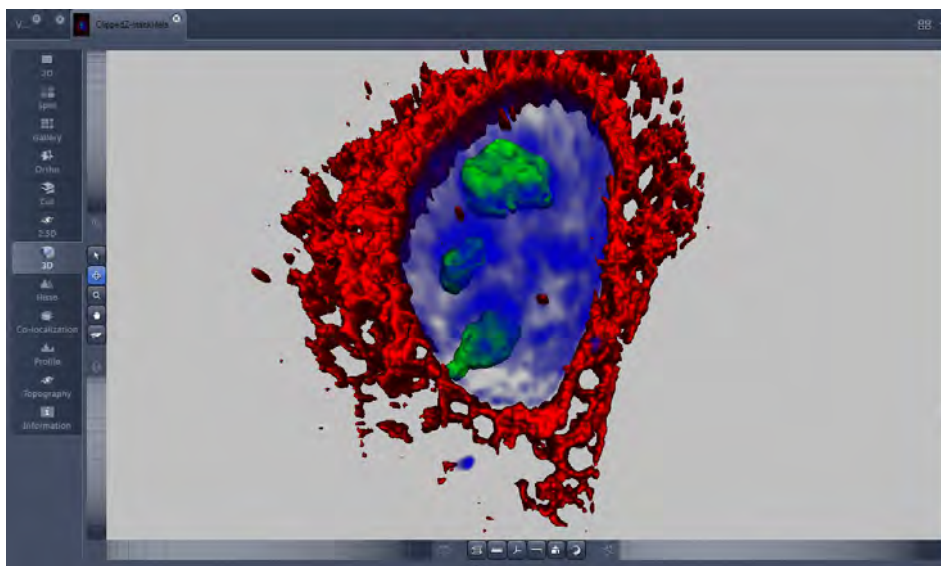
In **Mixed** mode both, transparency and surface mode, can be mixed in one volume display.

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.

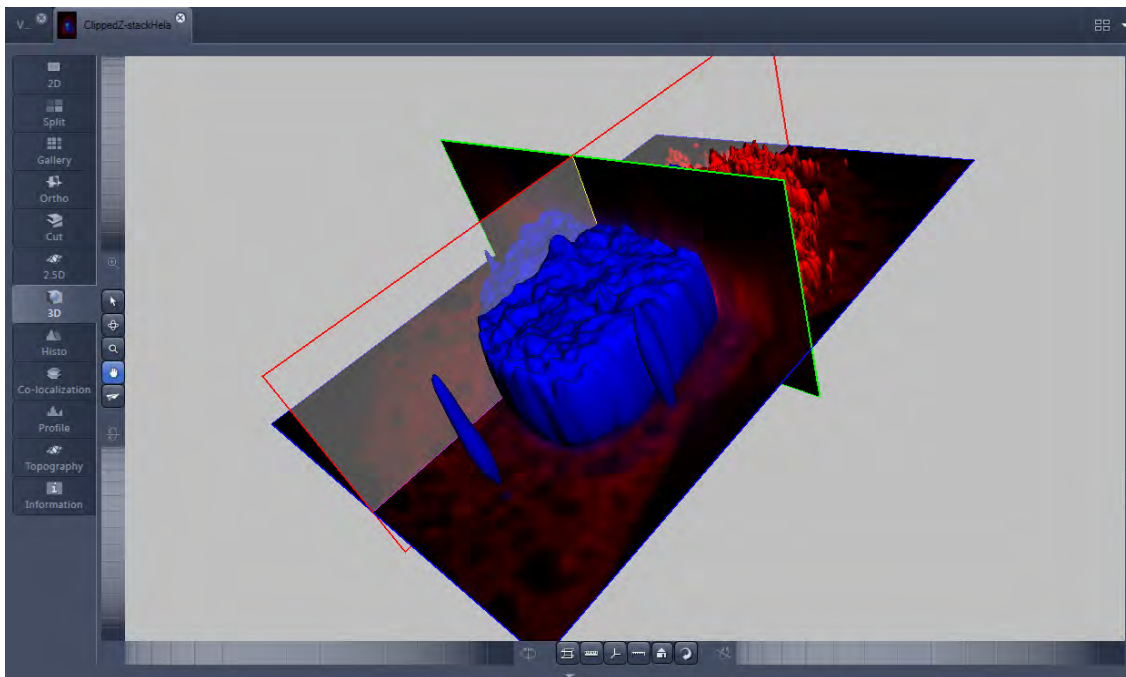
The render mode (**Transparent**, **Surface**, **Surf.+Transp.**) can be chosen per channel.

 The channels can also be switched off individually by de-activating the channels in the Dimensions view control panel.



**Fig. 196**      Image Display window, 3D display, Mixed projection

### 6.8.6 Clipping Planes

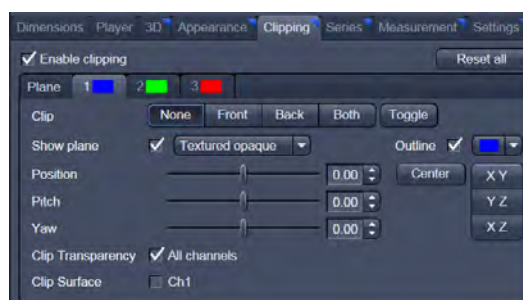


**Fig. 197** Image Display window, 3D display, Clipping planes

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. 

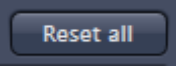


In **Surface** mode clipping planes can be used in a channel specific manner. This means, that in a multichannel image one can selectively cut away portions of the volume in one channel in order to unveil other structures from other channels. This technique is also applicable in **Mixed** mode.

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.

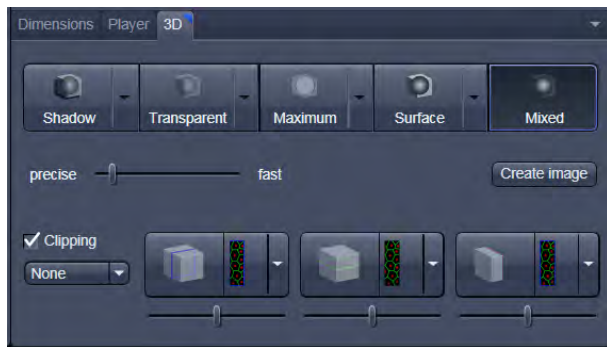



Fig. 198 Toggle button

### 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!

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.

### 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.

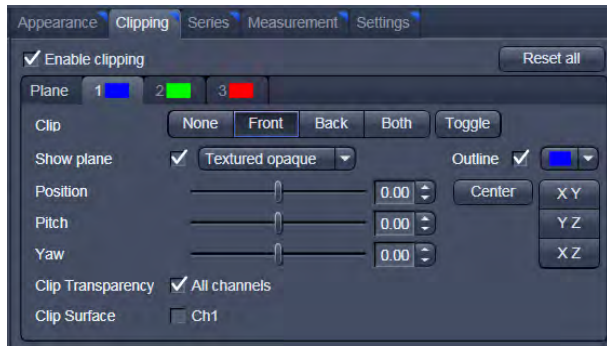


Fig. 199 Clipping planes

### 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.

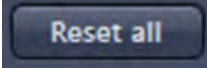
 These movements can also be "recorded" using the render series function.



**Fig. 200 Clipping planes settings**

### 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).

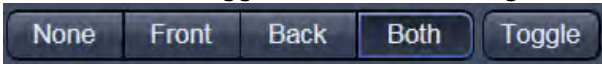
With the **Plane** tabs the planes can be addressed individually and their settings can be adjusted independent from each other.

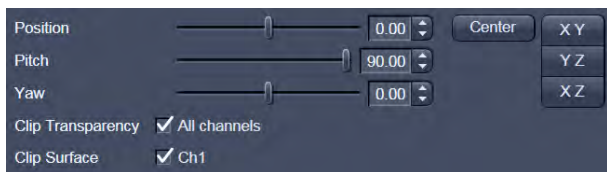
**Outline** displays or hides the colored outlines. The actual color can be chosen from a color chooser for each plane individually.

**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. 199).

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




**Fig. 201 Sliders Pitch, Yaw and Position**


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


### 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"

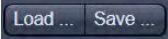
the operator. Pressing the  button switches back into the normal mode.

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.

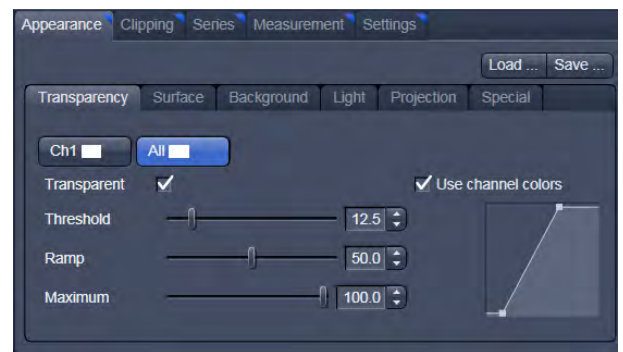
### 6.8.8 3D Rendering Settings in VisArtplus

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.



**Fig. 202 3D Rendering window (e.g. Surface Advanced rendering mode)**

- **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°).

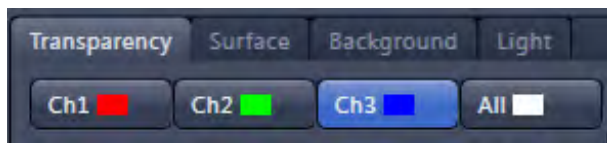



Fig. 203 Channels

- **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).

- **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 .

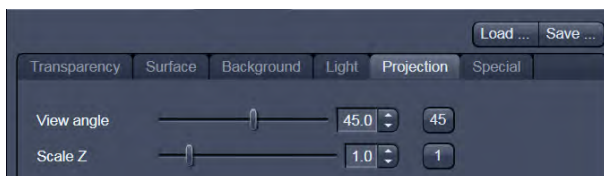


Fig. 204 Viewing angle / Z – scaling

- **View angle / Scale Z:** In hardware accelerated modes, you can change the projection angle used to view the scene anywhere between 10° and 80°. Use the slider or enter the **Angle** into the input field. The effect of this on the display perspective is similar as if you were viewing the 3D image through a telephoto or wide-angle lens. The Z-Scale of the dataset can be altered interactively by using the slider. The slider ranges from a factor of 0,1 to a factor of 5.

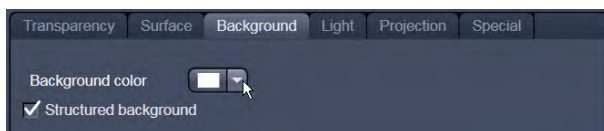


Fig. 205 Background

- **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.

### 6.8.9 Series

The **Series** tab (Fig. 206) 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. 208).

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.

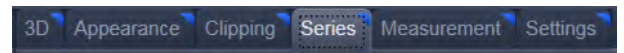


Fig. 206 Series tab



Fig. 207 Render Series window (e.g. Turn around X mode)

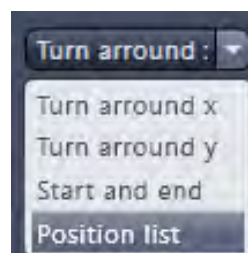


Fig. 208 Render Series modes

#### (1) Turn around X and Turn around Y mode

In this mode, the image is turned around the X-axis or the Y-axis exclusively.

The values for **Number of Views**, **Difference Angle** and **First Angle** can be selected accordingly).

#### (2) Start and End mode

In the **Start and End** mode, the image is reconstructed between a start position and an end position.

The rotation angles for X, Y and Z and the distance (zoom) can be determined using the sliders.

The value for **Total frames** can be varied. The frames are distributed equally between the start and end points (Fig. 209).

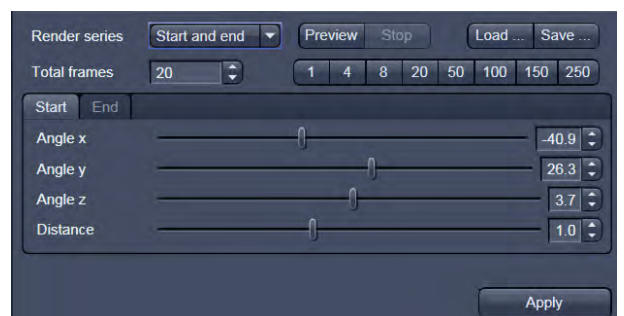


Fig. 209 Start panel in the Render View control block



**Fig. 210** Render Series window - Position List mode

### (3) Position List mode

In the **Position List** mode, the image is reconstructed between any required number of interim positions to be determined individually. Just rotate the reconstructed image with the mouse or do any other adjustment and add this as a "position" in the position list. The module will create a movie using those positions as a guideline. It will interpolate all the parameters ticked in the list.

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

Every required interim position is included in the list of the **Render Series** window with a click on the **Add** button.

**Remove** permits the contents of the list to be deleted. **Insert** inserts a position above the highlighted list entry.

The value for **Total frames** can be varied. A value of 20 produces a render series with 20 frames in total.

**Interpolate** lists variables that are interpolated during the Render Series.

Pressing the **Preview** 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.

### 6.8.10 Interactive Measurements

VisArtplus 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 looks for the highest local gray level maximum.

As a sign, that the mouse cursor has "found" a structure, the cursor shape changes from  into .

The found measurement point is drawn as a blue circle .

#### Interactive 3D-Measurement

The measurement dialog contains a toolbar with the available measurement tools.

The **Line** tool measures the length along a line in  $\mu\text{m}$ . First click on a starting point and move the mouse to the desired end point while keeping the left mouse key depressed. The measurement is concluded upon releasing the left mouse key.

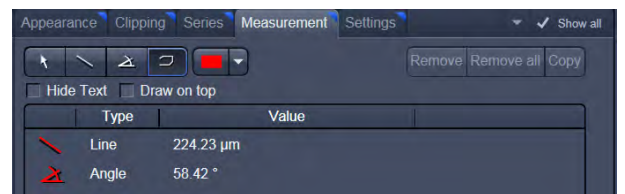


Fig. 211 3D Measurement View control tab

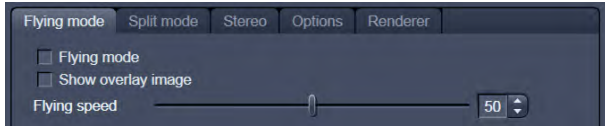
The **Angle** measurement tool defines an angle between two connected line segments. First define the starting point by clicking, and then draw the first line segment until you click on the first endpoint. Now draw the second line segment and conclude the measurement by clicking onto the second end point. This measures the angle in degree.

The **Curve** tool measures the total length ( $\mu\text{m}$ ) along a variable multi-segmented polygon line. Please click from point to point and finish the measurement by clicking with the right mouse key.

Measurement values are compiled in a list. In order to delete a measurement, select it in the list and press the **Remove** button. One can also delete all measurements by clicking on **Remove all**.

The measurements can be copied into the clipboard with the **Copy** button.

Measurements are only transiently drawn into the volume and are not stored automatically with the image.



**Fig. 212 3D Renderer Settings – Flying mode**

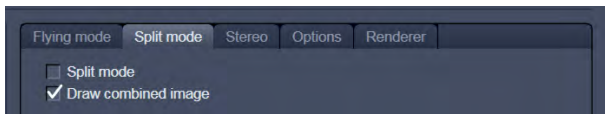
### 6.8.11 Settings

#### 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.

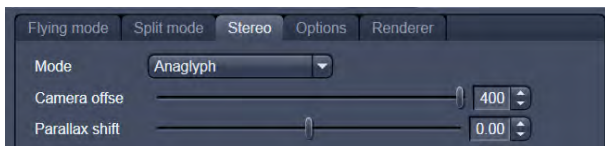
The fly-speed can be adjusted in a range from 1 to 100.



**Fig. 213 3D Renderer Settings – Split 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. 214 3D Renderer Settings – Stereo**

#### Stereo

Two Stereo views can be chosen from this sub-menu:

**Anaglyph** is a view in which the data can be examined in 3D using red/green glasses. The image is built up twice (once each for the red and green colors), resulting in a stereoscopic image.

The stereoscopic effect can only be seen with the aid of red / green 3D goggles. The red lens is to be used for the right eye and the green lens for the left eye.

**Camera:** 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.



### 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  $\alpha$ -values of the dataset can be adjusted. The rendering is getting more or less transparent using this setting.

**Reset:** Sets all render settings back to the factory standard settings.

#### Renderer Options:

**Renderer:** OpenGL Software is rendering the 3D data with the CPU; OpenGL Hardware uses the Graphics card to do this. Especially when having trouble with non Zeiss-certified graphics cards it is sometimes useful to switch to a pure CPU based rendering.

**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.

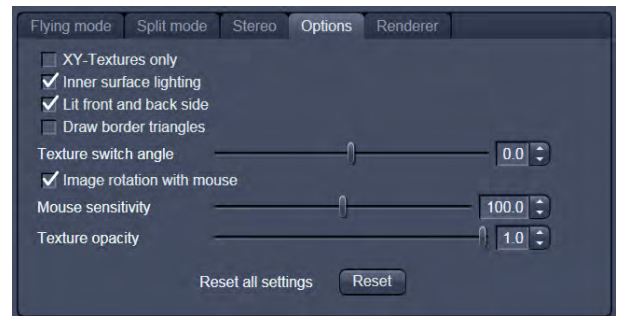


Fig. 215 3D Renderer Settings – Options

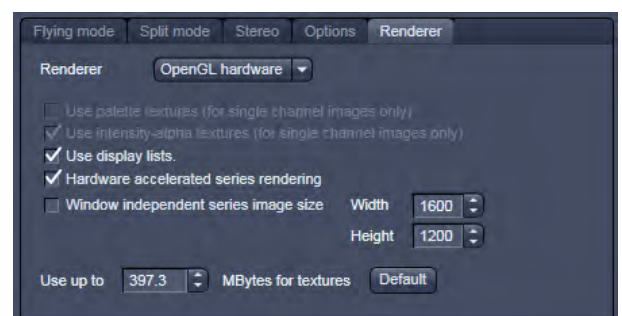


Fig. 216 3D Renderer settings: Renderer

**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 dialog 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 *VisArtplus* 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.

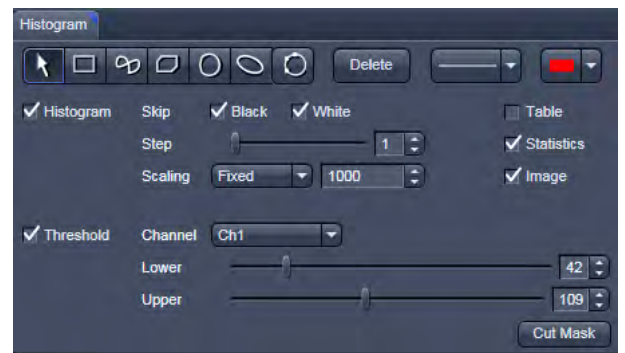
## 6.9 Histogram View

The **Histogram** function (**Histo View**) allows to:

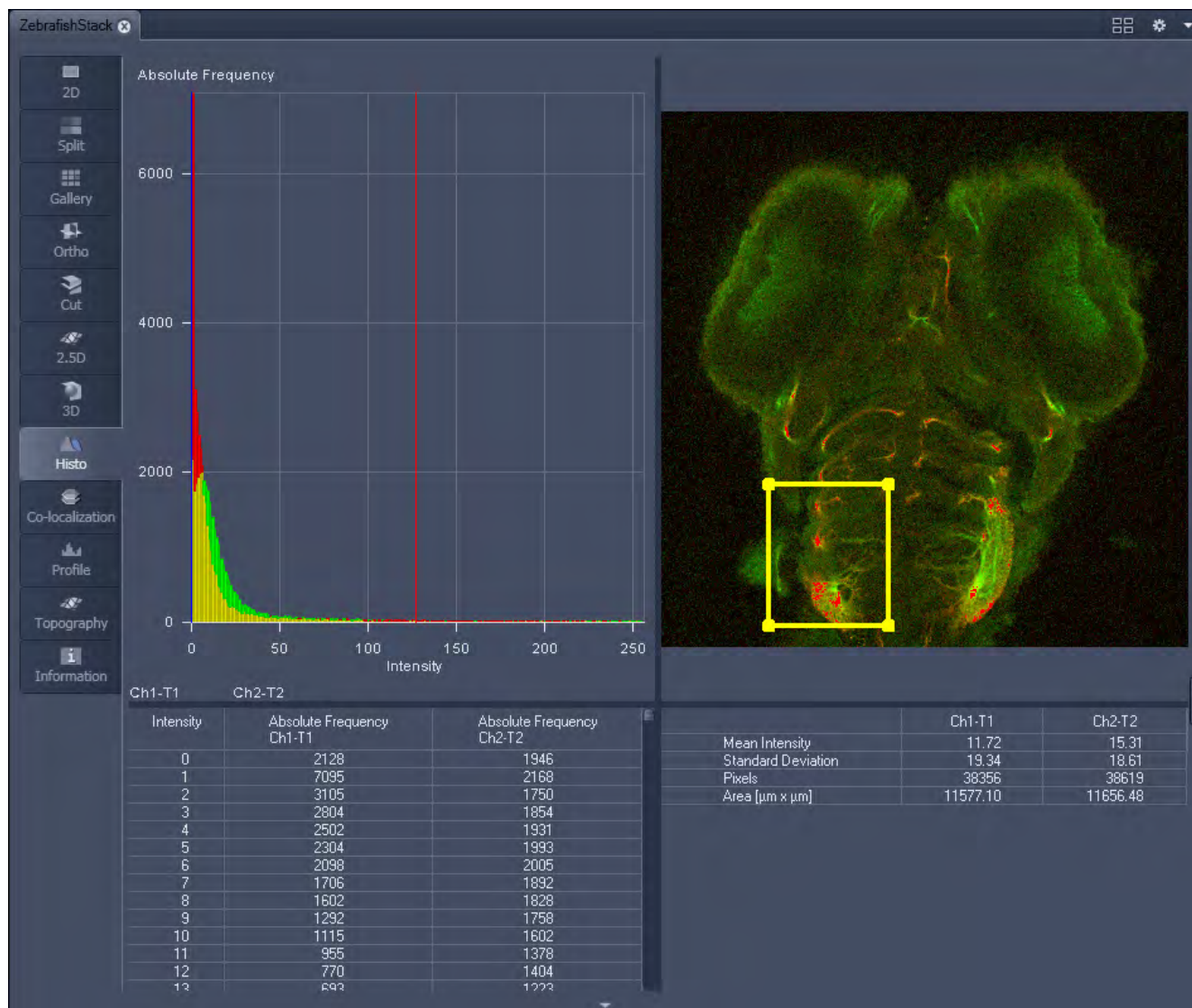
- display a histogram (distribution of pixel intensities) of an image or Region of Interest,
- show the histogram values in table form,
- copy table to clipboard or save as text file,
- measure area and mean gray value and standard distribution in an area.

The settings of the **Dimensions, Display, Player** and **Graphics** view options control blocks apply. The additional view-specific **Histo View Option** control block is shown in Fig. 217.


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



**Fig. 217** View Option control block - Histo View



**Fig. 218** Image Display, Histogram view

 The **Histo** button can also be used online during scanning.

**Histogram functions:**



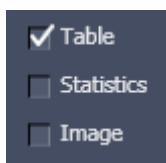
**Skip Black** and **Skip White** tick boxes: Ignore black pixels (gray value 0) and ignore white pixels (gray value 255 or 4096) in the histogram.



**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.



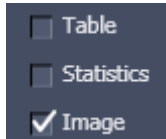
**Scaling pull down menu:** Sets the histogram graph scaling to either dynamic (adjusting to the maximum value) or fixed



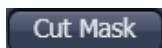
**Show Table** tick box: A table of the intensity values is shown in the **Image Display** window.



**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:**

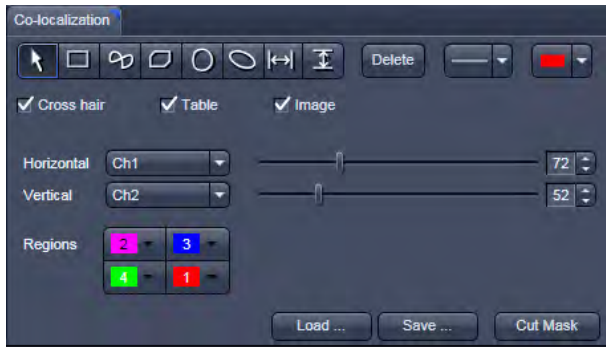
The functionality of the **Graphics View Option** control block fully applies to the **Histo View** Type. If a ROI is marked/selected in the Histo View Image display, only this ROI will be taken into account for the histogram display, the table and the statistics. If there are several ROIs, but none is marked/selected, the histogram display, the table and the statistics analyze all the pixels included in all ROIs.

**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. 219). The threshold can be set for each channel (channel selection pull down menu).



**Fig. 219** Histo View Option control block - Threshold



**Fig. 220 Colocalization View Option control block**


## 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 **Graphics** view options control blocks apply. The additional view-specific **Colocalization View Option Control Blocks** is shown in Fig. 220.

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. 221 and Fig. 222).

 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.

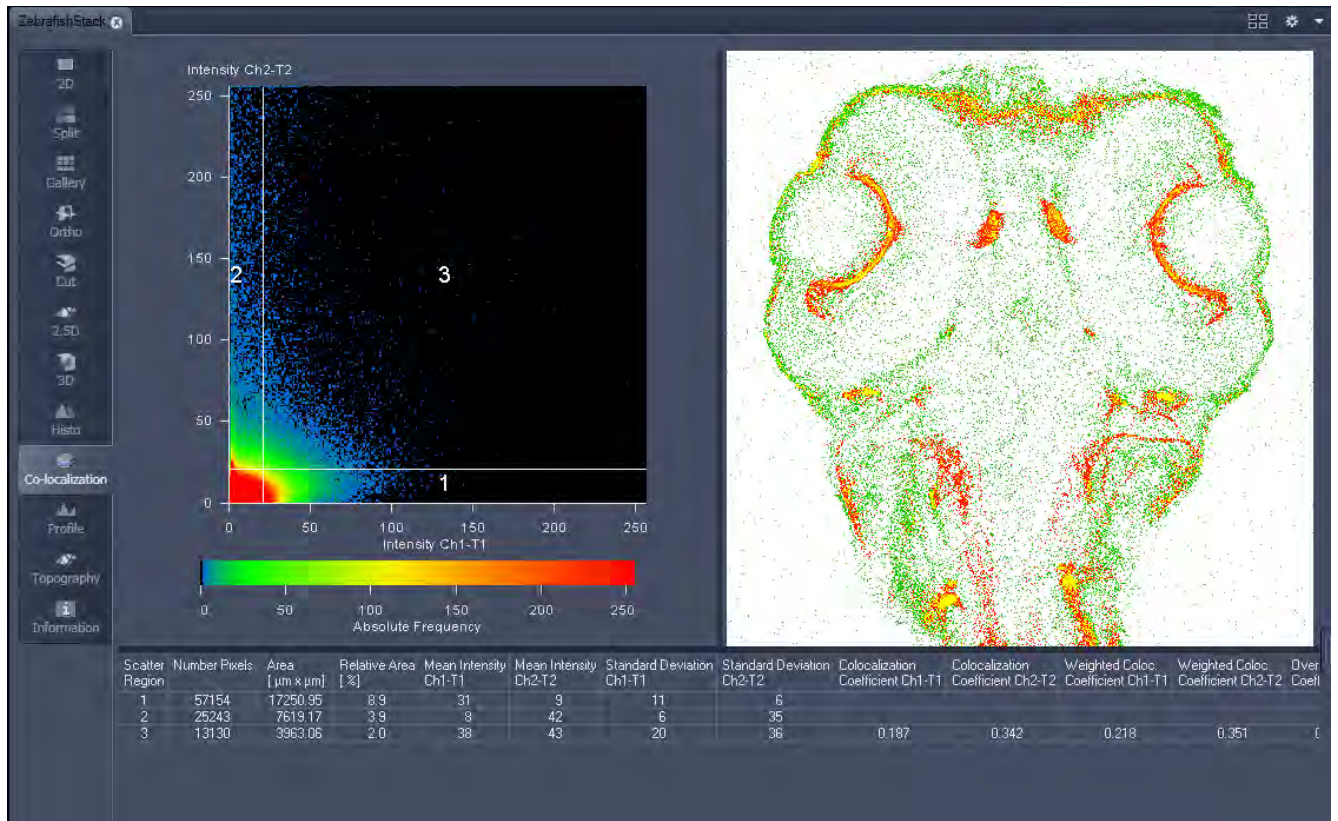


Fig. 221 Image Display, Colocalization view

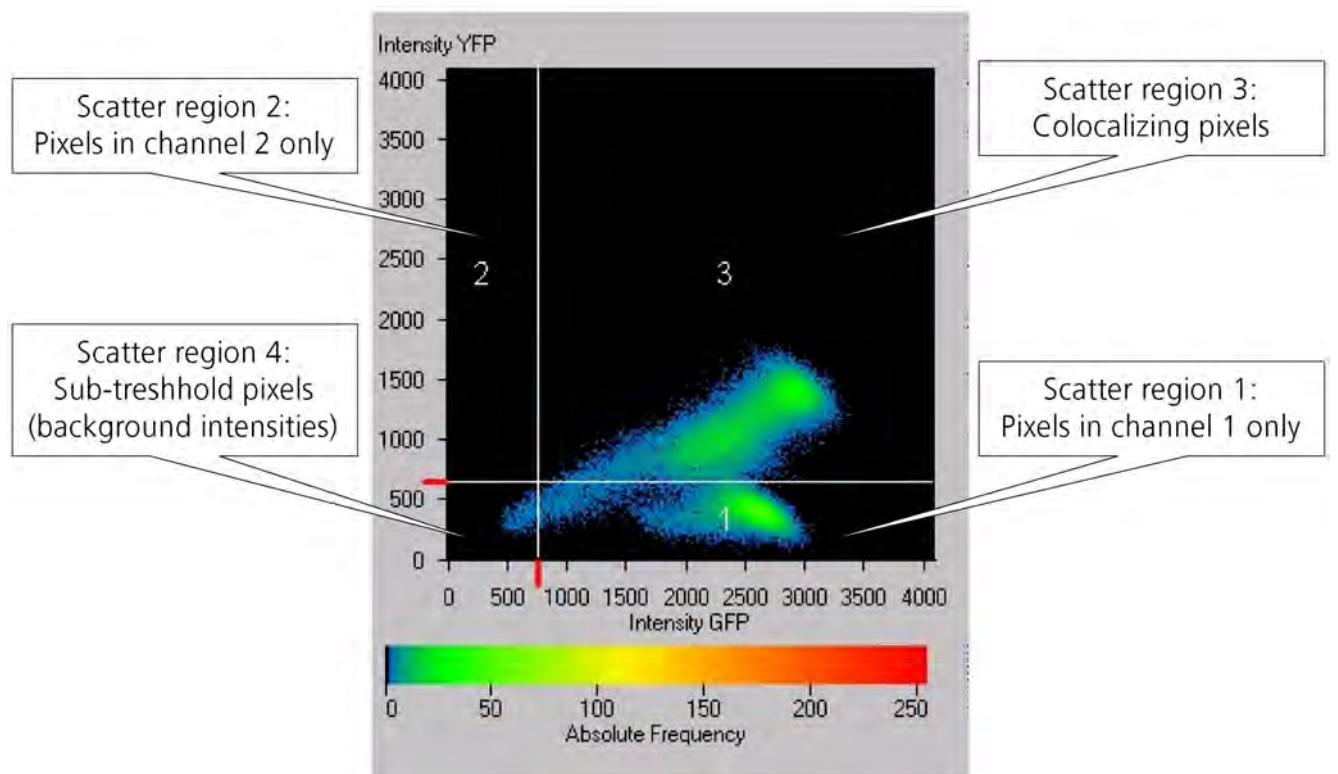




Fig. 222 Scatter diagram and threshold with crosshair

### 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.

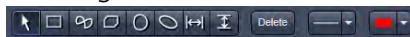
 Differences between the images cause irregular spots in the scatter diagram.

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.

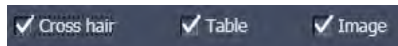
 Scatter diagram, image display and data table are interactively linked and immediately updated when settings are changed.

#### Available Controls:

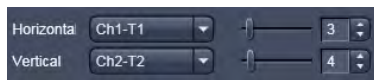
Drawing tools



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.

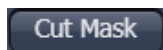


Scaling pull down menu: Sets the histogram graph scaling to either dynamic (adjusting to the maximum value) or fixed



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 R<sup>2</sup>)

### 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

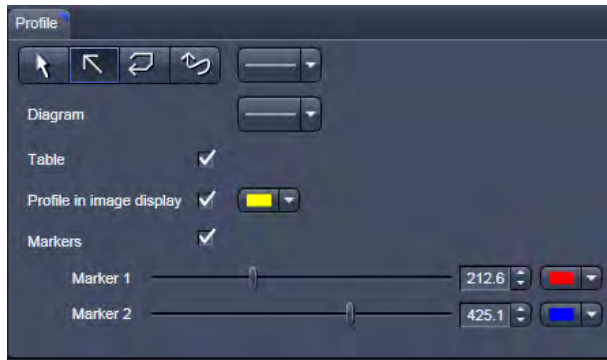
**6.10.4 Overlap Coefficient, Overlap Coefficient after Manders**

(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)



**Fig. 223 View Option control block - Profile View**

**6.11 Profile View**


This function allows to:

- display the intensity distribution of an image along a straight or curved line,
- show the intensity values in table form and copy table to clipboard or save as text file,
- show separate profiles for each channel in a multi channel image.

The settings of the **Dimensions**, **Display**, **Player** and **Graphics** view options control blocks apply. The additional view-specific **Profile View Option** control block is shown in Fig. 223.

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. 224).
- 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. 225).

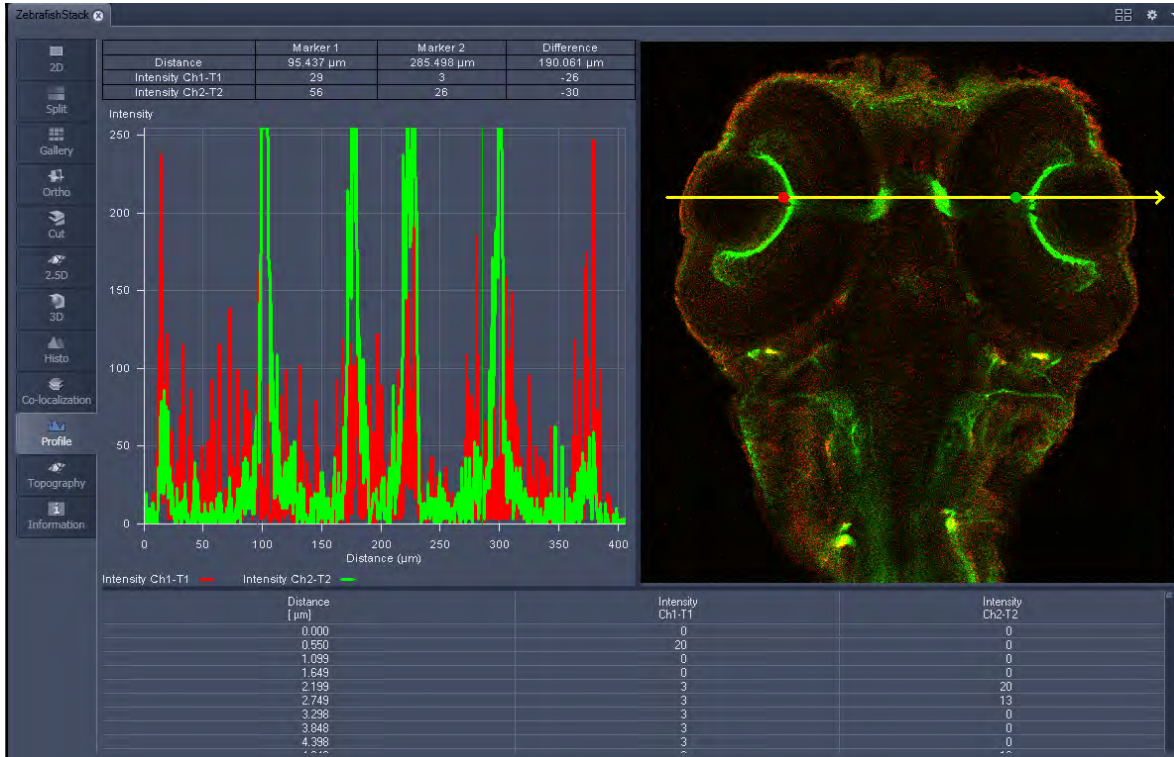


Fig. 224 Image Display - Profile View, Line Profile with markers

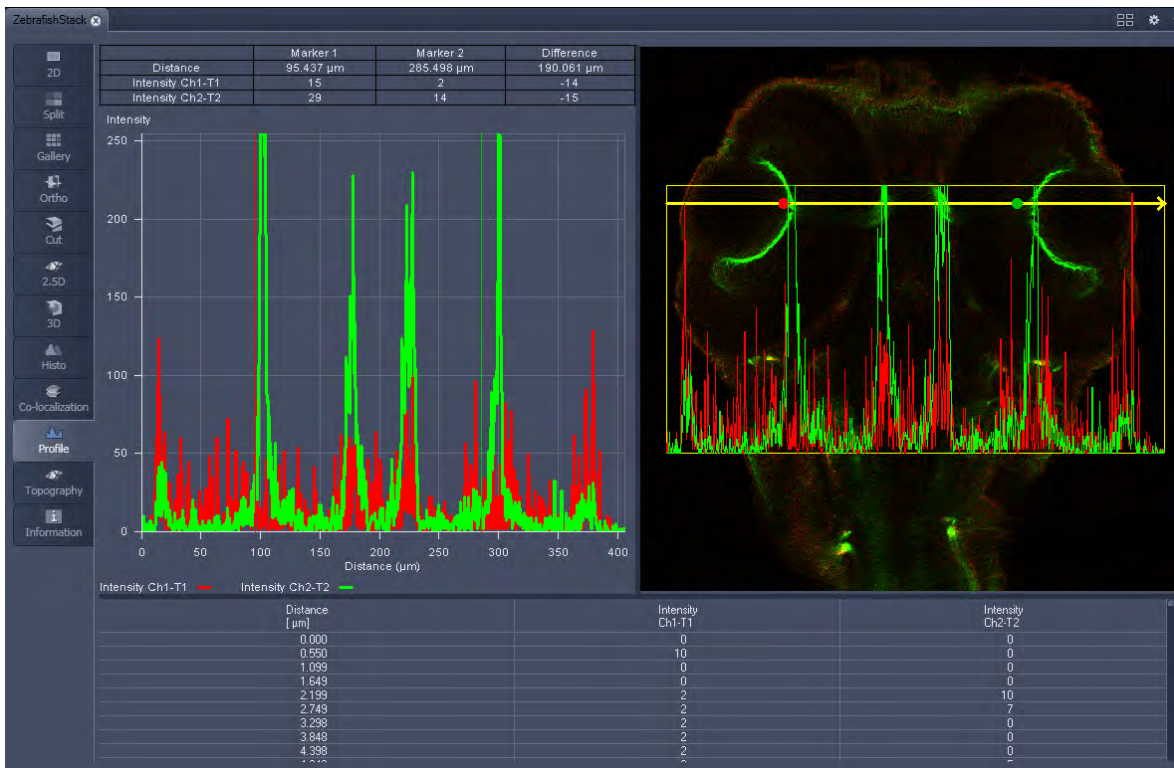


Fig. 225 Image Display - Profile View, Profile displayed in Image

## 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 Graphics** 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.

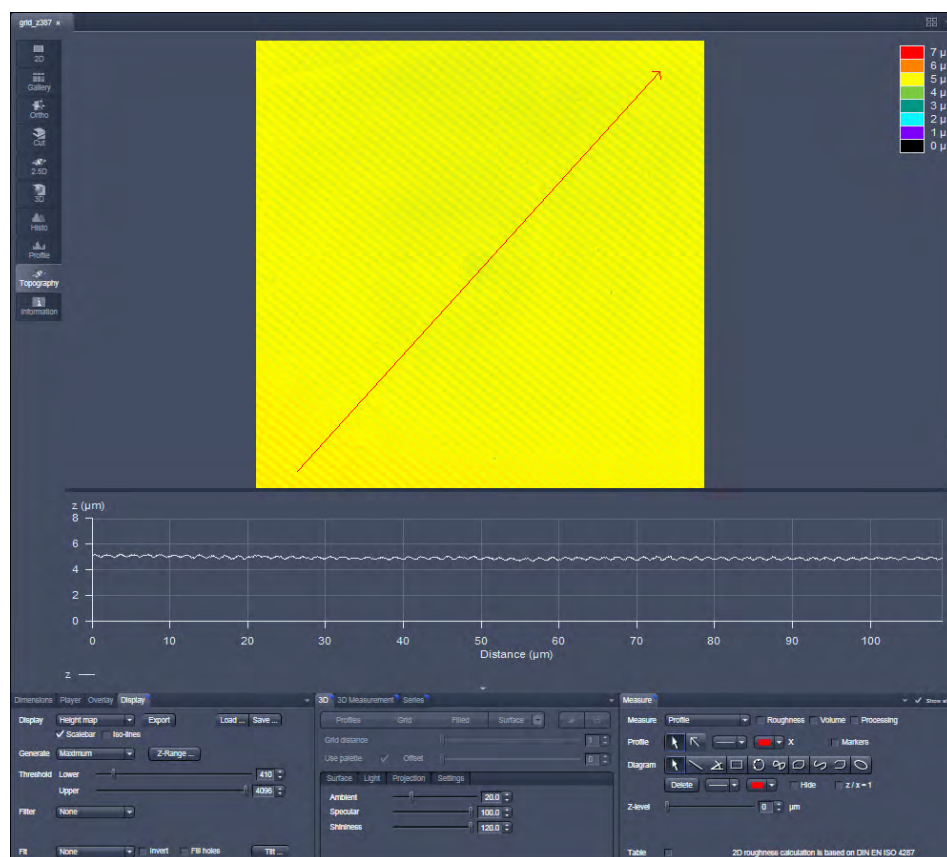


Fig. 226 Center Screen Area, Topography view

The Topography view contains the following image view control blocks:

<b>Display</b>	Set 2D or 3D, kind of generation, thresholds, filters and fits
<b>3D</b>	Set options of different 3D views.
<b>Measure</b>	Includes all measurement functionality, like profiles, roughness etc.
<b>3D Measurement</b>	Includes additional measurement functionality for 3D views.
<b>Series</b>	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:
    - Scalebar:** Show / hide scalebar
    - 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

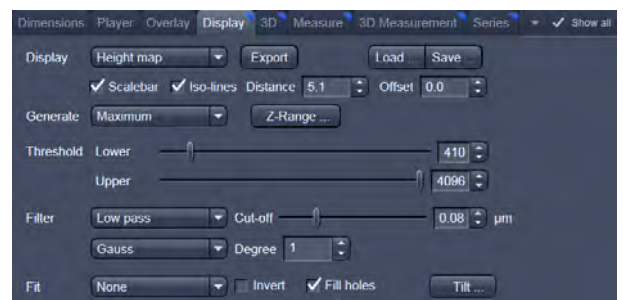
- Click on the **First maximum** button to calculate the topography surface by using the first maximum coming from the top.
- This mode provides better result for surfaces of semitransparent materials with inclusions of higher reflectivity or transparent multilayers with subsurface layers of higher signal intensity.
- Click on the **Last maximum** button to calculate the topography surface by using the last maximum coming from the top.
- Click on the **First/Last maximum** button to calculate both topography surfaces by using the first and last maximum coming from the top at the same time. Settings on thresholds and filters can be chosen individually for first and last maximum resp.

### 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.**
- Click on **Z-Range...** to calculate the topography surface by using the lower and the upper height thresholds for image display. Use of this function is recommended to get rid of unwanted peaks and valleys taken into account for parameter calculation. All topographic data with height values less or higher than the thresholds set are ignored for the display and parameter calculation. This threshold applies both for 2D as well as for 3D topography display modes.



**Fig. 227 Image view control block - Display**



**Fig. 228 Z-Range pop up to define height thresholds**

By clicking on the **Peak - valley** button the surface topography is displayed in that way that it is automatically normalized to the lowest and highest Z value of the current 3D topography. Ticking the 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.

- Choose a **Filter**:
  - **None**
  - **Median**
  - **Low pass**
  - **High pass**
  - **Band pass**
  - **Gauss**
  - **Average**

In case of **Low**, **High** and **Band** 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 or both at one time 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  $\mu\text{m}$ . Select a position of the **Degree** slider. The filter functions can be calculated from 1st order to 5<sup>th</sup> order accuracy. **Gauss** and **Average** filters can be set in pixels.

### 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.



### 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. 229).

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)

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. 230).

 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.

In case of **Surface** the renderer item can be set, clicking into the right part of the **Surface** button (Fig. 231).

#### OpenGL - software

The graphics calculation is performed using the installed software.

#### OpenGL - hardware

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

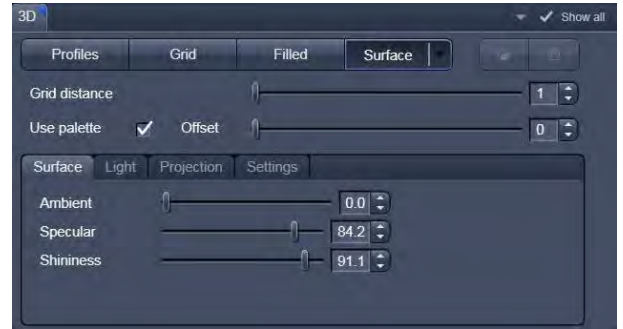


Fig. 229 Image view control block - 3D

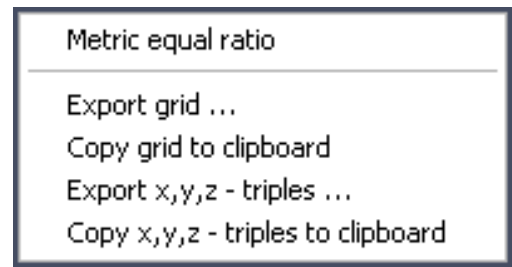


Fig. 230 Context menu using 3D Grid



Fig. 231 Surface renderer items

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



Fig. 232 Surface renderer: Surface tab



Fig. 233 Surface renderer: Light tab



Fig. 234 Surface renderer: Projection tab

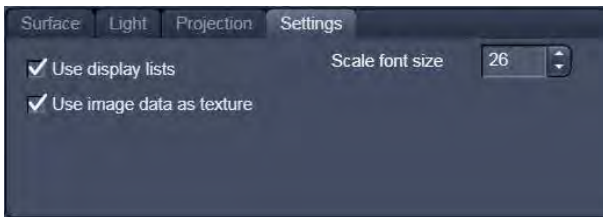


Fig. 235 Surface renderer: Settings tab

**Ambient/Specular:** Material properties; how many % of the light component are projected by the material into which spectral ranges.

**Shininess:** Suitable to specular light. Shininess equal to 25 % determines diffuse light.

**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.

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

**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 maximum intensity projection is used as texture of the 3D model.

**Scale font size:** Sets the font size for the 3D model.



**Load / Save** (see Fig. 229) 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):

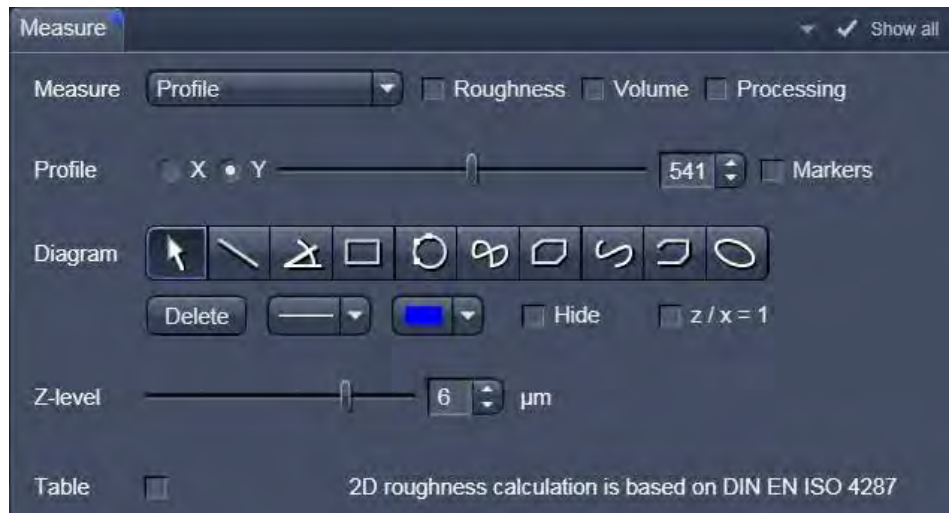
- Click in the image and hold down the mouse button. The perspective is changed by moving the mouse button in horizontal or vertical direction.

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. 236). The measurement functions can be performed in Height map, Maximum intensity and 3D display modes.



**Fig. 236 Image view control block: Measure**

Automated convention in height statistics analysis:

<b>Topo Filters</b>	None, median,	FFT High	FFT Low
Data formats	<b>P</b> Primary profile	<b>R</b> oughness	<b>W</b> aviness
2D profile	Pxx	Rxx	Wxx
3D topography	PSxx	RSxx	SWxx

The following measurement functions are available in the **Measure** drop down menu:

- **No diagram**
- **Profile**
- **Z-histogram**
- **Bearing area ratio**

Also these 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

- Choose **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



Click this icon to draw parallel profiles within the height map (not available in 3D view)

If **3D** is chosen in the Display block, the following tools are available to set the profile line within the image:





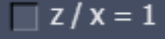
With these sliders the profile line can be moved on the 3D topography, in X and Y direction as well as rotary.


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:

	<b>Move</b> the markers. <b>Zoom</b> 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 <b>straight line</b> . 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 <b>free angle</b> . 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 <b>rectangle</b> . 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 <b>circle</b> . 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 <b>closed Bezier</b> 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 <b>closed polyline</b> 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 <b>open Bezier</b> 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 <b>open polyline</b> 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 <b>ellipse</b> . 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.
	<b>Deletes</b> all drawing elements or the one just selected.
	Change of the <b>line width</b> of the drawing elements.

	Clicking on the <b>Color</b> 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 <b>hide</b> all drawing elements, but not the markers.
	Sets the <b>z/x</b> ratio in the profile diagram to the value <b>1</b> . 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

- Choose 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

Choose **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

- Choose **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
	Maximum peak to valley	Rz	Pz	Wz
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 elements  $R_c$

$$R_c = \frac{1}{N_y} \cdot \sum_{j=1}^{N_y} \cdot z_{tj}$$

where  $z_{tj}$  is the peak to valley height of the profile element j.

$N_x, N_y$  ... number of pixels in X- or Y-direction

- Arithmetic mean deviation of all profile height values  $R_a$

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

- 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  $R_{KU}$

$$R_{KU} = \frac{1}{N_y \cdot R_q^4} \cdot \sum_{j=1}^{N_y} z^4(x, y_j)$$

- Maximum peak height  $R_p$

$$R_p = z_{\max} - \bar{z}, \quad \bar{z} = \frac{1}{N_y} \cdot \sum_{j=1}^{N_y} z(x, y_j)$$

- Maximum valley depth  $R_v$

$$R_v = \bar{z} - z_{\min}$$

- Maximum roughness depth  $R_t$  (= Peak to Valley / PV)

$$R_t = z_{\max} - z_{\min}$$

maximum height difference of the overall topography.

- Roughness depth along a profile  $R_z$

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

maximum height difference of the profiles.

 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
	Maximum peak to valley	RSz	PSz	WSz
If chosen filters are:		FFT High	No, M	FFT L

**(4) Calculation of roughness parameters:**

The following roughness parameters are calculated:

- Mean height of surface elements  $RS_c$

$$RS_c = \frac{1}{N_x \cdot N_y} \cdot \sum_{i=1}^{N_x} \cdot \sum_{j=1}^{N_y} \cdot z_{tij}$$

where  $z_{tij}$  is the peak to valley height of the surface element  $i,j$ .

Surfaces are split into 5 x 5 surface elements.  
 $N_x, N_y$  ... number of pixels in X- or Y-direction

- Arithmetic mean deviation of all surface height values  $RS_a$

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

- 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  $RS_{KU}$

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

- Maximum peak height  $RS_p$

$$RS_p = z_{\max} - \bar{z}, \quad \bar{z} = \frac{1}{N_x \cdot N_y} \cdot \sum_{i=1}^{N_x} \cdot \sum_{j=1}^{N_y} \cdot z(x_i, y_j)$$

- Maximum valley depth  $S_v$

$$RS_v = \bar{z} - z_{\min}$$


- Maximum roughness depth  $RS_t$  (= Peak to Valley / PV)

$$RS_t = z_{\max} - z_{\min}$$

maximum height difference of the overall topography.

- Maximum roughness depth  $RS_z$  of surface elements

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

 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  **Volume** within the Measure line:
  - The volume parameters are calculated and displayed to the right of the image.
  - To copy / save the content displayed, click with the right mouse button into the shown tables and decide to copy or save. The values can be imported to other programs (e.g.: MS Word or MS Excel) via the **Paste** function.
- Setting the **Z-level** slider enables you to change the height level of the topography. The portion of the topography lying below the set height level is filled with "water" (blue color) and the volume parameters are calculated online only for the projecting part of the topography.
- To use the **Z-level** function, load the **Profiles** 3D display mode containing the **Glowscale** or **Grey** channel color to obtain optimum display.
- If the **Measure** function **Bearing area ration** is also activated, a red marker line shows the position of the height level in the percentage of contact area curve.

Within the Volume measurement also the ratio of **valid** data points (means signal intensities within a given intensity threshold) is displayed.

#### Parameters

The following parameters are calculated:

**Z:** height level (selectable with the **Z-Threshold** and **Fill Level** sliders). The setting of this value influences the following parameters.

**Vm (z):** material volume above chosen height level

**Vv (z):** void volume below chosen height level

**Vmr (z):** material volume ratio

$$V_{mr}(z) = \frac{V_m(z)}{V_m(z_{min})}$$

**Vvr (z):** void volume ratio

$$V_{vr}(z) = \frac{V_v(z)}{V_v(z_{max})}$$

**Sr:** surface bearing area ratio of the topography at Z  
percentage of contact area (=  $A_u / (x * y) * 100 \%$ )

$A_u$  - surface bearing area of the topography at Z (= projection area of those parts which are situated above chosen height level)

**Sda:** true surface = sum of all triangles formed by adjacent data points of the surface reconstruction

**Sdr:** developed surface area ratio:

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

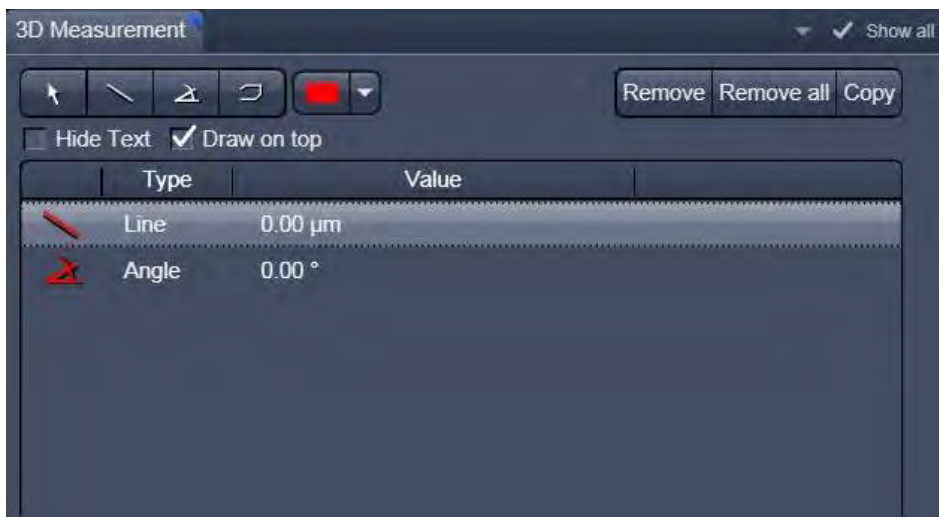
$$\text{projected area} = x * y$$

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. 237 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 (VisArtplus).

### 6.13 Mean of ROI: Additional View Type for Time Series

The **Mean of ROI** View Type allows to.

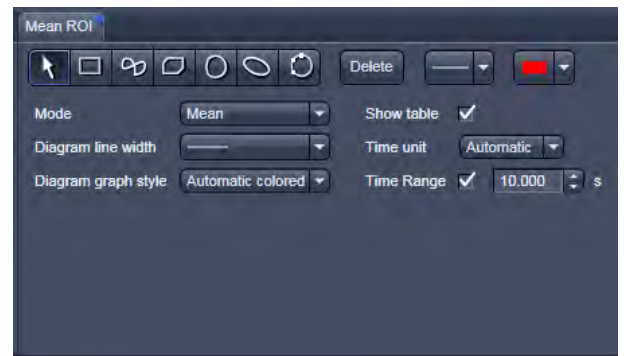
- display the intensity - time diagram (mean intensity in user defined ROIs over time),
- use frame - time series and frame - Z-Stack - time series as input,
- show the intensity values in table form and copy table to clipboard or save as text file.

The **Mean of ROI** function permits interactive analysis and monitoring intensity over time.

The settings of the **Dimensions, Display, Player** and **Graphics** View Options control blocks apply. The additional view-specific **Mean of ROI View Option** control tab is shown in Fig. 238.

Any changes done with this View Option control tab 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. 239 and Fig. 240).



**Fig. 238 Mean of ROI View Option control tab**

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. 239).

The **Mean of ROI** analysis always refers to the currently displayed z-section of a Z-Stack (see **Dimension** View Option control tab for which section it is).

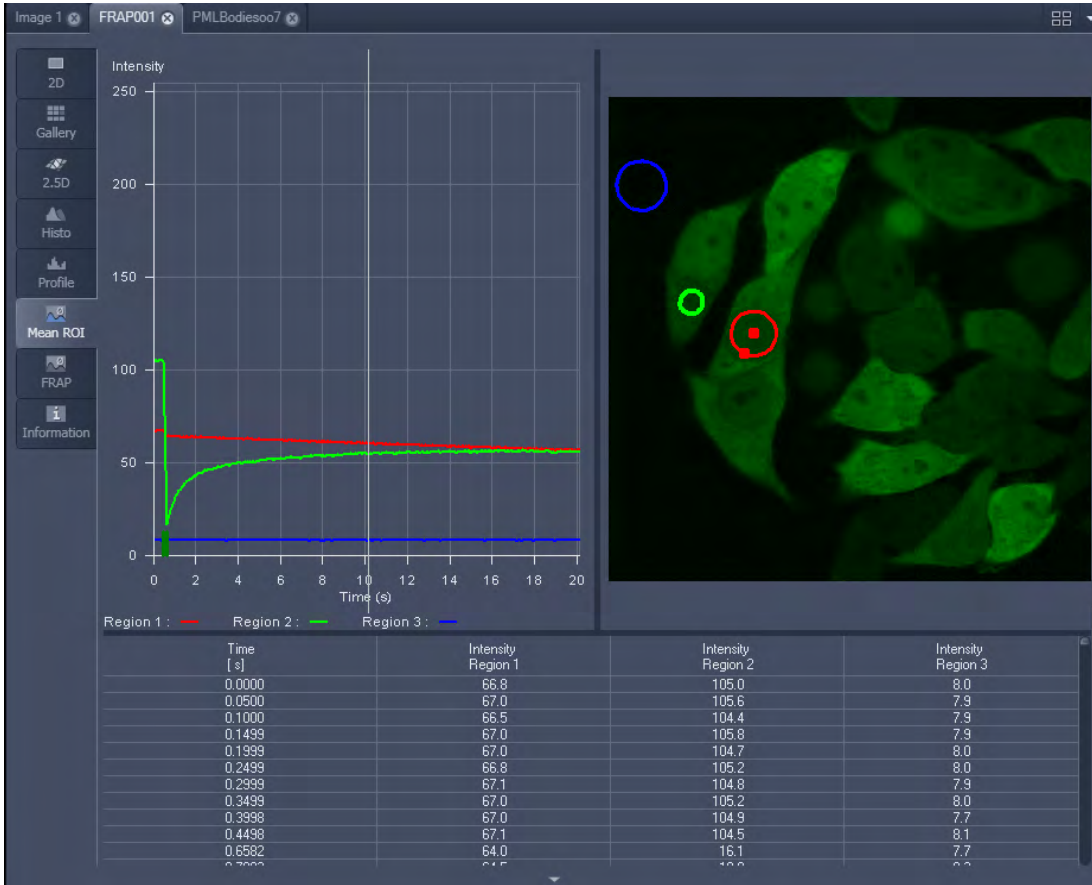


Fig. 239 Image Display window, Mean ROI display for time series in single plane

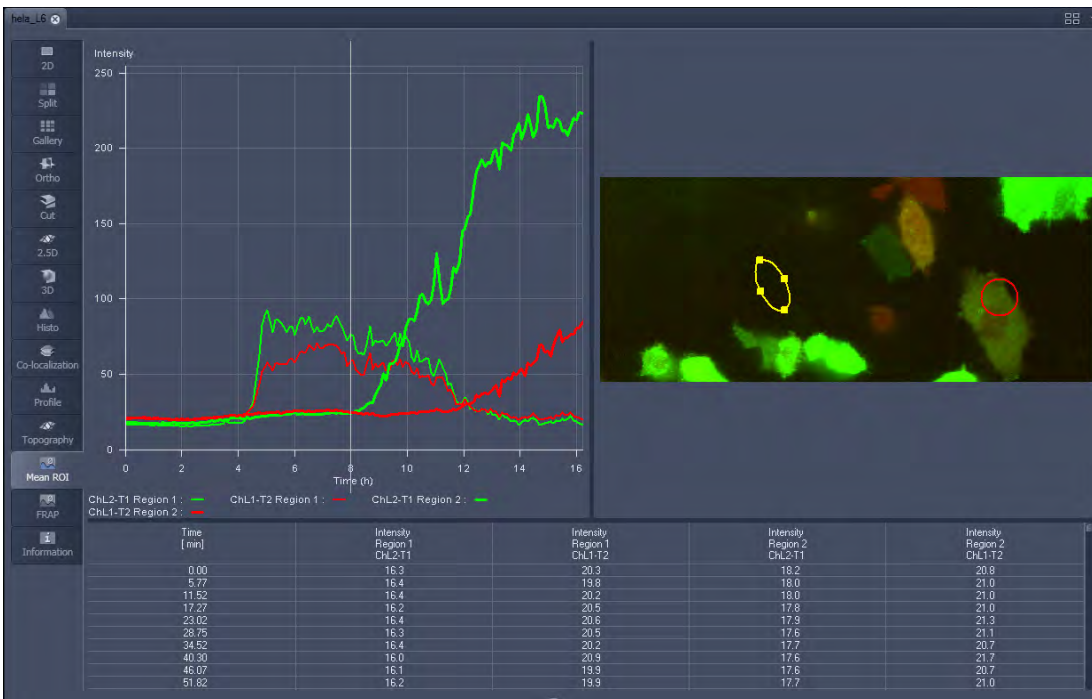





Fig. 240 Image Display window, Mean ROI display for time series of Z-Stack

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

#	Type	X	Y	Width	Height	Lock
1		147	65	51	37	
2		239	176	40	40	
3		376	121	84	113	

**ROIs** can be managed in the **Graphics** View Option control tab.



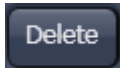
**Arrow** button: Activation of the mouse button for resizing or movement of the ROI in the **Image Display** window.



**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.



**Delete** button: All the ROIs to the image are deleted.



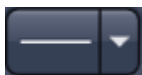
**Rectangle and 3D Rectangle** button: Activates the rectangle drawing mode. Click and hold down the mouse button, drag the rectangle in any direction, release the mouse button to end the procedure.



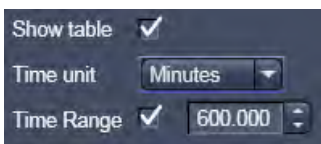
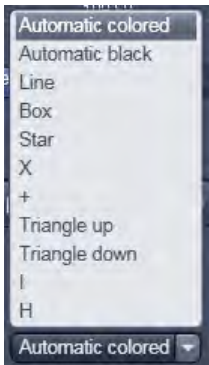
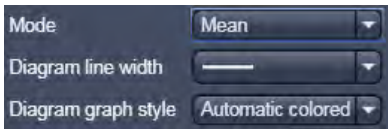
**Ellipse and 3D Ellipse** button: Activates the ellipse drawing mode. The first click sets the center point, the displayed line permits the determination of the first dimension, the second click sets the first dimension, the second dimension and the rotation direction can then be determined; the third 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.

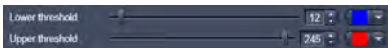


**Buttons for diagram display options:**

- **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** tick box and spin box: reduce the displayed time range to the selected value



**In Area Mode: Ch1 / Ch3 / Ch4** pull down: Selection of the channel to be used.



**In Area Mode: Threshold low and high** slider: The intensity values below /above threshold are not displayed for the **Area** function.



### 6.14 Kinetic / FRAP View: Additional View Type for Time Series

The **FRAP** function permits interactive analysis of bleaching experiments, including.

- Correction of FRAP data for bleaching and background,
- Fitting of FRAP data to a mono exponential or double exponential model for intensity.

The settings of the **Dimensions, Display, Player** and **Graphics** View Options control tabs apply. The additional view-specific **FRAP View Option** control tab is shown in Fig. 241.

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. 242, Fig. 244 and Fig. 245).

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

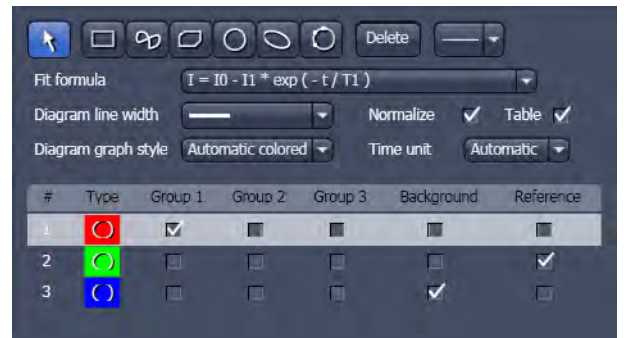


Fig. 241 FRAP View Option control block

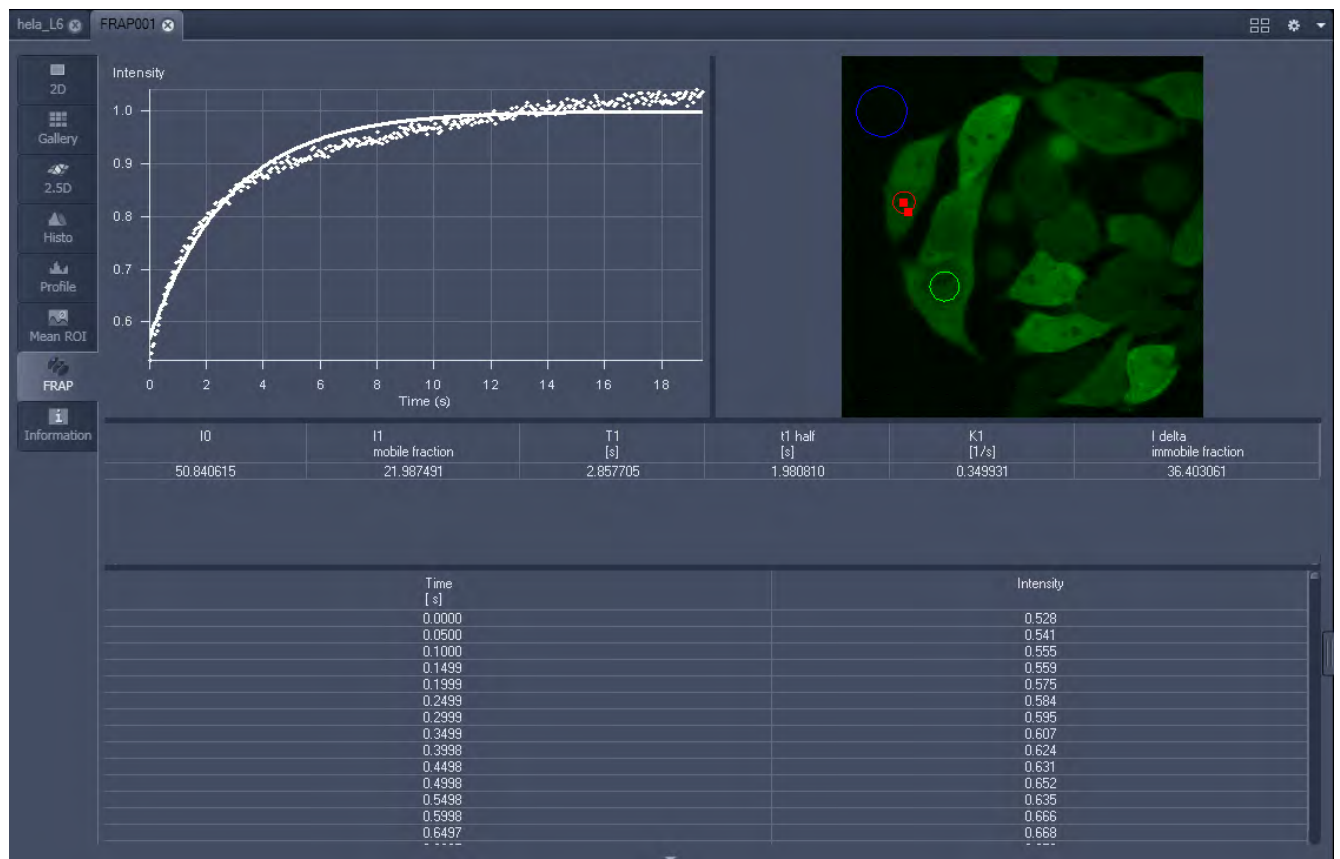


Fig. 242 Image display of a time series in the FRAP View tab

Available tools in the FRAP View:

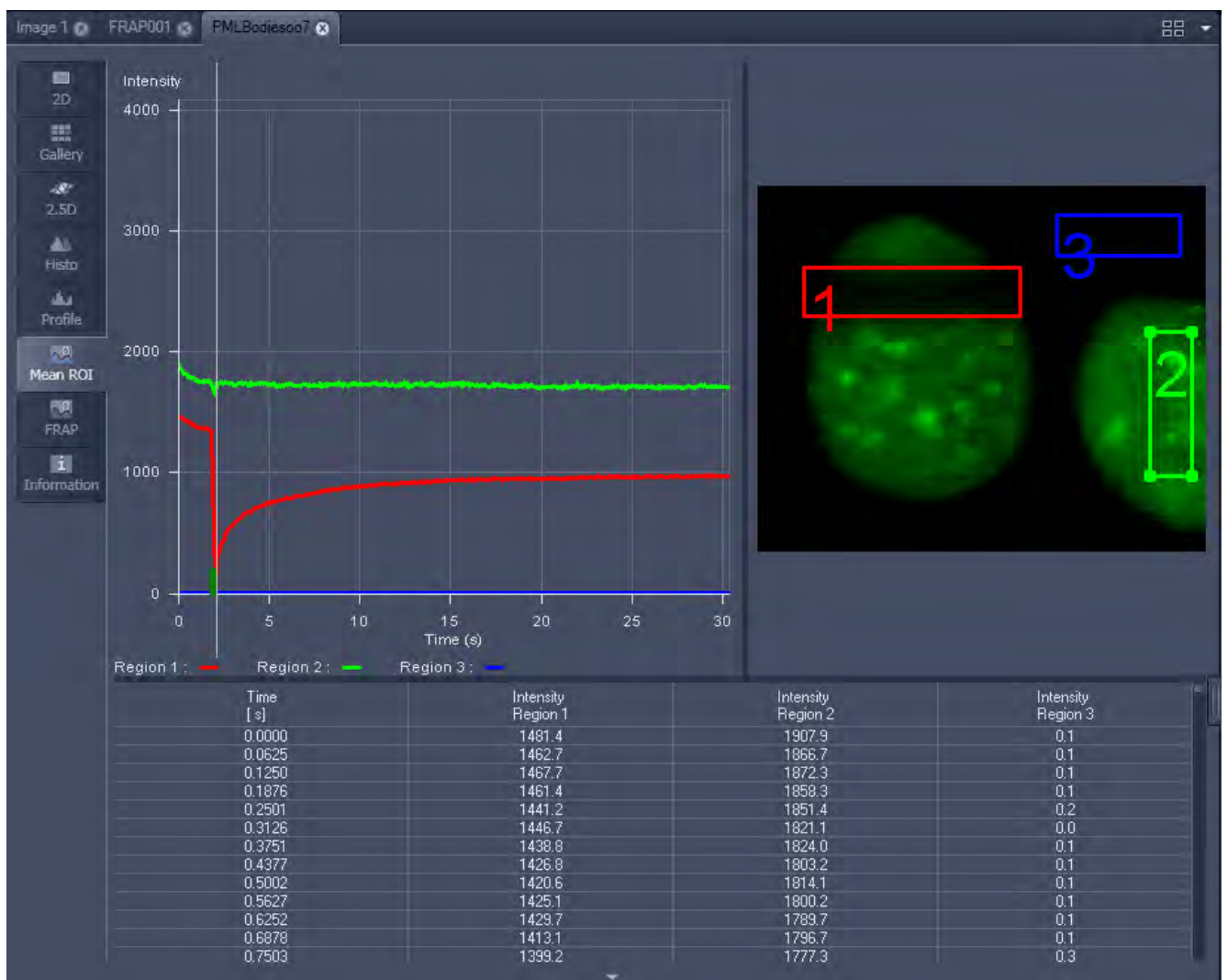
- Channels** Select single channels or all channels for analysis in the general **Dimensions** view options control tab.
- Kinetic Model / Fit formula** Pull down menu to select the mathematical model (mono or double exponential model) for fitting the data.
- Analysis Areas / Regions of Interest (ROIs)** The **FRAP** View Options control tab includes drawing tools that work identically to the **Graphics** 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 3), and the reference ROI (ROI 2) (see Fig. 243). 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 tab 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 **Graphics** view options control block.

- Define your ROIs for analysis in the **Mean of ROI** View tab.



**Fig. 243** 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.


- Choose 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**).

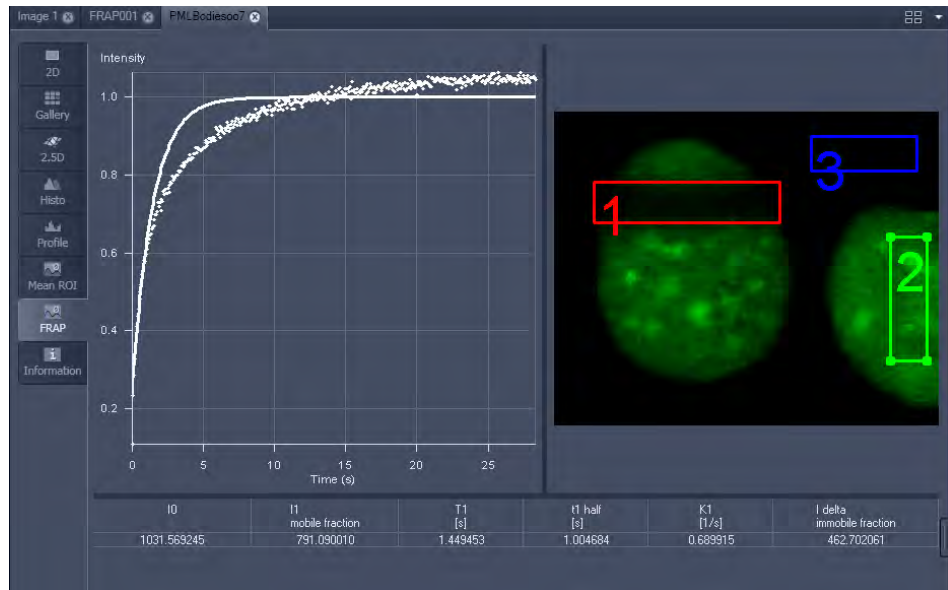
The data can be normalized optionally when marking the check box **Normalize** in the FRAP View Options control block.

 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.

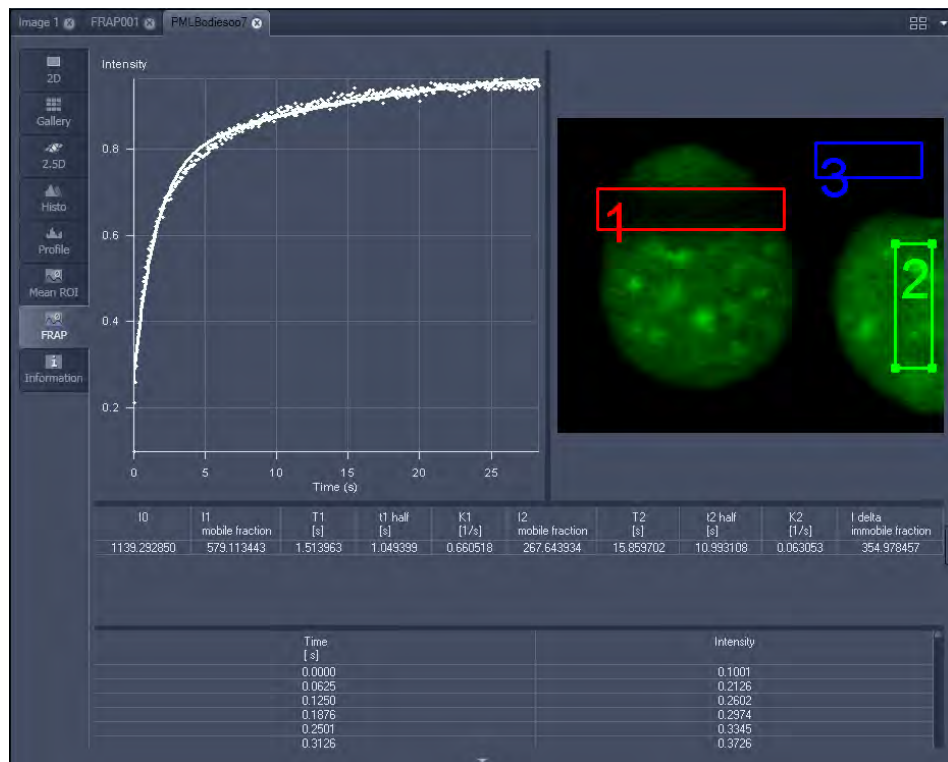
 The analysis is not stored with the image.

 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:  $-t_{\text{half}} = (\ln 0.5) * T1$



**Fig. 244** Image window displaying the analysis of FRAP data using a mono exponential fit



**Fig. 245** 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:

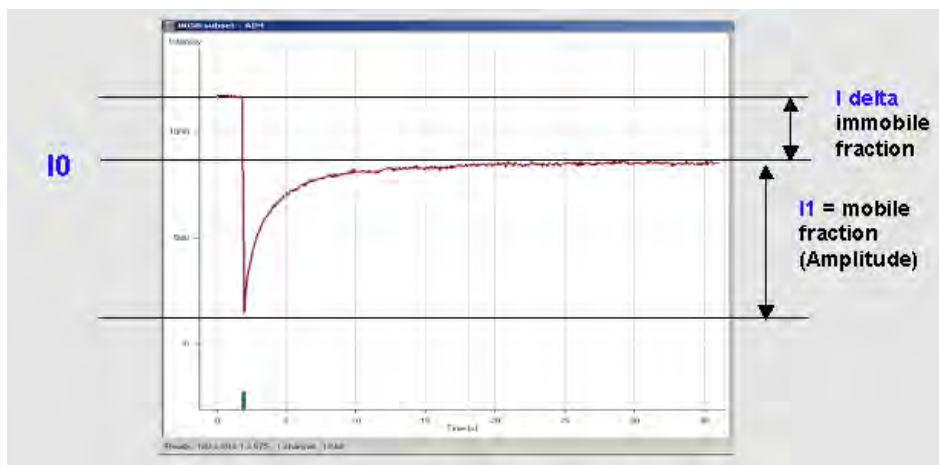
- The amplitude of the two curves, displayed as one (which corresponds to each part of the mobile fractions **I1** and **I2**).
- The fitted parameters **T1** and **T2** (seconds) for each mobile fraction.
- The rate constant for the exchange of molecules between the bleached region and the surrounding 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 dialog, 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. 246. 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. 246** Schematic FRAP curve with marks at the relevant data points. **I0**, **I1** and **I delta** which are calculated performing the Kinetic Analysis

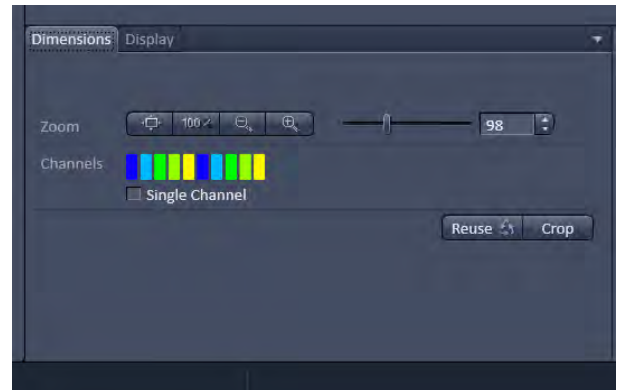
### 6.15 Lambda Coded: Additional View Types for Lambda Mode

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. 248).

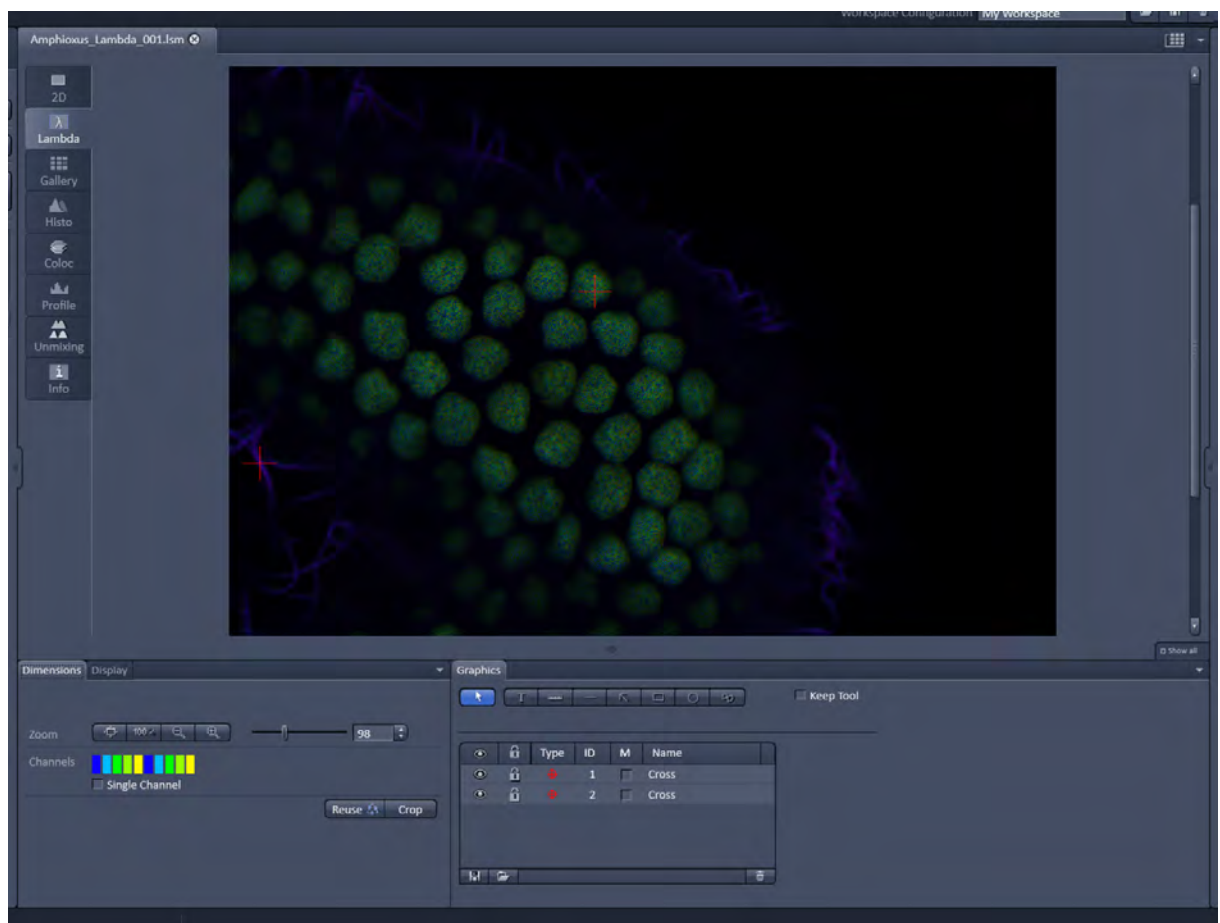
The settings of the **Graphics** View Options control block apply as in 2D view. In the **Display** View Options control block, the channel-specific settings of brightness, contrast and gamma don't apply since there are no channels.

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. 247.

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



**Fig. 247** Lambda Coded - specific Dimensions View Option control block



**Fig. 248** Dimensions view option control block for Lambda Coded View

### 6.16 FRET View

In the **FRET** View type (Fig. 249), 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.

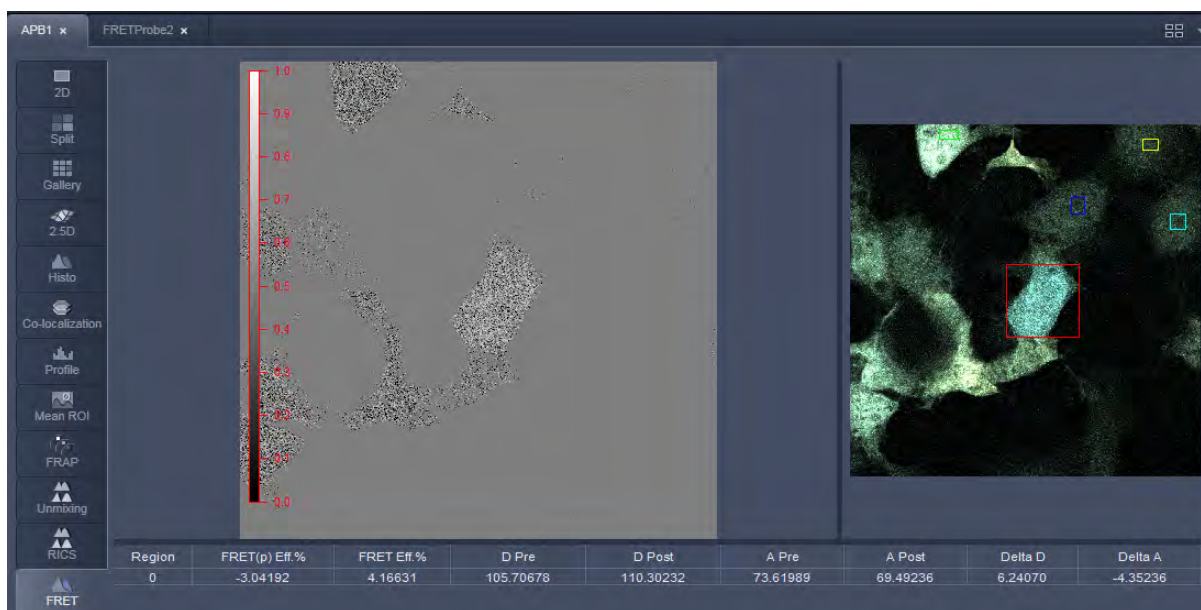
The **FRET** View will only be present if either a time series with a bleach event or a three channel image is present in the image container.

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.

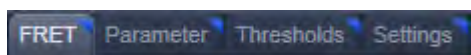
To access the **FRET** View, load or acquire either a multichannel data set (minimum 3 channels) or a time series with bleach event and click on the **FRET** tab.

 **Tables can always be saved or copied** to the clipboard by right-mouse clicking on the table display!



**Fig. 249 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. 250):

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 **Graphics**).

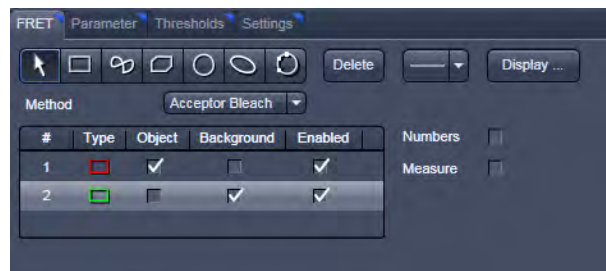
As **Method** for FRET Analysis, only **Acceptor Photobleach** is available in the drop down list.

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**.



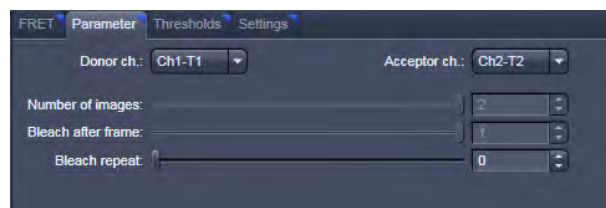
**Fig. 250 FRET View Options Control Block, FRET tab**

#### Parameter tab (Fig. 251):

In case the image series for Acceptor Photobleaching has not been acquired with the FRETplus Macro, various Parameters of the image series can be set in this tab.

The image of the Donor and the Acceptor are assigned to the appropriate channel using the drop down menu next to **Donor ch.:** and **Acceptor ch.:**.

The total **Number of images**, the time for the bleaching event (**Bleach after frame**), and the action of **Bleach repeat** is set using the slider next to each action or by editing the field next to the slider.



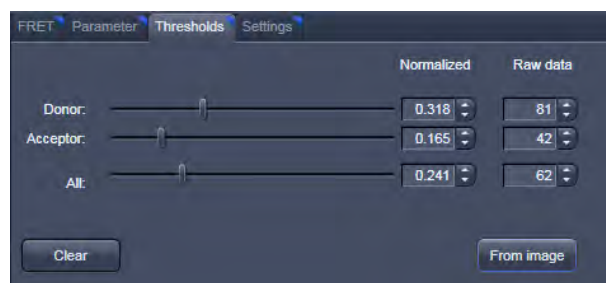
**Fig. 251 FRET View Options Control Block, Parameter tab**

#### Thresholds tab (Fig. 252):

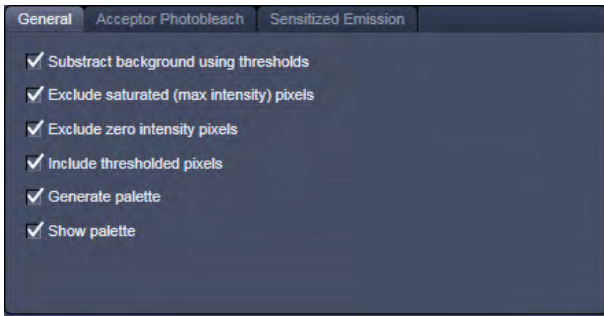
The threshold for the image analysis can be set manually using the slider or editing text box next to **Donor**, **Acceptor** or **All**, where **All** moves both sliders to the same value.

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.

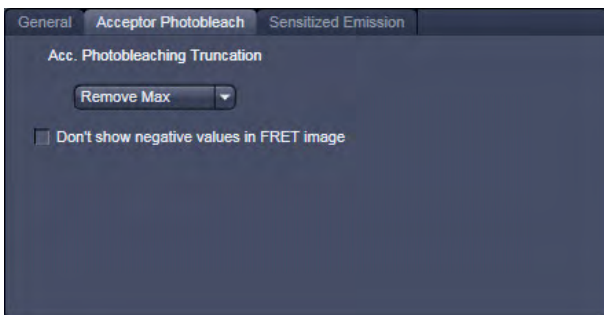
The values for the thresholds are either displayed as grey value levels (**Raw data**) or **Normalized** to the value 1.



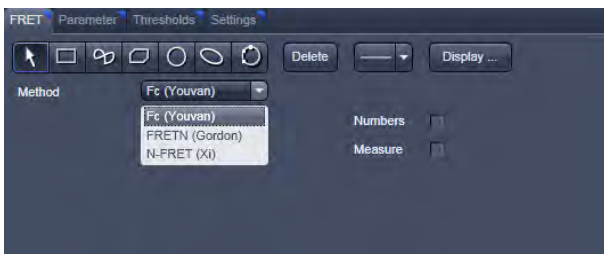
**Fig. 252 FRET View Options Control Block, Thresholds tab**



**Fig. 253 FRET View Options Control Block, Settings tab, General**



**Fig. 254 FRET View Options Control Block, Settings tab, Acceptor Photobleach**



**Fig. 255 FRET View Options Control Block, FRET tab**

**Settings tab (Fig. 253):**

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. 254) 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. 255):**

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

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. 256):**

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**).

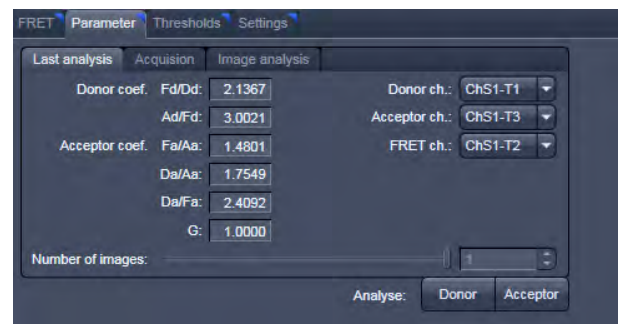
The values for the **Acceptor coefficient** include the FRET signal detected in the Acceptor channel / Acceptor signal detected in the Acceptor channel (**Fa/Aa**), the Donor signal detected in the Acceptor channel / Acceptor signal detected in the Acceptor channel (**Da/Aa**), the Donor signal detected 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.:**

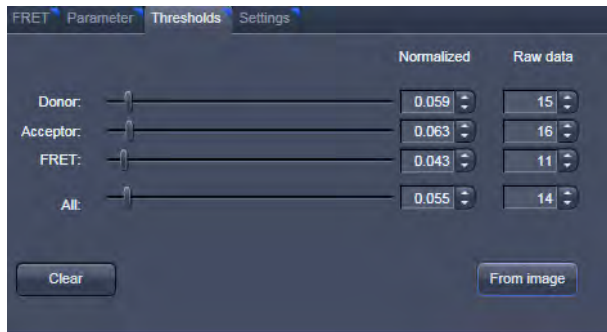
The total **Number of images** is set using the slider or by editing the field next to the slider if necessary.

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.



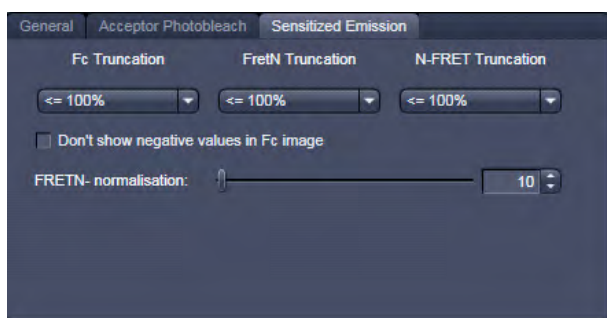
**Fig. 256 FRET View Options Control Block, Parameter tab, Last Analysis**



**Fig. 257 FRET View Options Control Block, Thresholds tab**

**Thresholds tab (Fig. 257):**

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. 258 FRET View Options Control Block, Settings tab, Sensitized Emission**

**Settings tab (Fig. 253 and Fig. 258):**

This tab provides access to the definition of the parameters used for the image analysis. The tab **General** (Fig. 253) 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. 258) sets additional Truncation parameters for each method to analyze the Sensitized Emission experiment such as the choice for **No truncation**, **Remove maximum** or the percentage of truncation to choose between 100, 200 and 300 %. For the Fc analysis method, the option "**Don't show negative values in the Fc image**" can be selected using the check box. Also included is a slider to set the value for normalization of the results of the FRETn-method.

## 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 **Graphics** 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. 259 and Fig. 260.

Any changes done with this View Option control block 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. 261). The spectra from images which are acquired on an LSM 700 are shown as integrated spectra. This reflects the way, how Lambda stacks are acquired with the LSM 700 (see the respective description in the LSM 700 Manual).

To access the Unmixing analysis view type, load or acquire a Lambda stack data set and click on the **Unmixing** tab.

 The Unmixing View is available (→ displayed) for all Lambda stacks and for all multi-channel images acquired on hardware with calibrated detectors. This includes all LSM 710, LSM 780, LSM 710 NLO, LSM 780 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. 259 Unmixing View Option control block



Fig. 260 Unmixing - Extract - View Option control block

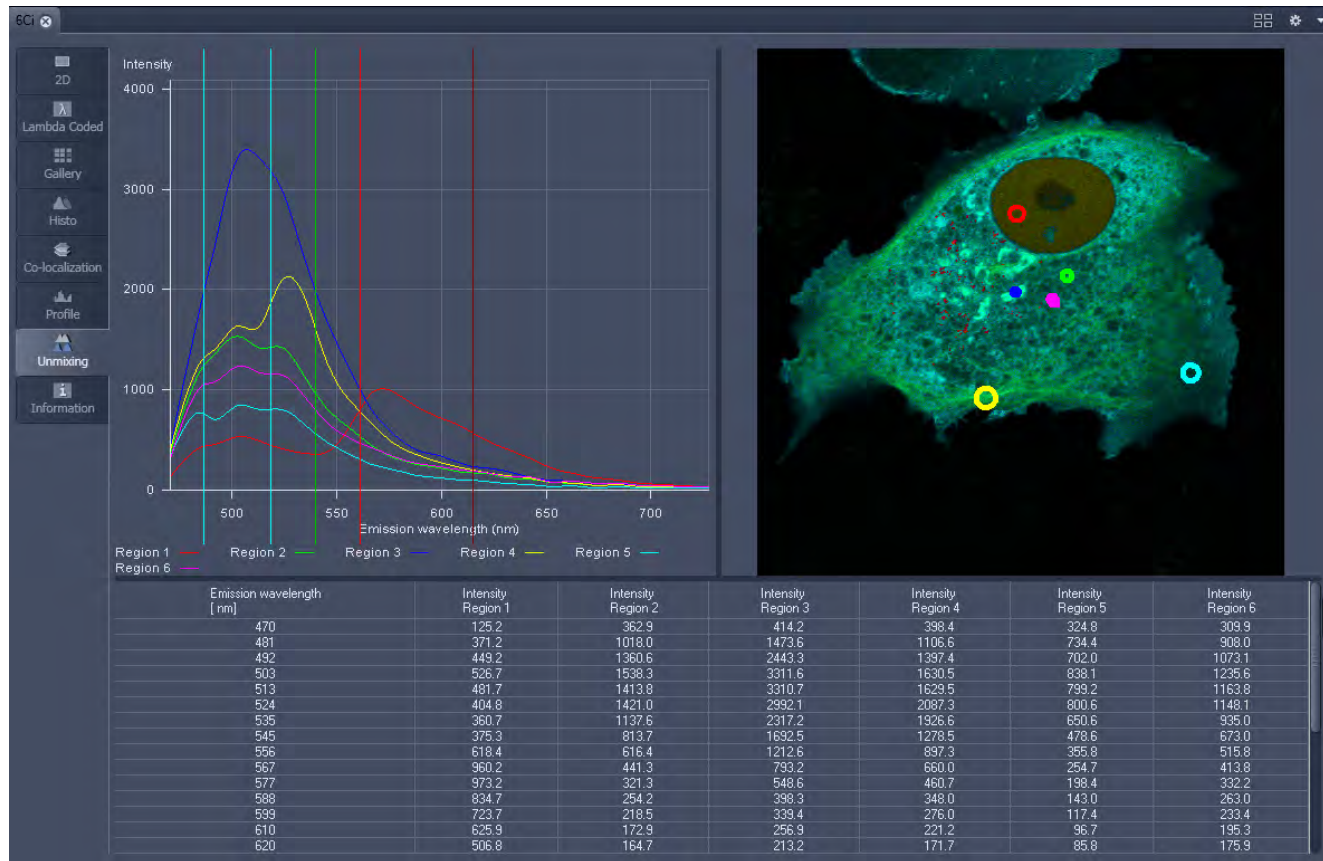
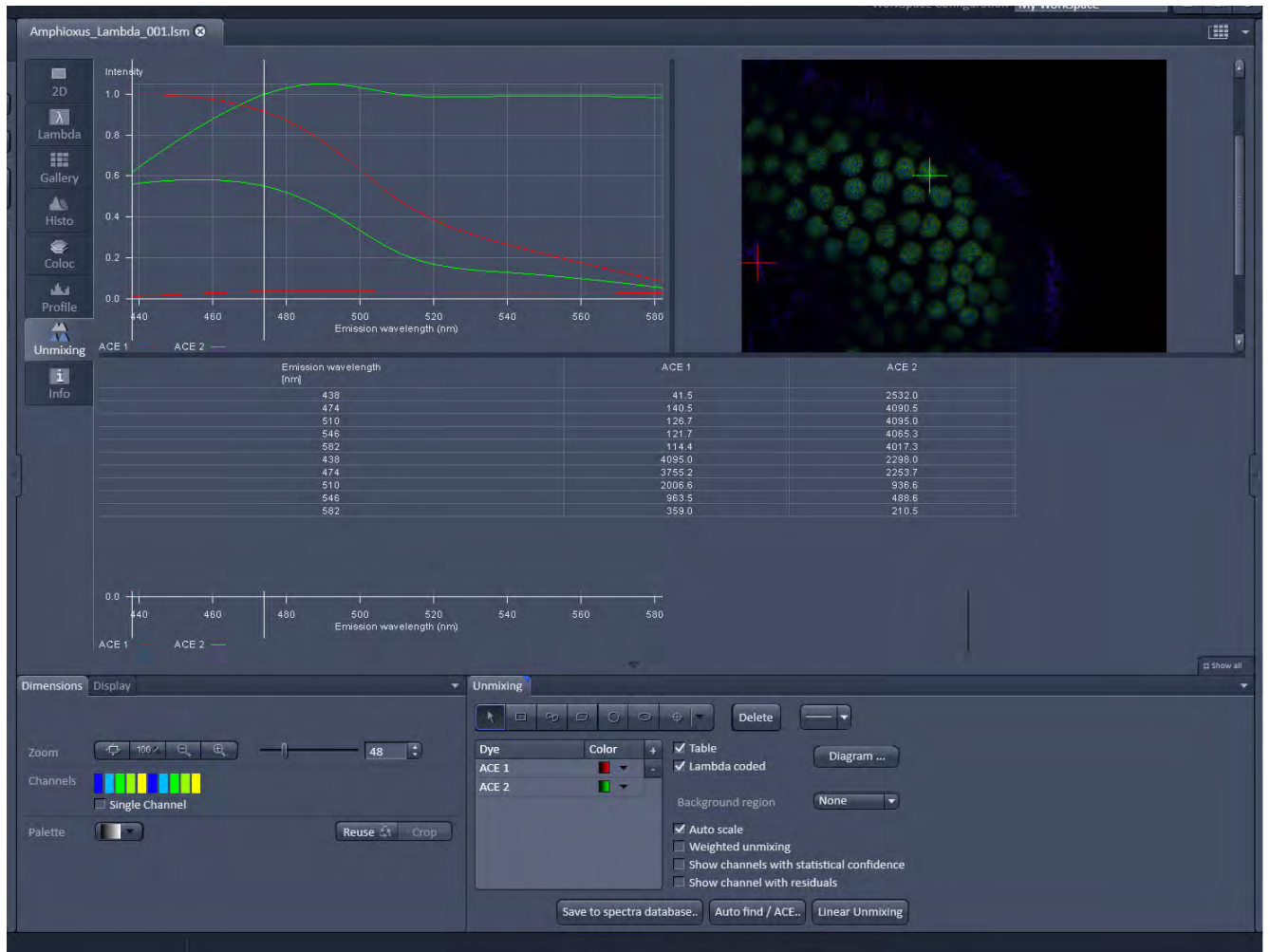


Fig. 261 Unmixing Display of a Lambda stack on a LSM 710 / 780



**Fig. 262** 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

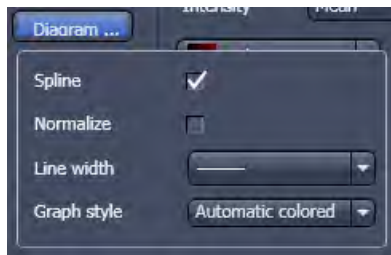
– Tools for display customization and drawing:

#### Drawing tools

Drawing tools work the same as in the **Graphics** View Option control block

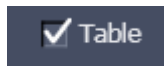


**Check box for image display:** show the image in grayscale or Lambda coded




#### 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!

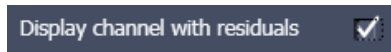
– Tools for unmixing:



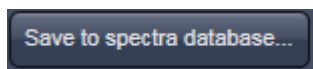
Select ROI for background subtraction



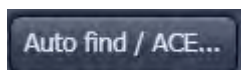
Automatically balances the intensity of unmixed channels to equal levels.



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 **Processing – 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. 263).



#### **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.



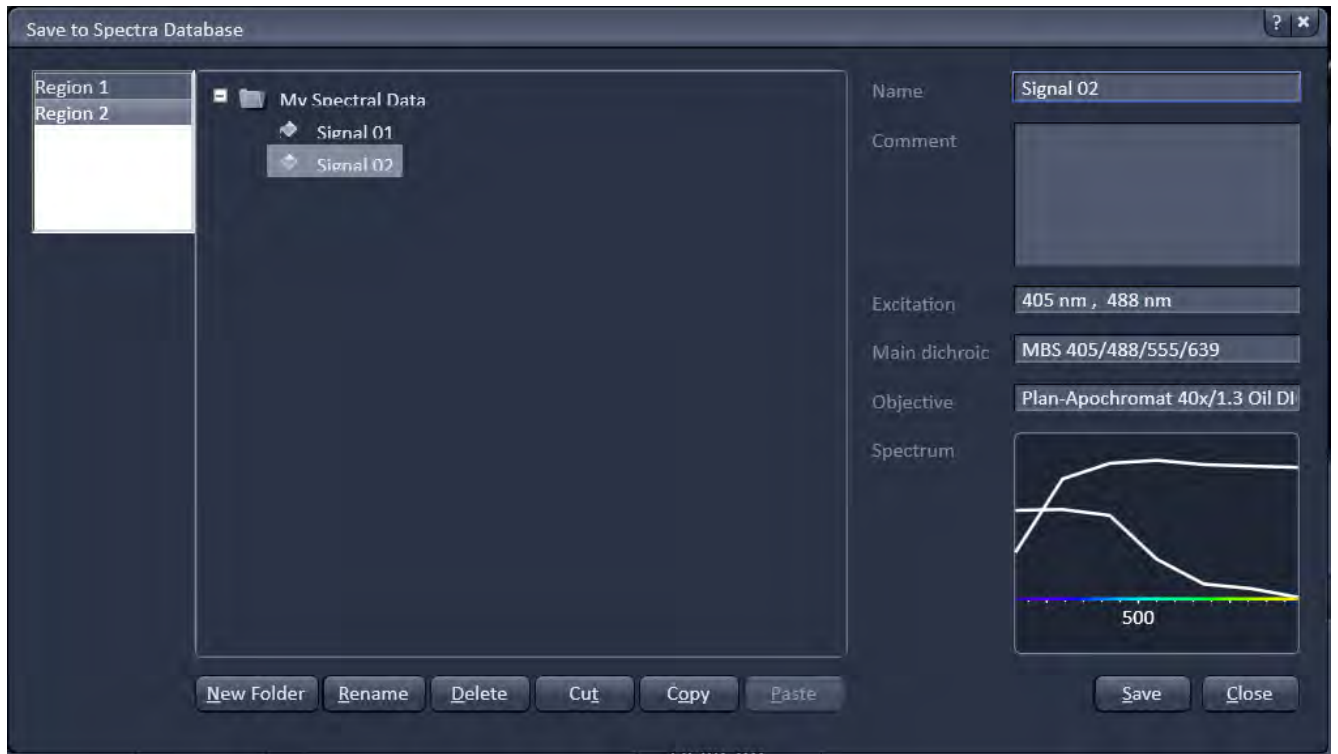
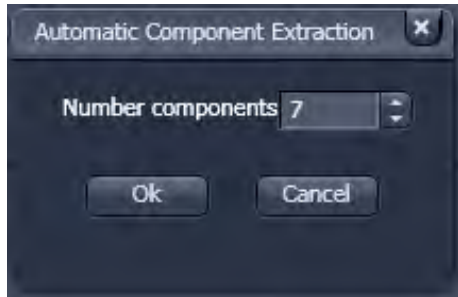
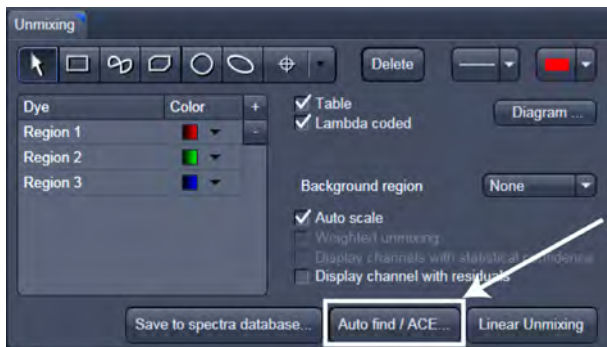


Fig. 263 Save to Spectra Database: database interface with subfolder structure



**Fig. 264 ACE, number of components selection window**



**Fig. 265 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 **Unmix View** tab.
- Click on the **Auto find / ACE...** button in the **Unmixing** View Option control tab (Fig. 265).
- Select the number of components in the sample and click **Ok** (Fig. 264).
- Review the results in graph.
- When the obtained spectra appear reasonable, unmix the data set by clicking the **Linear Unmixing** button (Fig. 265).
  - 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.

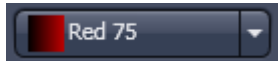
However, it provides a solution for Emission Fingerprinting in those cases where reference spectra are not accessible via single-labeled controls.

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 and LSM 780 system with 34 channel Quasar detector. Hence, they are not available for LSM 700 or LSM 7 LIVE Hardware (tool tab grayed).

Button:



Slider:



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.



Add or reduce the number of channels displayed and available for assignment. Up to 8 channels labeled after the assigned LUT are available.

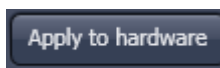


Sum signals from selected detector elements  
Mean signals from selected detector elements  
Autoscaling of output channels (individually)



**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 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  $\mu\text{m}$
- 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

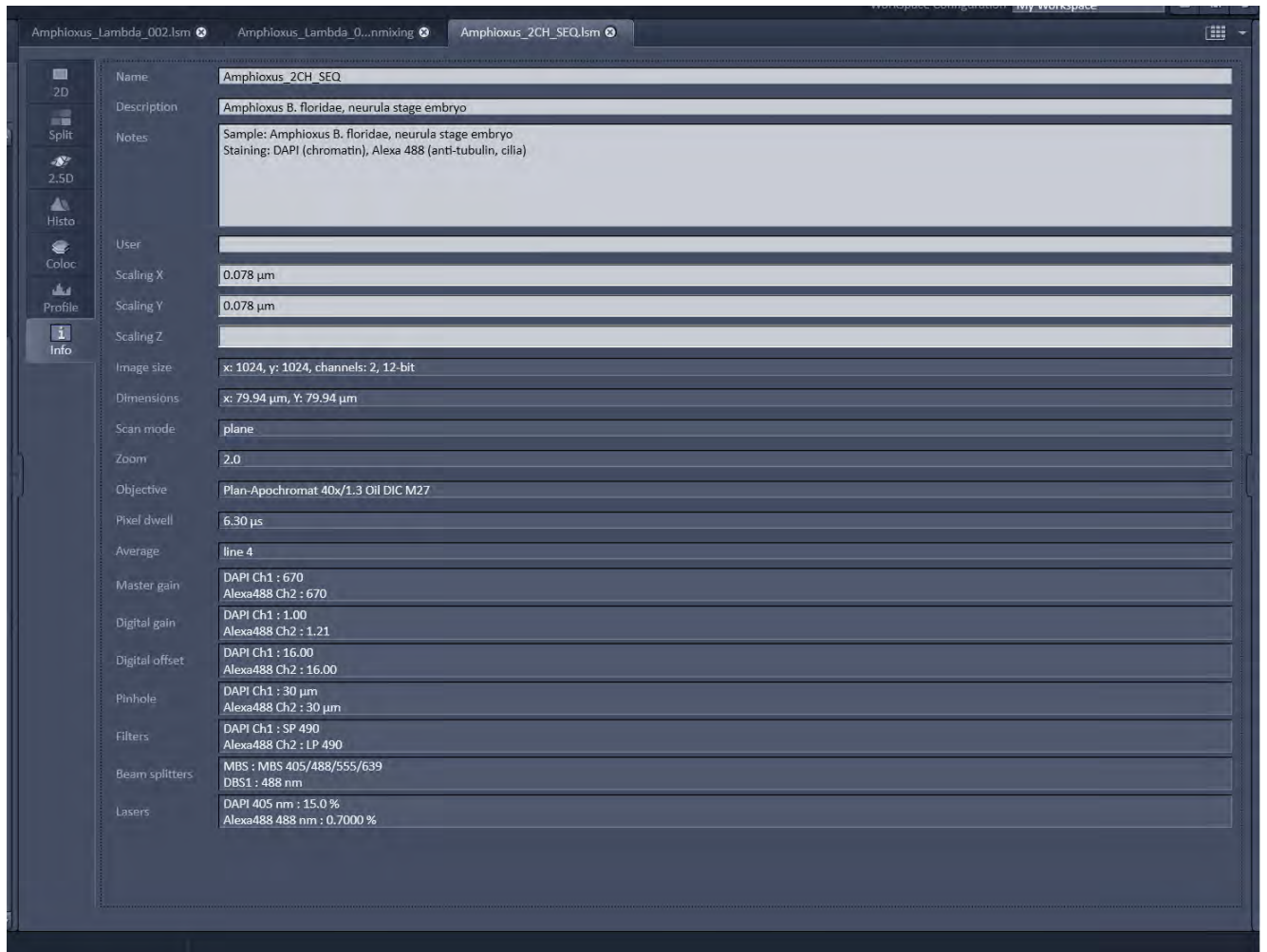


Fig. 266 Information View of a 2 channel Z-Stack

## 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** dialog in the **File** menu these formats can also be chosen as file type.

## 7.2 ZEN File Browser

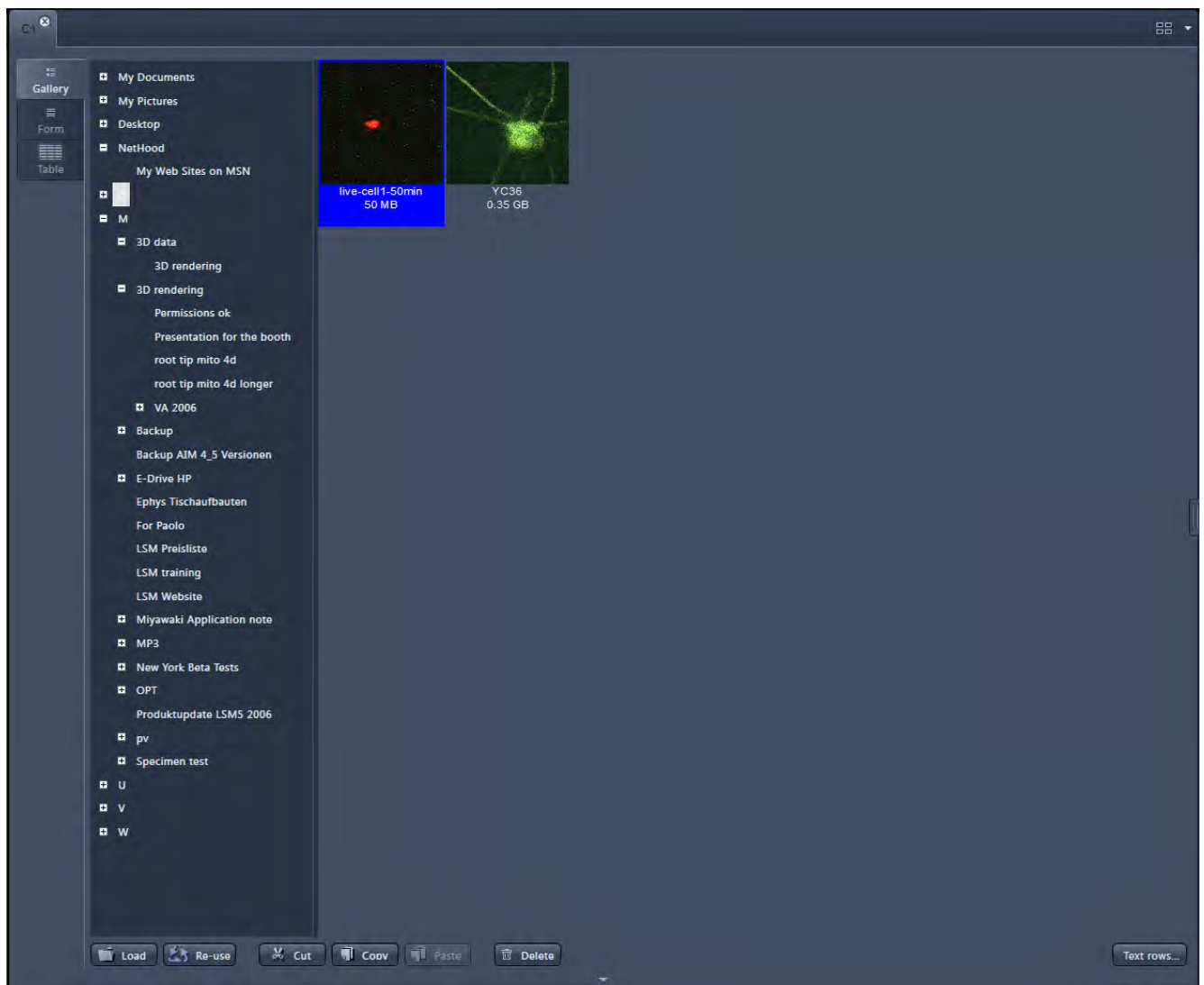
The ZEN File Browser is a versatile tool to access your image data.

- In the menu bar, click on **File** and select the **New File Browser ...** item to open the **ZEN File Browser**.

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. 267** 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. 268) fading in the right hand corner inform about missing hardware if applicable. The message box disappears after ~10 seconds.

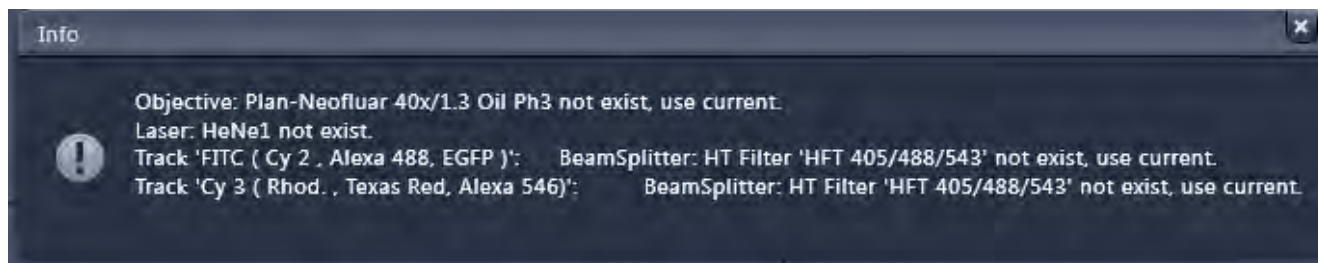


Fig. 268 Error message

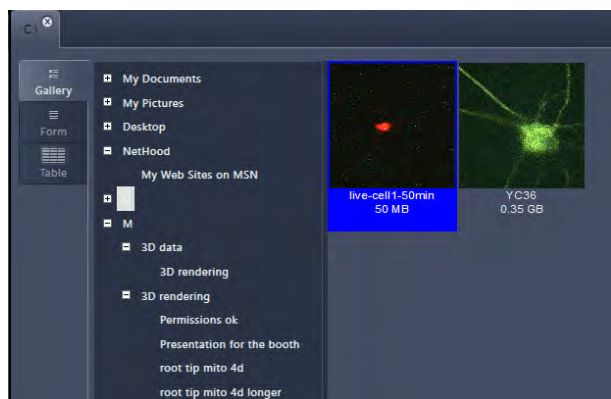


Fig. 269 Gallery view

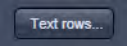
## 7.2.1 Gallery View of the ZEN File Browser

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.



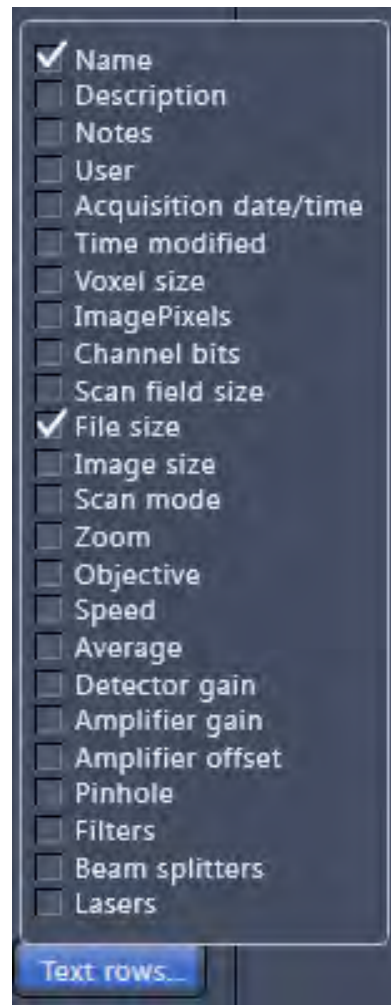
**Opening Images:**

- To open an image, double click on it.
- For opening multiple images at a time, select the desired images and press **Load**.

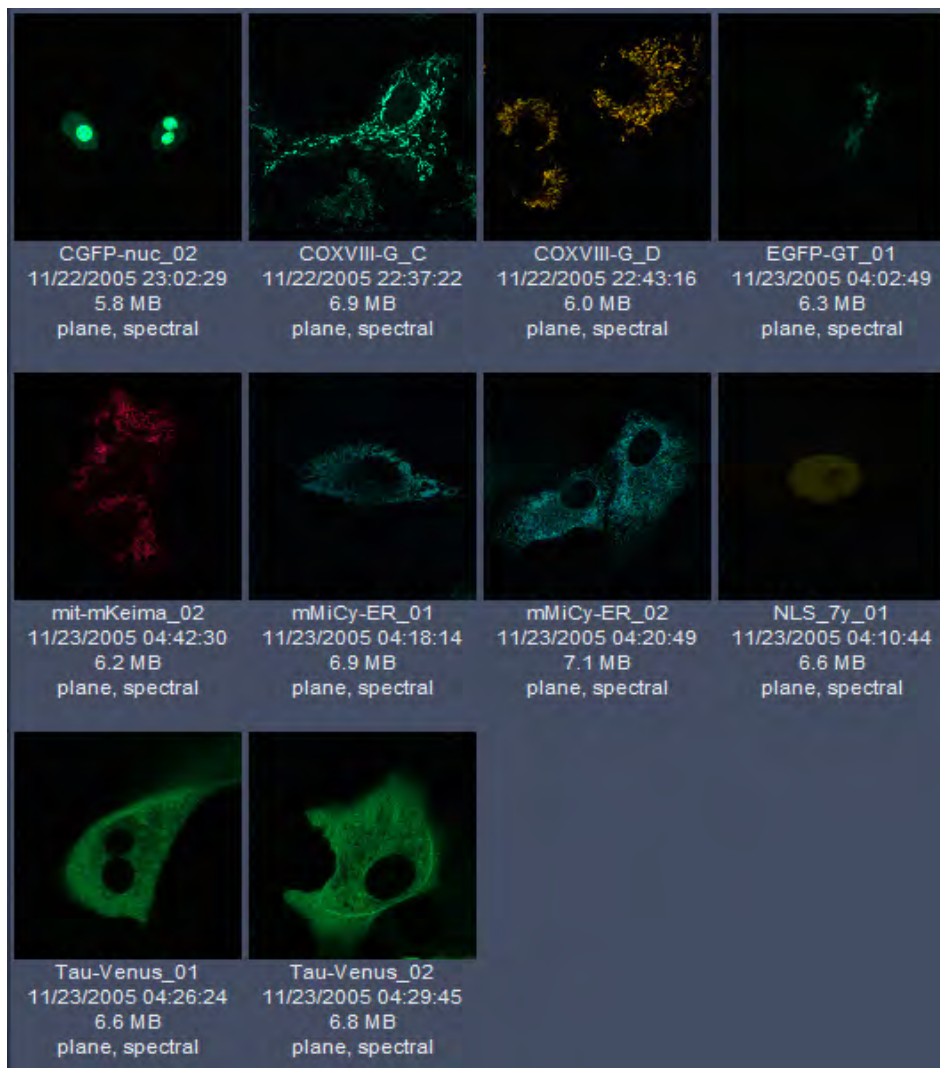
The information stored in the .lsm file format can be displayed underneath the thumbnail. By pressing the  button a choice of information is available (see Fig. 270).

The example below (Fig. 271) shows the use of the display of:

- file name,
- acquisition date,
- file size and
- scan mode.



**Fig. 270** Image information selection


**Fig. 271** Images with information

## 7.2.2 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



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.

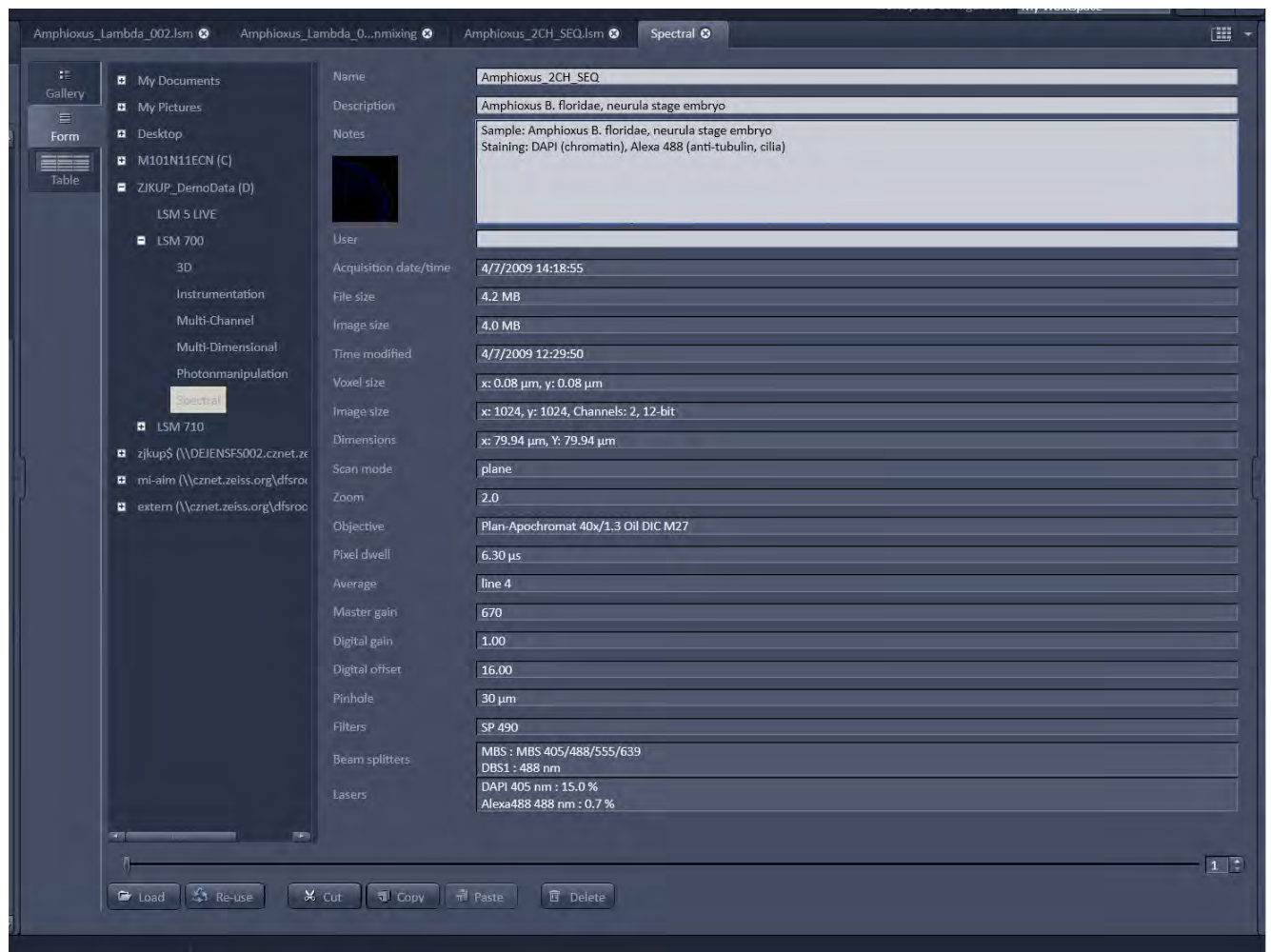


Fig. 272 Form view

### 7.2.3 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 file
555-nuc_01	458	Administrator	11/22/2005 23:09:36	11/22/2005 14:10:57	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_555_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_555_01_unmix	458	Administrator	11/23/2005 07:58:25	11/22/2005 22:59:21		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_555_01_unmix_BK	458	Administrator	11/23/2005 07:59:56	11/22/2005 23:02:56		512 x 512 x 1 x 1	9 Channels, 16-bit	
7_555_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	58.38 µm x 58
7_555_02_unmix-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_555_02_unmix	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_555_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	72.98 µm x 72
7_555_03_unmix-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_555_03_unmix	458	Administrator	11/23/2005 08:16:33	11/22/2005 23:17:05		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_555_04	458	Administrator	11/23/2005 08:21:30	11/22/2005 23:23:56	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_555_04_unmix-BK	458	Administrator	11/23/2005 08:25:01	11/22/2005 23:25:16		512 x 512 x 1 x 1	9 Channels, 16-bit	
7_555_04_unmix	458	Administrator	11/23/2005 08:23:19	11/22/2005 23:24:05		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_555_05	458	Administrator	11/23/2005 08:31:14	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	60.82 µm x 60
7_555_05_unmix-BK	458	Administrator	11/23/2005 08:34:51	11/22/2005 23:35:33		512 x 512 x 1 x 1	9 Channels, 16-bit	
7_555_05_unmix	458	Administrator	11/23/2005 08:33:18	11/22/2005 23:34:07		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_555_06	458	Administrator	11/23/2005 08:40:11	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	72.98 µm x 72
7_555_06_unmix-BK	458	Administrator	11/23/2005 08:42:48	11/22/2005 23:43:17		512 x 512 x 1 x 1	9 Channels, 16-bit	
7_555_06_unmix	458	Administrator	11/23/2005 08:41:58	11/22/2005 23:43:06		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_555_07	458	Administrator	11/23/2005 08:46:29	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_555_07_unmix-BK	458	Administrator	11/23/2005 08:49:34	11/22/2005 23:50:10		512 x 512 x 1 x 1	9 Channels, 16-bit	
7_555_07_unmix	458	Administrator	11/23/2005 08:48:30	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: 0.19 µm, y: 0.19 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	97.31 µm x 97
7_CG_01_unmix-BK	458	Administrator	11/23/2005 06:02:07	11/23/2005 02:04:32		512 x 512 x 1 x 1	9 Channels, 16-bit	
7_CG_01_unmix	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_unmix2	458	Administrator	11/23/2005 06:17:04	11/22/2005 21:18:54		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_CG_02	458	Administrator	11/23/2005 06:20:50	11/22/2005 21:26:37	x: 0.15 µm, y: 0.15 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	76.82 µm x 76
7_CG_02_unmix	458	Administrator	11/23/2005 06:24:20	11/22/2005 21:28:33		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_CG_02_unmix_BK	458	Administrator	11/23/2005 06:27:36	11/22/2005 21:28:44		512 x 512 x 1 x 1	9 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	112.28 µm x 1
7_CG_03_unmix	458	Administrator	11/23/2005 06:36:24	11/22/2005 21:36:56		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_CG_03_unmix_BK	458	Administrator	11/23/2005 06:38:09	11/22/2005 21:38:25		512 x 512 x 1 x 1	9 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 1
7_CG_04_unmix	458	Administrator	11/23/2005 06:52:14	11/22/2005 21:52:41		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_CG_04_unmix_BK	458	Administrator	11/23/2005 06:54:36	11/22/2005 21:55:08		512 x 512 x 1 x 1	9 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	72.98 µm x 72
7_CG_05_unmix	458	Administrator	11/23/2005 07:08:22	11/22/2005 22:09:39		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_CG_05_unmix_GT-BK	458	Administrator	11/23/2005 07:11:32	11/22/2005 22:12:23		512 x 512 x 1 x 1	8 Channels, 16-bit	
7_CG_05_unmix_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_unmix_BK	458	Administrator	11/23/2005 07:10:21	11/22/2005 22:10:40		512 x 512 x 1 x 1	9 Channels, 16-bit	
CGFP-nuc_01	458	Administrator	11/22/2005 22:58:37	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
CGFP-nuc_02	458	Administrator	11/22/2005 23:02:29	11/22/2005 14:03:23	x: 0.16 µm, y: 0.16 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	81.09 µm x 81
CDXVIII-G_C	458	Administrator	11/22/2005 22:37:22	11/22/2005 13:38:58	x: 0.17 µm, y: 0.17 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	85.86 µm x 85
CDXVIII-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	58.38 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	58.38 µm x 58
mit-mKeima_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	72.98 µm x 72
mit-mKeima_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
mMICy-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
mMICy-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	58.38 µm x 58
NLS_7y_02	458	Administrator	11/23/2005 04:14:12	11/22/2005 19:15:25	x: 0.11 µm, y: 0.11 µm	512 x 512 x 1 x 1	22 Channels, 12-bit	58.38 µm x 58
SECFP-pm_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	72.98 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	72.98 µm x 72

Fig. 273 Table view

The columns displayed in the table are selectable from the **Table columns** button. The options shown in Fig. 274 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.

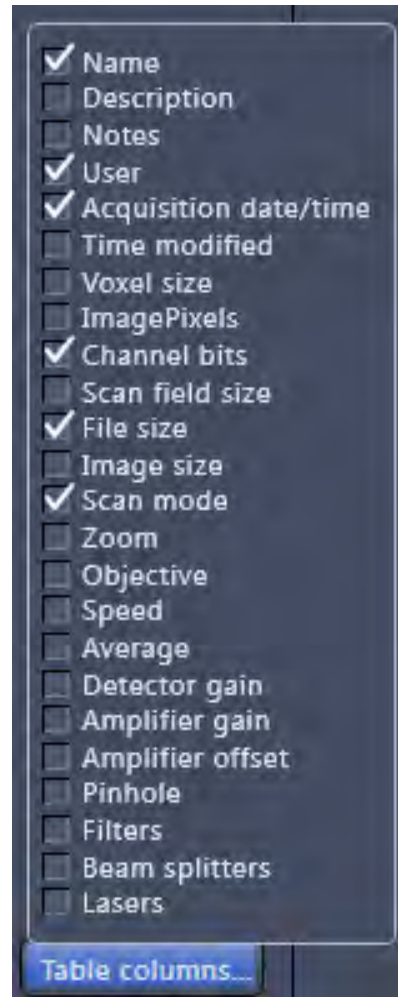


Fig. 274 Table view options

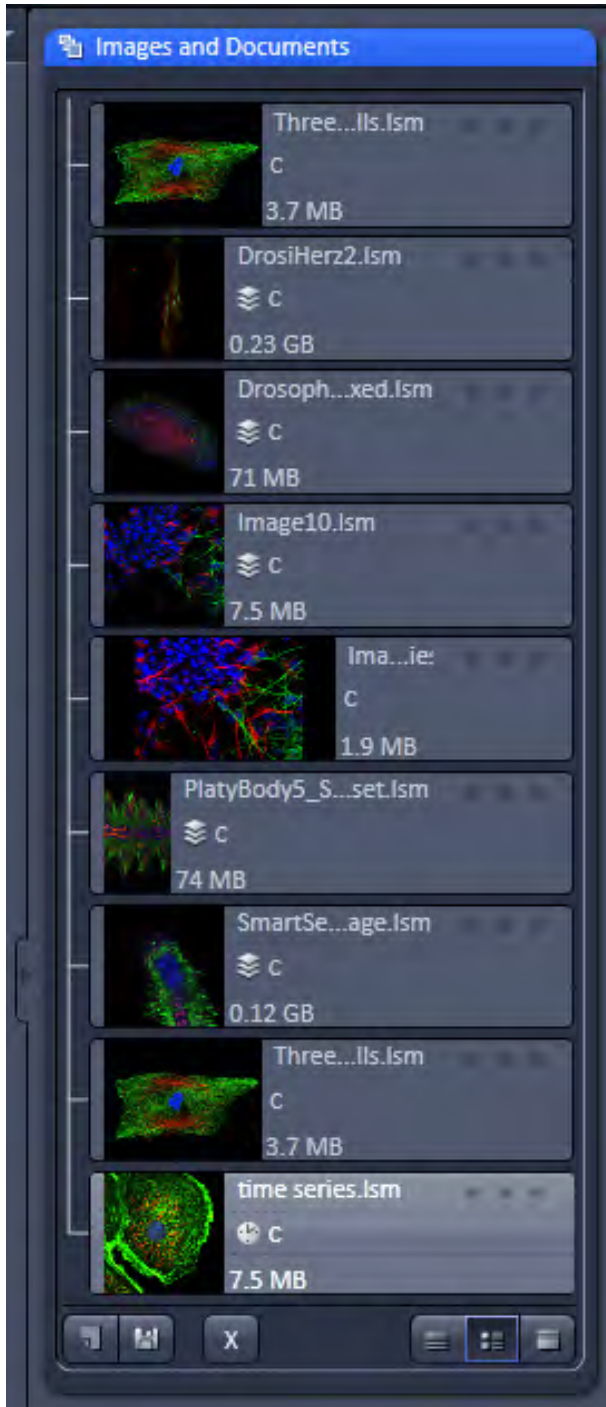





Fig. 275 Images and Documents panel

-  Textual View
-  Thumbnail View
-  Big View




The default setting is **Thumbnails view** as indicated on the screenshot.



### 7.3 Documents panel


The **Documents** panel (Fig. 275) 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 saved and closed using the following buttons:



- A new acquisition document can be opened using the  button.
- The  button saves the selected image.
- The  button closes the selected image.

 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 black triangle at the left side of the  panel.

Once hidden, a click on the right boarder of ZEN lets the **Documents** panel slide back into the application.

- All files can be displayed in three ways by using the  buttons underneath the section.

- The context menu of the **Open Image** section allows the following functions:
  - Selecting one of the view options **Textual View**, **Thumbnail View** or **Big View**
  - Selecting all opened image files by clicking on **Select All**
  - Opening a **New Acquisition Document**
  - Saving or closing the selected image files by clicking on **Save** or **Close**

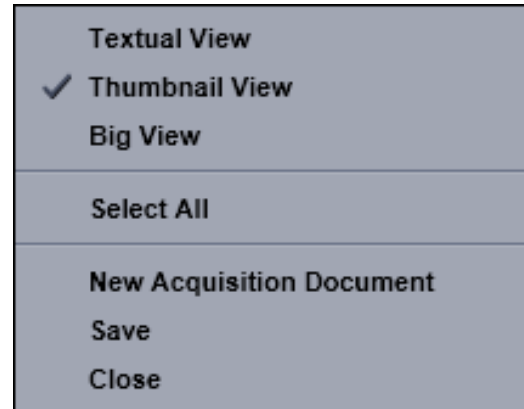






Fig. 276 Context menu of the Open Images section

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. 277).

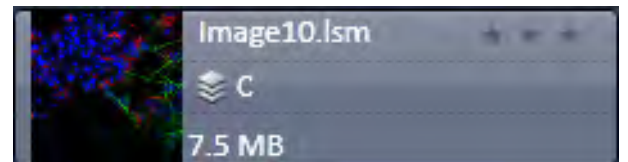


Fig. 277 2 Ch. Z-Stack image

- In case the image is not saved or has been processed, a warning symbol is displayed on the right side (Fig. 278).

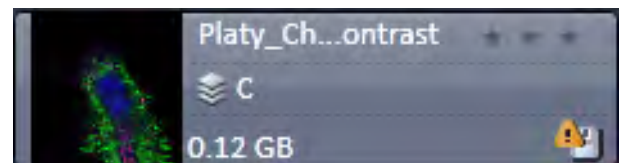
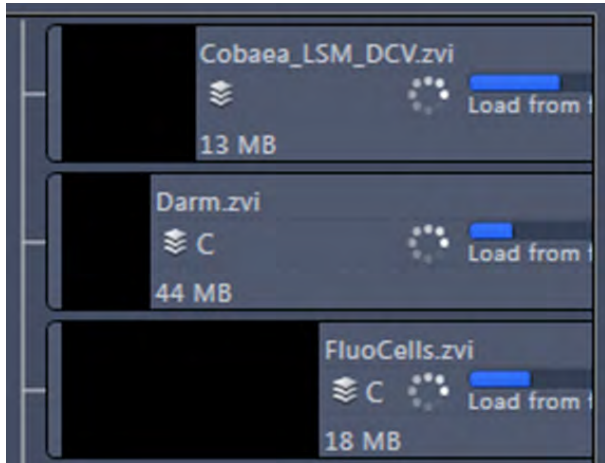


Fig. 278 Image with warning symbol



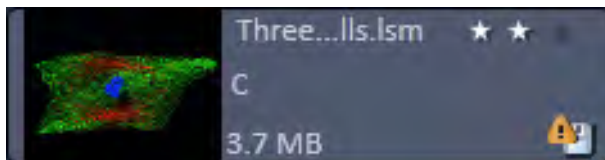
**Fig. 279** Image progress bar

- A progress bar (Fig. 279) is shown for each image facilitating the overview if e.g. many processing functions are running at the same time.



**Fig. 280** Enlarging the image view

- In case long filenames are used the whole section can be dragged to the left and thereby enlarged (Fig. 280).




**Fig. 281** Image rating star function

- The stars can be used to rate the pictures. They are activated by clicking on them.



---


## 7.4 Opening of Files via the "Open" Command in the File Menu

- Click on the **File** menu of the menu bar and select **Open** or click on the  icon of the main toolbar.
  - The standard windows open dialog appears.
- Select the desired file and click **Open**.

## 7.5 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 menu bar or click on the  icon of the main toolbar.
  - 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.

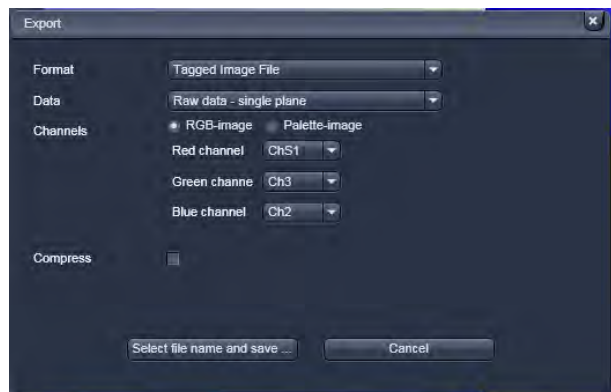


Fig. 282 Export window

## 7.6 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 menu bar.
  - This opens the **Export** window (Fig. 282).

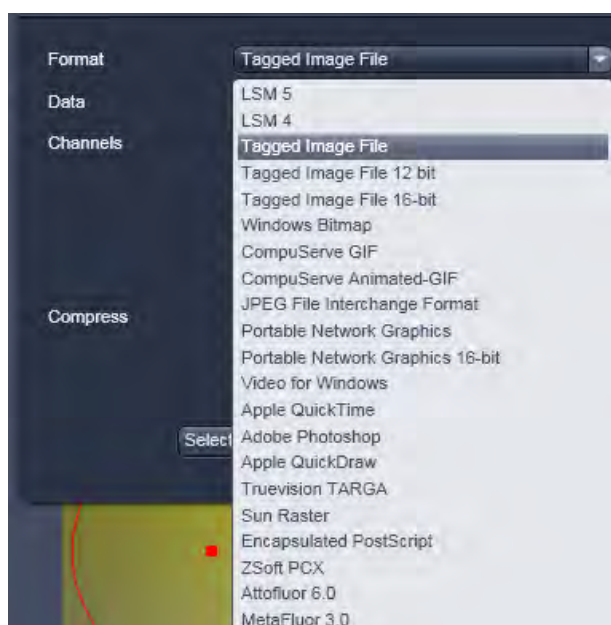


Fig. 283 Format selection



Fig. 284 Data type selection

- Under **Format**, select the format to which the image is to be exported to (Fig. 283).

- Select the data type which the image is to be exported under **Data** (Fig. 284).
- Choose 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 dialog appears. Click **Save** to complete the export process.

☞ When stacks or time series are exported, each frame is stored as an individual image.

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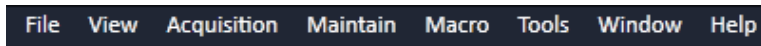
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# 1 Macros

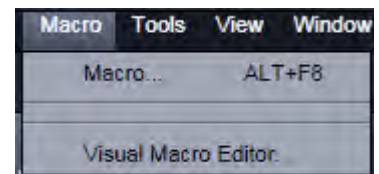
The macro function permits the recording, running and editing of command sequences and their allocation to buttons in the **Macro** menu. Macros can be set up in two different ways: The Visual Macro Editor or alternatively they can be programmed in Visual Basic directly.

- In the **Menu** bar (see Fig. 1), click on **Macro**.



**Fig. 1** Menu bar

- This displays the **Macro** menu (see Fig. 2).
- The Macro list is bipartite: **Macro** – editing and recording of macros; assign macros to list
- **Visual Macro Editor** – edit automatic workflows with pre-defined modules



**Fig. 2** Macro menu

Selecting **Macro** opens a dialogue for editing and recording Macros based on VBA programming in the **Macro Control** window (see Fig. 3). VBA programming is described in Section 2 of the printed manual.

Using the **Visual** Button (optional) allows to create automated work flows using the arrangement of symbols which depict the single steps within a work flow. See **Visual Macro Editor**, page 23 for further details.

## 1.1 Macro Language

"Visual Basic for Applications", called VBA in the following, is used as the Macro language. This language is well known and widely used as Macro language in the "Microsoft Word for Windows" and "Microsoft Excel for Windows" products. Experience with "Microsoft Visual Basic" would also be beneficial for macro-programming ZEN.

An Integrated Development Environment (IDE), the "Microsoft Visual Basic Editor", is available for editing and debugging of macros. The IDE includes an "online help" with a detailed description of the VBA language.

Macros are stored in project files. One project file can include several macros.



**The VBA functionality described in this paragraph refers to VBA as implemented in the Carl Zeiss LSM software 4.2. With the introduction of ZEN, the range of functions is and will be extended, while VBA can still be used with the functions described below. The extension of functionality applies mostly to display and access of image data. If you need assistance or further information please contact your local Carl Zeiss LSM specialist:**



Fig. 3 Macro Control window

## 1.2 Macro Control

To open the Macro Control, choose **Macro** from the **Macro List** in the main **Menu Bar**.

This opens the **Macro Control** window.

### 1.2.1 Edit Macro Function

This function allows you to manage project data. Macros can be recorded, stored, performed, edited and, if required, deleted.

- Press the **Edit Macro** tab to switch to the **Macro** and **Recording** panels (see Fig. 3).

### (1) Macro panel

**New** button: Creates a new project.

**Load** button: Opens an existing project.

**Save** button: Stores the project on the hard disk.

**Save As** button: Stores an existing project under a new name.

**Unload** button: Removes the selected macro from the **Macros** list.

**Edit** button: Allows macros to be edited and debugged. The editor (**Microsoft Visual Basic**) is automatically located at the beginning of the relevant macro.

**Run** button: Runs a macro.

**Step** button: Opens the editor for line-by-line editing / debugging.

**Delete** button: Deletes the selected macro.

**Editor** button: Opens the editor. Displays the processed area of the macro edited last.

Macros are stored and managed in project files (\*.lvb). Before you can record or edit a macro, you have to create a project as follows:

- Press the **New** button to create a project file.
  - A new project is created and displayed in the **Project** selection box. The project name is automatically default (LSM followed by a number), but can be edited afterwards.

To activate an existing project, proceed as follows:

- Press the **Load** button.
  - The **Open** window will be opened (see Fig. 5).
- Select the relevant project file (data extension: **\*.lvb**) from the **Macros** list box. Click on the **Open** button.
  - The project file will be opened and the macros contained in it are displayed in the **Project** selection box of the **Macro Control** window. You can use the pull down menu to load any listed Macro.

Recorded macros are stored in main memory first. Before the macros can be assigned to the list in the **Macro** submenu, the project must be stored on the hard disk.

- Press the **Save** button under the project name in the **Macro Control** window and determine the file name in the **Project** selection box, if required.



Fig. 4 Macro panel with new Macro assigned

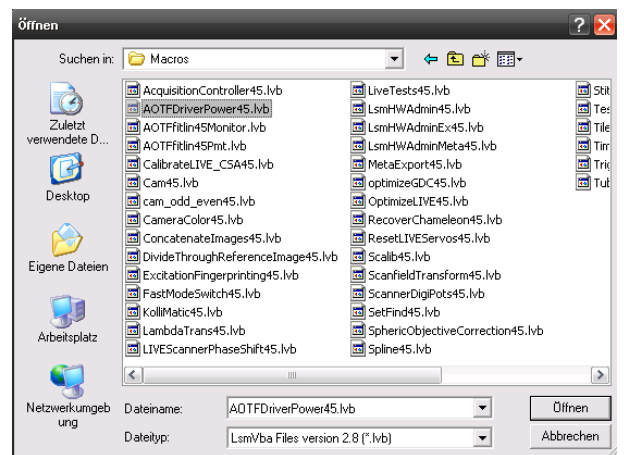


Fig. 5 Open window

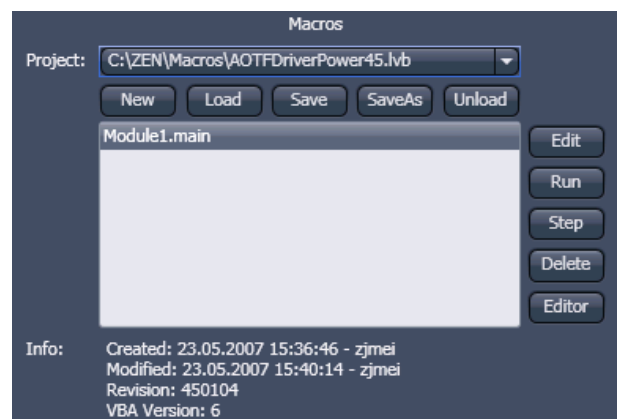


Fig. 6 Macro panel with existing Macro assigned

## (2) Recording panel

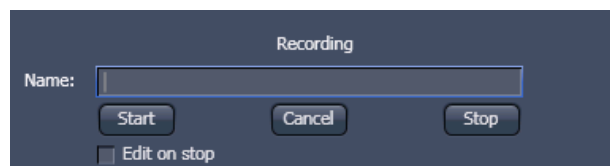
Before recording a command sequence, you can enter the name for the macro to be created in the **Name** input box of the **Recording** panel.

**Start** button: Starts recording.

**Cancel** button: Cancels the recording procedure.

**Stop** button: Stops recording.

**Edit On Stop**: On stopping the recording procedure, the macro editor is automatically opened at the relevant position.




**Fig. 7** Recording section

### Proceed as follows to record a macro:

- Enter a name for the macro to be created under **Name** in the **Recording** panel.
- Click on the **Start** button to start recording the macro.
- Then perform the operations to be stored, e.g.:
  - Click on the LSM **Auto Exposure** action button. A find scan will be performed.
  - Click on the LSM **New** action button. A new **Image Display** window will be opened.
  - Click on the LSM **Snap** action button. A scan will be performed.
- Then click on the **Stop** button to end the recording. (**Cancel** enables you to cancel recording)
  - If recording was successful, the entered **Name** will then also be available in the **Macros** list box of the **Macro** panel. The new macro is automatically assigned to the current project. It is possible to assign as many macros as required to a project.
- Click on the **Save** button to store the new macro.

### Proceed as follows to run a macro:

- Select the required macro from the **Project** list box of the **Macros** panel.
- Click on the **Run** button to start performing the macro.

 Provided that a macro is linked to a list in the **Macro** section of the **Menu Bar**, you only need to click on its name to perform it.

### Proceed as follows to delete a macro:

- Select the required macro from the **Project** list box of the **Macros** panel.
- Click on the **Delete** button. The macro will be removed from the list.

### Proceed as follows to edit a macro:

- Select the required macro from the **Project** list box of the **Macros** panel.
- Click on the **Edit** button. The Microsoft Visual Basic editing window will be opened.
- Make the required changes.

### 1.2.2 Assign Macro to the Macro list

This function permits stored macros to be linked to the assigned Macros list in **Macro** of the **Menu Bar**.

- Press the **Assign Macro** tab to switch to the **Assign Macro** panel (see Fig. 8).

#### Define Buttons panel

Proceed as follows to link a macro to the **Macro** list:

- Select the button number corresponding to the position in the list from the **Button** selection box.
- Enter the button labelling in the **Text** editing box.
- Select the name of the project file from the **Project** box using the "... " button.
- Select the macro name from the **Macros** box.
- Press the **Apply** button to assign the relevant macro to the specified list position in the **Macro** toolbar.

#### Delete a macro from the Macro list

Proceed as follows to delete the linking between a **Macro** list position:

- Select the button number from the **Button** selection box.
- Press the **Delete** button to delete the list entry.



Fig. 8 Macro Control window – Assign Macro

### 1.2.3 Editing and Debugging of Macros

The **Edit** in the **Edit Macro** panel button activates IDE (Integrated Development Environment), which allows macros to be edited and debugged. Under the **Help - Microsoft Visual Basics Help** menu item, IDE contains detailed "online" help on its operation and on the VBA macro language. Therefore, only a few hints are provided in the following:

You should activate the required toolbars. We would recommend you to activate the **Debug** toolbar via the **View - Toolbars -Debug** menu item.

The following buttons in the toolbar can help you when debugging macros:



Starts running the command lines.



Stops running the command lines.



Interrupts processing of the command lines (pause).



Sets a breakpoint in the line with the text cursor.



Processes a command line and steps into subprocedures.



Processes a command line and steps over subprocedures.



Exits the subprocedure (step out).



Displays the value of the marked expression (Watch). If nothing is marked, the value of the variable above the text cursor is displayed.



Activates the Watch window in which values of variables and expressions can be displayed. For this, text is marked in the code window and dragged into the Watch window. Variables can be modified in the Watch window.


In the left-hand edge of the code window you will find an arrow beside the current command line. A new current command line can be determined by moving the arrow via the mouse. This makes it possible to skip command lines or to process command lines several times.

### 1.3 Overview of Available Macros

Documentation files (\*.rtf, \*.doc) of advanced macros will be located in the macro directory.

Name	Description
AOTFfitlin50Monitor.lvb	Linearize laser attenuation (AOTF or mechanical)
AOTFDriverPower50.lvb	Attenuate laser light by the AOTF by additional dampening factors
Autofocus50.lvb	Automatic focusing according to a set configuration
CameraColor50.lvb (also Button in Maintain)	Color balance of AxioCam HRC
Center50.lvb	Centers the field of view around the position marked with the cross tool
ConcatenateImages50.lvb	Enables the combination of all kinds of images irregardless of the size and data depth to be presented in a time series. Imported images can also be included. Type of concatenation (fitted to the smallest frame or ROI, fixed size or fitted to the largest frame) can be defined by the user.
ExcitationFingerprinting50.lvb	Automated generation of excitation lambda stacks for generating excitation spectra which can be used for linear unmixing to separate overlapping dyes. Any detector can be used, also NDDs. Description as guide tour on the installation CD-ROM or DVD.
FRET plus.lvb	(Option) Enables the user to perform FRET experiments based on sensitized emission or acceptor photo bleaching (depending on the type of LSM) including commonly accepted analysis algorithms.
Kollimatic50.lvb	Automatically generates the calibration matrix of a LSM 7 LIVE system for optimal confocal aperture and collimator settings
Lambdatrans.lvb	Time series alternating between lambda and transmission mode
LsmHWAdmin.lvb (also Button in Maintain)	Direct service hardware access (password protected)
LsmHWAdminEx.lvb	Calibration service macro (password protected)
ModifySeriesZEN.lvb	Modifies Z Stacks and Time Stacks like Rotation of the stacks, being mirrored, Conversion of time stacks into z-stacks and vice versa;
StitchArtPlus.lvb	StitchArt Plus macro ( <b>Option</b> )
MultiTime.lvb	Set up of time series experiments including repeated imaging, bleaching and autofocusing with defined configurations at multiple locations and for various views at each location ( <b>Software option</b> )

OptimizeLIVE50.lvb	Temporarily adjusts the confocal aperture position of a LSM 7 <i>LIVE</i> without changing the general calibration values
RecoveryChameleon.lvb	Imitates recovery of chameleon NLO laser in case the laser is falling into CW lasing and does not get to mode lock anymore.
ScanfieldTransform50.lvb	Adjusts image match relative to each other or to ROIs.
SphericObjectiveCorrection50.lvb	Calibrates the spheric component of objective error by automatic acquisition of a Z stack on a flat surface and a spherical fit of the topographic data.
TestGrid50.lvb	Projects a testgrid into the image window (password protected).
TileScanRotation50.lvb	Defines rotation angle of scanners during tile scan


 During installation, default macros will be installed according to their type either in ZEN\ZEN\HWT or ZEN\Macros\. Self generated Macros will be in ZEN\Macros. In case of a new installation, old macros will be stored in ZEN\Macros\BackupMacros or ZEN\Backup\, to avoid problems with identical names of existing and new macros.

## 1.4 Sample Macros

The ZEN software package includes a set of basic macros free of charge

Other macros from the list (previous page) are part of the product range of Carl Zeiss MicroImaging GmbH. Please contact your sales representative for a quote.

In the following paragraphs you find a short description of some representative and common macros. Please contact your local LSM specialist for more detailed and extensive information.

 **Please check the C:\ZEN\Macros folder on your system for separate documentation in the more extensive macros in .pdf format.**



## 1.5 Kollimatic

This VBA Macro is used to calibrate the LSM 7 *LIVE* on the current system configuration (e.g. objectives, filters, etc.)

### Prerequisites

- System Warm-Up (at least 2 hours; for procedure please read the User's Manual) and environmental conditions according to setup requirements.
- "OptimizeLIVE.lvb" Macro: ensure both channel sliders set to 0
- Proper adjustment of collimators and Confocal Slit Aperture (CSA) 1 + 2 (this has to be done by the local Service Representative)
- AOTF RGB + V linearized properly with Macro AOTFFitlin.lvb
- All used objectives are stored in the database according their exact order in the nose piece.
- Necessary calibration slides are available
  - 1x Bead Slide 1  $\mu\text{m}$  for dry objectives, # **000000-1371-460**,
  - 1x Bead Slide 200 nm for immersion objectives, # **000000-1371-461**;to be ordered from Carl Zeiss Jena.

### Basic Functions of the Macro

The Kollimatic macro features two main parts, Basic Calibration and Calibration Matrix:

#### A - Basic Calibration

- Needs to be done only once with one of the recommended objectives (should be part of the shipment in order to be able to repeat the Basic Calibration without calibrating all objectives again (see objective list down below))
- Is for calibrating both collimators RGB + V
- Is for calibrating the Y-position of the Beam Shift Compensator (BSC) for all present NFT positions




On currently delivered LSM 7 *LIVE* Systems, the Basic Calibration is done in the factory (This can be confirmed by checking that **BasicCalibrationLog.txt** is present in C:\ZEN\Bin).

Objective list (objectives are listed in order of most to least recommended for this calibration task)

- Plan APOCHROMAT 20x / 0.8
- Plan Neofluar 40x / 1.3 Oil
- C-APOCHROMAT 10x / 0.45W
- C-APOCHROMAT 40x / 1.2W corr
- Plan APOCHROMAT 5x / 0.16
- Plan Neofluar 10x / 0.3
- Plan APOCHROMAT 63x / 1.4 Oil
- C-APOCHROMAT 63x / 1.2W corr
- Plan Neofluar 20x / 0.5

## B - Calibration Matrix

- Is based on the calibration results of the Basic Calibration
- Needs to be performed for each objective separately
- Is for calibrating the collimator position of both collimators RGB + V only for the selected Objective depending on:  
Laser Line, Zoom + EF Filter Position
- Is for calibrating the BSC Y-position depending on:  
Laser Line, Zoom + EF Filter Position

 On currently delivered LSM 7 *LIVE* Systems, the Calibration Matrix is set up in the factory for every objective belonging to the system (This is done together with the Basic Calibration).

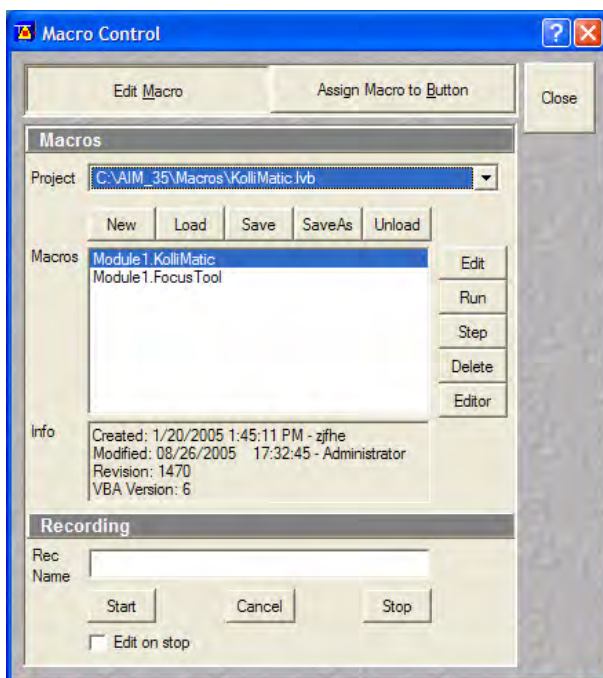
Without running Calibration Matrix, the positions of the collimators and BSC Y should be ok. This is due to the Basic Calibration, but the results are not fully optimized.

### Installation of the Macro:

If not already done, copy the required files to the following destinations:

File Name	Destination
KolliMatic.lvb	C:\ZEN\Macros
PotentialKollimaticConfig.ini	C:\ZEN\Bin
KolliMatic_Instructions.pdf	C:\ZEN\Macros

### Starting the Macro:



- Start the LSM software and check first, if all prerequisites have been fulfilled (see section Prerequisites)
- Put the appropriate calibration slide (depending on current objective: immersion or non immersion) onto the microscope stage
- Activate all lasers, and using the and focus with the 488 nm line start scanning and focus onto the beads
- Put the appropriate calibration slide (depending on current objective: immersion or non immersion) on the microscope stage, start single scan
- Use the **Macro** menu of the main LSM software to **Load** and **Run** the Macro **KolliMatic** (see Fig. 9)

Please wait a few seconds, the Macro will set up beam path and all necessary settings automatically.

**Fig. 9** Macro Control Window of the main LSM Software

### Preparations for using the Calibration Slides:

- Follow the steps shown in the macro **Sample and Configuration Setup** window.
- Use the arrows or the drop down menu in order to select one track for each available laser line (no need to select several tracks for the same laser line), start with a track for the lowest wavelength laser (i.e. typically the 405 nm laser line.).
- Select an area of the bead sample that provides as many beads as possible, at least 4 (fit into the green rectangle)
- In Fig. 10, the Basic Calibration has been selected (Check box **Basic Calibration: Requires selecting preferred objective lens.**), therefore some more tracks are available
- Use the **Laser Power** as a first option in order to set the intensity, second option is **Integration Time**.
- In order to start / stop the scan, just select / deselect the check box **Continuous Scan**.
- Focus on the beads.
- Defocus with the **Collimator** slider until the beads become line shaped.
- Focus with the microscope focus drive to get the vertical lines as thin as possible (Fig. 11).
- Now use the **Collimator** slider to get a round spot out of the line (Fig. 12).
- Repeat these steps for a track with the 488 nm laser line.
- After one track for each laser line has been adjusted, click **Next**.

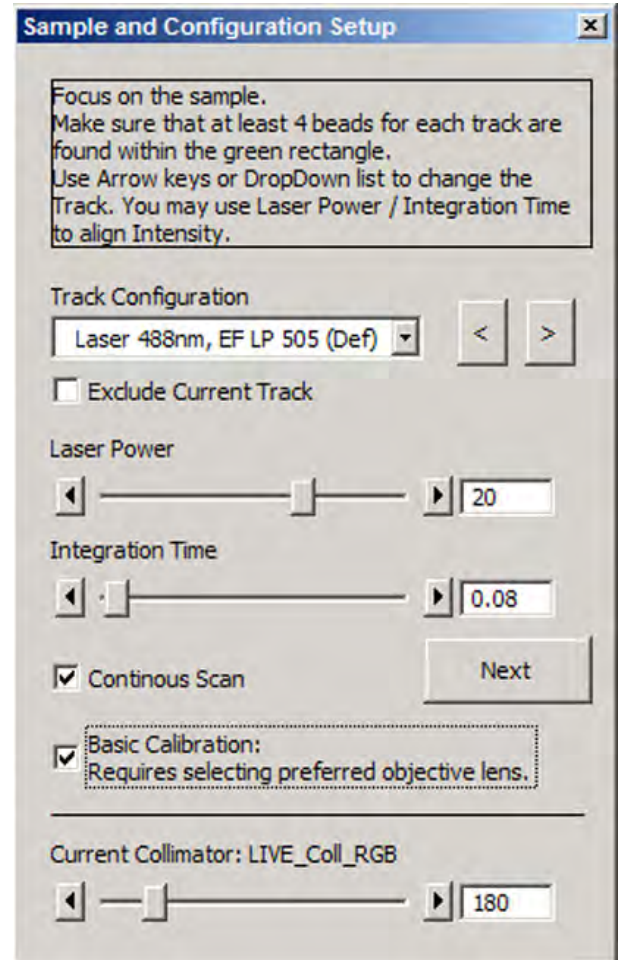
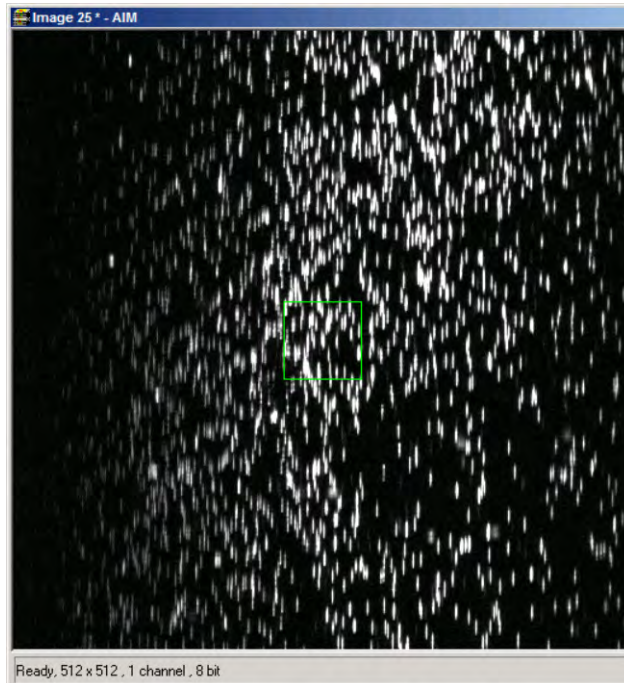
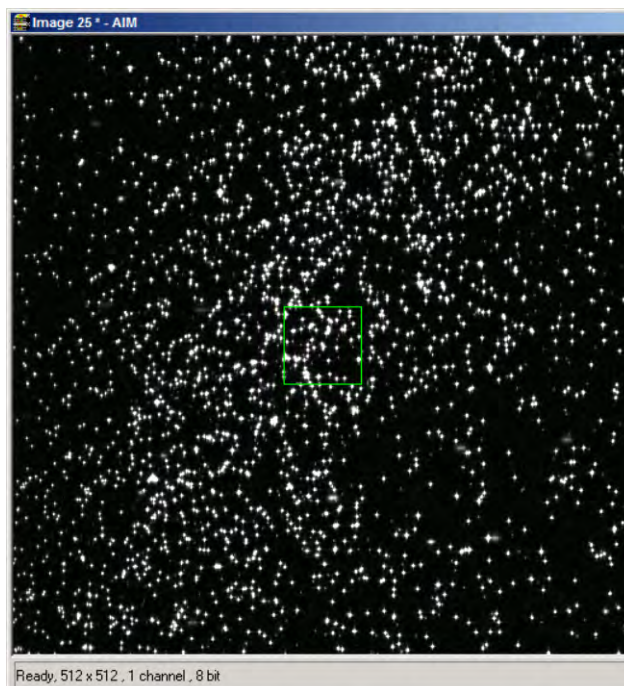


Fig. 10 Figure Sample and Configuration Setup Window of the Kollimatic Macro



**Fig. 11** Beads focused for maximal vertical thinness



**Fig. 12** Beads optimally focused

## Running the Basic Calibration



If you have selected the check box for **Basic Calibration**, please follow these instructions. In order to run the **Calibration Matrix** only, move on to Running the Calibration Matrix.

- Make sure that both check boxes have been selected (Fig. 13)
- Double check if the objective settings (shown at the bottom of the macro window) match the currently used objective
- Click the **Calibrate** button
- In order to follow the calibration process, just click into the **Message** window; a scroll bar will appear
- The **Basic Calibration** takes about 15 min
- The displayed curve should resemble a Gaussian profile
- After the macro has finished, a message will pop up with the request to restart the LSM software to make the changes effective.
- Press **Exit** and restart the LSM software

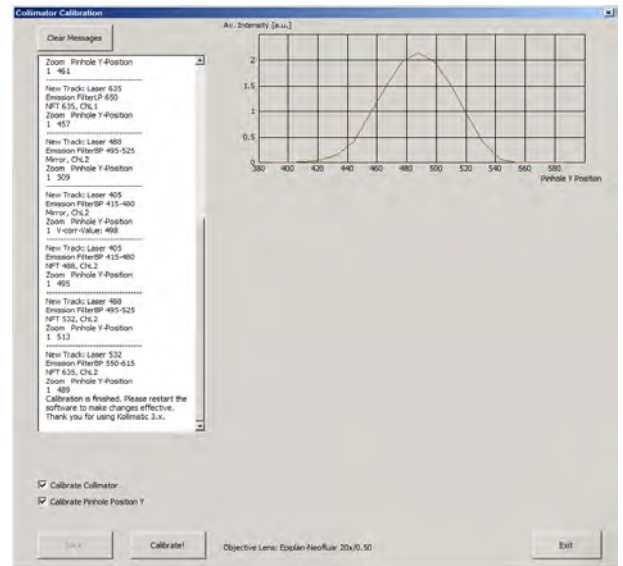

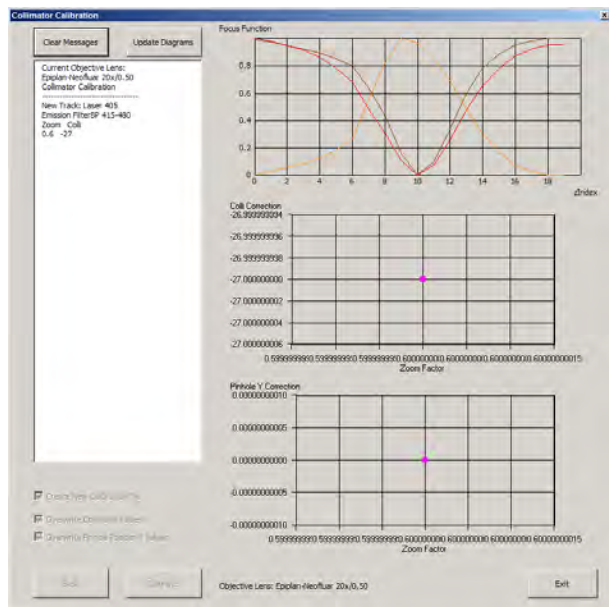


Fig. 13 Collimator Calibration Window

### Running the Calibration Matrix

 If you haven't selected the check box for Basic Calibration, the **Calibration Matrix** will be selected automatically. In this case, when the Calibration Matrix is running, the Basic Calibration is disabled.



**Fig. 14** Running the Calibration Matrix

- This window provides three check boxes (Fig. 14)
- The recommended setting is to select **Create new calibration file**. All previous files will be deleted.

Exceptions:

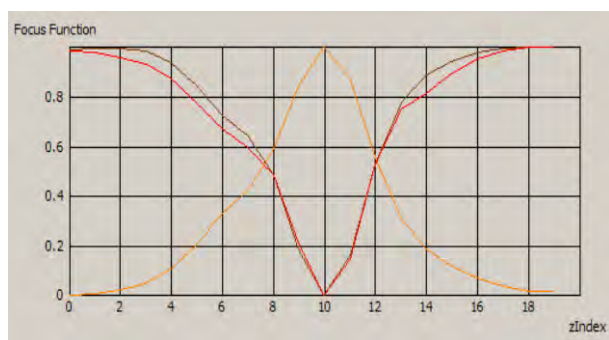
#### No check box checked

- Especially after an unexpected interruption of the program, it is possible to continue from the position where the process stopped (saving time).

#### Overwrite Collimator values

- The values for the chosen objective will be added or updated.
- After selecting the collimator option, the Pinhole Y option will be selected automatically.

- Double check if the objective settings (shown at the bottom of the macro window) match the currently used objective.
- Click the **Calibrate** button.



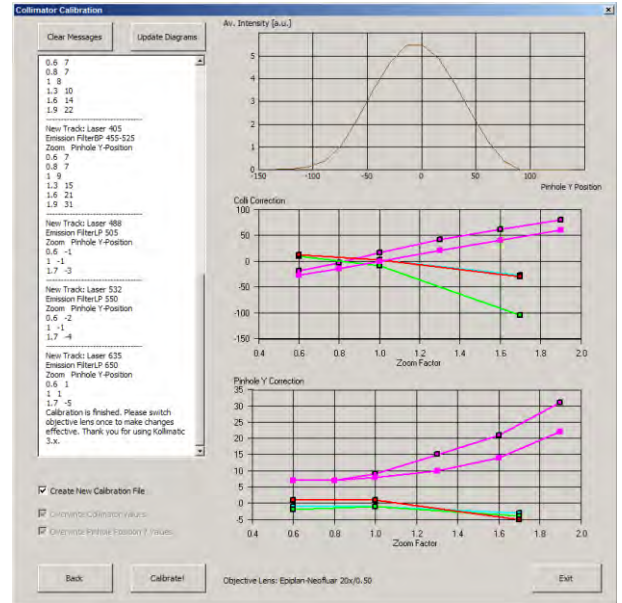
**Fig. 15** Graph of Focus Function

Meaning of the Graph (Figure):

- Brown: Focal plane of the CCD Line Detector
- Red: Focal plane of the collimator
- Orange: Estimated focal plane

The brown and red graphs should be close to this curve.

- In order to follow the calibration process, just simply click into the Message Window and; a scroll bar will appear (Fig. 16).
- The Calibration Matrix takes about 25 min for each objective.
- After the macro has finished, a message will pop up with the request to switch the objective lens in order to make the changes effective.
- If necessary, repeat the procedure for the other objectives.
- Use the **Update Diagrams** button in order to check, if an objective has already been calibrated.
  - Select an objective, press the **Update Diagrams** button
  - If no graphs appear, this particular objective has yet needs to be calibrated.
- Click **Exit** to finish.



**Fig. 16 Collimator Calibration window after calibration**

Meaning of the Graph (Figure):

- Each coloured line represents an individual laser line.
- Each coloured calibration point represents an emission filter.
- Standard graphs are displayed with black points.

Meaning of the Colours:

- <420 nm: → Pink
- 421...475 nm: → Blue
- 476...495 nm: → Turquoise
- 496...550 nm: → Green
- 551...590 nm: → Yellow
- >590 nm: → Red

## Important Information!

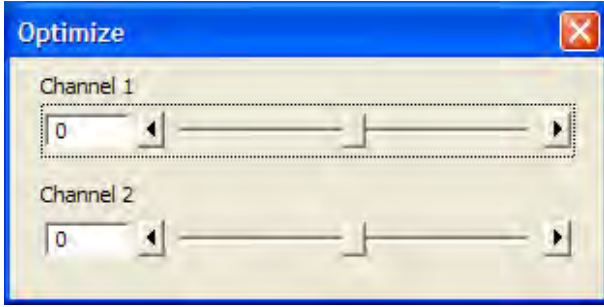


Fig. 17 Optimize Macro

☞ If the calibration has been completed successfully with the Calibration Matrix, do no longer use the pinhole and collimator functionality of the LSM software in the **Maintain** menu.

The use of the pinhole sliders to raise the quality of the images is well known from the other LSM Systems.

On the LSM 7 *LIVE*, only the **Optimize** sliders of the **OptimizeLIVE.lvb** macro have to be used (Fig. 17) to compensate for misalignments (e.g. temperature influences).

After the Calibration Matrix has optimized a particular objective, the results will be stored automatically in a file as shown below. These files can be found in C:\ZEN\BIN.

☞ Do not change any of the values!


```

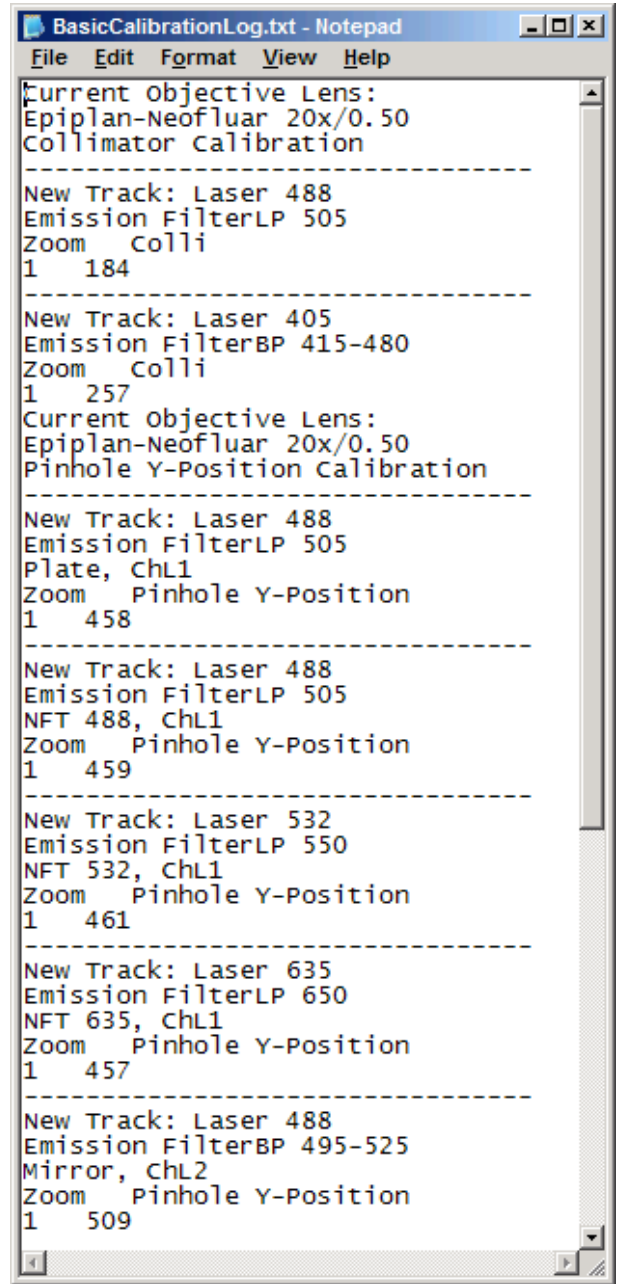
442340.pos - Notepad
File Edit Format View Help
LSM 5 LIVE Calibration file. Created or modified: see File date and time.
;This file is valid for Objective lens Epiplan-Neofluar 20x/0.50
;wl, ef, zoom, phy, coll11, coll12
0,9,1,7,-27,-27
0,9,3,7,-15,-15
0,9,5,8,-1,-1
0,9,8,10,21,21
0,9,11,14,41,41
0,9,14,22,60,60
0,0,1,7,-19,-19
0,0,3,7,-3,-3
0,0,5,9,16,16
0,0,8,15,42,42
0,0,11,21,62,62
0,0,14,31,79,79
1,0,1,-1,12,12
1,0,5,-1,2,2
1,0,12,-3,-27,-27
2,0,1,-2,10,10
2,0,5,-1,-9,-9
2,0,12,-4,-105,-105
3,0,1,1,12,12
3,0,5,1,2,2
3,0,12,-5,-30,-30
  
```

Fig. 18 Calibration file



The calibration results of the Basic Calibration will be saved in a file named **BasicCalibrationLog.txt** (to be found in the BIN-Folder as well).

 Changes won't effect the stored values.



```

BasicCalibrationLog.txt - Notepad
File Edit Format View Help
Current Objective Lens:
Epiplan-Neofluar 20x/0.50
Collimator Calibration
-----
New Track: Laser 488
Emission FilterLP 505
Zoom Colli
1 184
-----
New Track: Laser 405
Emission FilterBP 415-480
Zoom Colli
1 257
Current Objective Lens:
Epiplan-Neofluar 20x/0.50
Pinhole Y-Position Calibration
-----
New Track: Laser 488
Emission FilterLP 505
Plate, ChL1
Zoom Pinhole Y-Position
1 458
-----
New Track: Laser 488
Emission FilterLP 505
NFT 488, ChL1
Zoom Pinhole Y-Position
1 459
-----
New Track: Laser 532
Emission FilterLP 550
NFT 532, ChL1
Zoom Pinhole Y-Position
1 461
-----
New Track: Laser 635
Emission FilterLP 650
NFT 635, ChL1
Zoom Pinhole Y-Position
1 457
-----
New Track: Laser 488
Emission FilterBP 495-525
Mirror, ChL2
Zoom Pinhole Y-Position
1 509
    
```

Fig. 19 BasicCalibrationLog file

## 1.6 OptimizeLIVE Macro

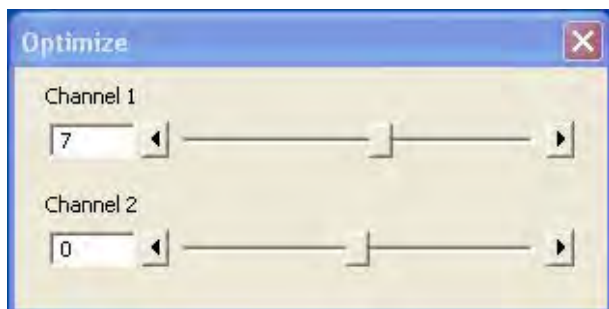


Fig. 20 Optimize window

For temporary optimization of the LSM 7 *LIVE* beampath, try to avoid a modification of the general adjustment in the pinhole dialogue. Use the **Optimize** slider instead. The general setting of the confocal aperture might still be correct for other scan parameters.

The Optimize slider can be set independently for each channel. The slider is moved to the one or the other side while scanning continuous. The brightest image result is the optimal setting. This setting does not affect the general adjustment of the LSM 7 *LIVE* collimator and confocal aperture, but can enhance image brightness efficiently.

## 1.7 Image Match (Scanfield Transformation) Macro

Adjusts the Image match between two scanning imaging systems or a manipulation and a scanning imaging system.

To adjust the image match, set up a multitasking that creates one channel for each scanning and/or manipulation system. In case of the LSM *DuoScan* in combination with LSM 7 *LIVE*, use a T-PMT to create an image with the *DuoScan* system.

For LSM 7 *MP* with two scanning systems, chose the right beam combiner in the reflector turret when adjusting the scanfields with the macro. It has to be confirmed, that both laser beams are optimally aligned to the optical axis of each scanner respectively before adjusting the scanfields. The alignment of the laser beams to the optical axis is performed by the field service engineer at the set up of the system.

- Start the Image Match (Scanfield Transformation) Macro (Fig. 21).
- Use the Image match calibration sample delivered with the system. This sample has a fluorescent layer and a reflective grid, useful to create images in fluorescent, reflective or transmission light mode.
- Start continuous scan, and set laser power and gain to appropriate levels. The point scanning system will be the speed limiting system, so choose a fast scanning speed to get a faster image refresh.
- Use unidirectional scan to avoid adjustment errors due to bidirectional image jitters.
- For LSM 7 *LIVE* with LSM *DuoScan* set the matching zoom factors with one of the **Match DuoScan zoom/offset** or **Match LIVE zoom/offset** buttons relative to each other. It is recommended to adjust the LSM zoom.

These buttons set the two scanheads at a matching scanfield diameter.

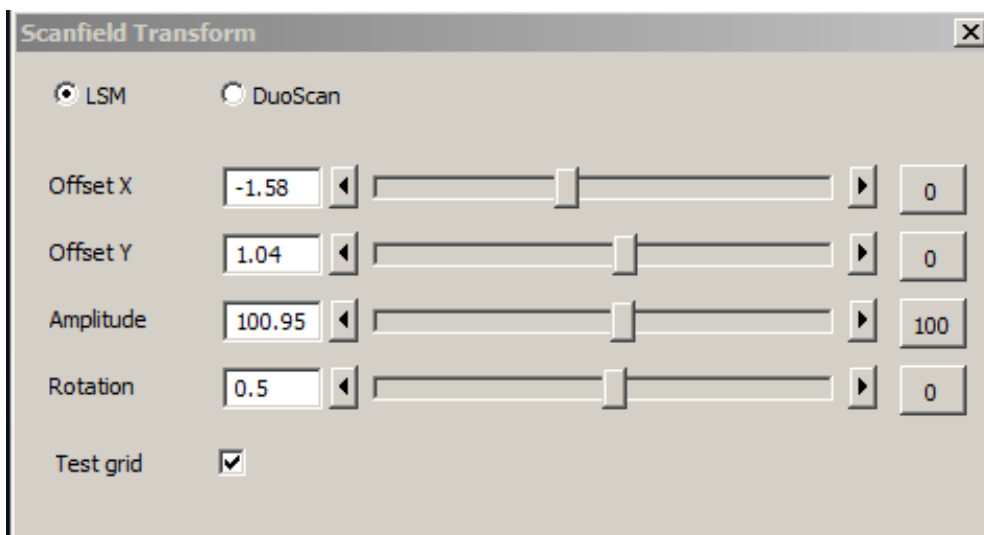
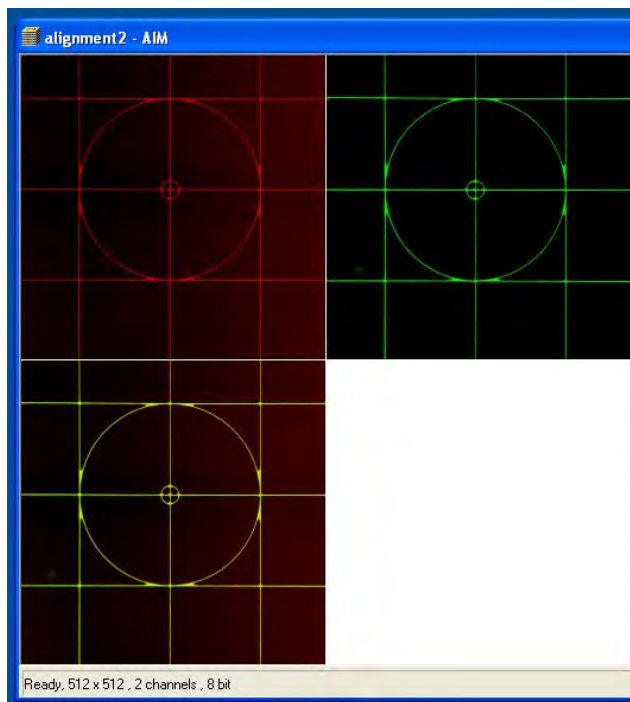



Fig. 21 Scanfield Transform macro interface

- Choose split image for the image window display. You will see the scanned areas of both scanning systems and an overlay.



**Fig. 22** Split image

- Choose LSM (710) or *DuoScan* to adjust the image match.
- While scanning, move the sliders of the Image match macro to get a perfect overlay of the scanning and/or manipulation system relative to each other.
- Offset X/Y adjusts the horizontal and vertical image shift
- Amplitude adjusts a size difference between the images
- Rotation compensates for a tilt in one of the images.


 Store the final image, and use the ReUse function for future image matching. The Multitrack setup and the Zoom factors will then be set automatically.

- For LSM 7 MP with two scanners, focus onto the grid using the imaging scanner (choose "LSM" in the Macro window) and position the center of the grid sample into the center of the image. To do this project the virtual grid into the image by checking the box next to **Test grid** in the macro window (Fig. 21) and try to overlay the grid sample with the Test grid in the image moving the sample stage accordingly. Fine adjust the image of the grid sample to the Test grid using the buttons for Offset, Amplitude and Rotation.
- Next switch to "DuoScan" and adjust the image of the grid sample to the Test grid using Offset, Amplitude and Rotation. Do not move the sample grid during this step. Take care to use moderate laser powers of only a few % as the Multiphotonlaser can destroy the grid sample.

## 1.8 Visual Macro Editor

The Visual Macro Editor is an optional software module which allows the user to program a variety of scanning procedures, image calculations or image processing routines as well as the combination of these functions.

### 1.8.1 Open / Close the Visual Macro Editor Window

- Choose the **Visual Macro Editor** from the **Macro** menu in the Main Menu Bar.
- Click on the  button in the upper right side of the window to quit.

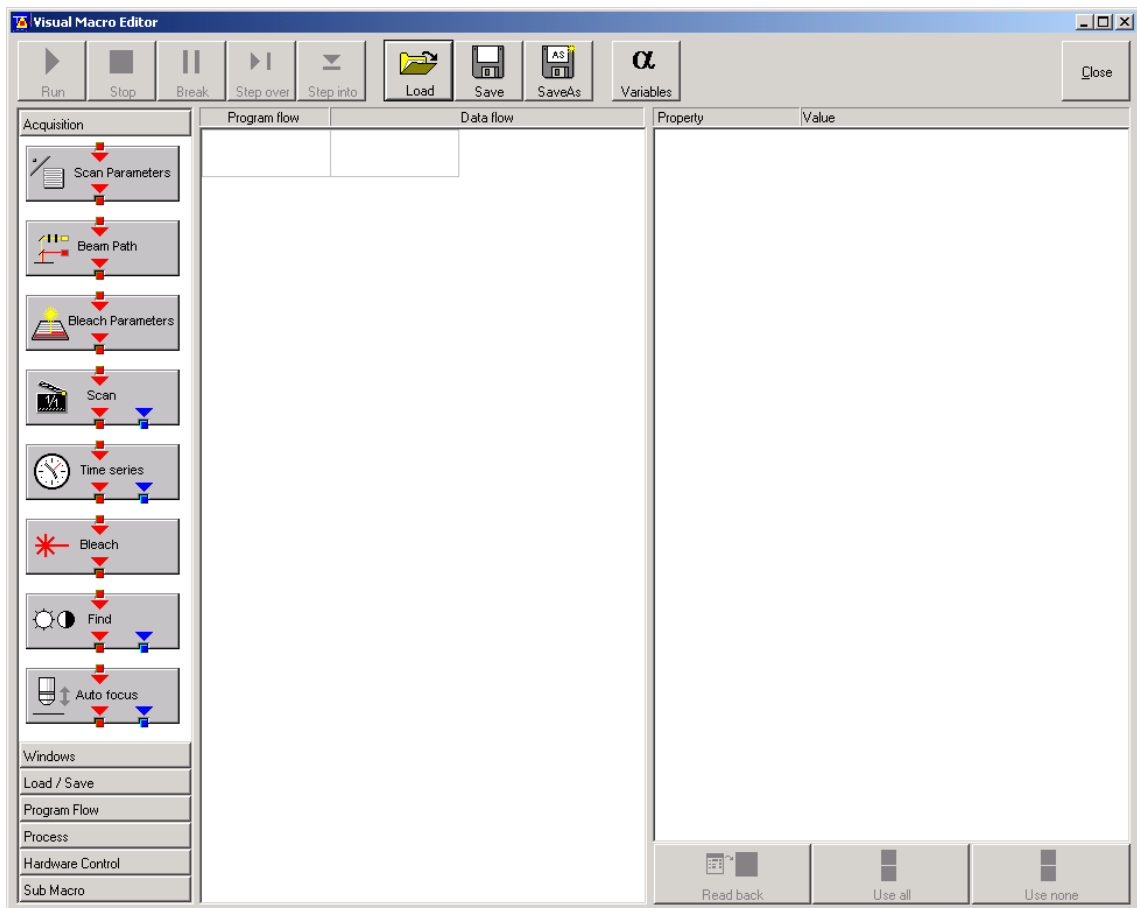


Fig. 23 Visual Macro Editor window

---

## 1.8.2 Function Description

### Toolbar buttons

<b>Run</b>	Starts the macro starting with the first block in the program flow column.
<b>Stop</b>	Stops the macro at any time.
<b>Break</b>	Interrupts the macro. Clicking the button again will resume the actual program flow of the Macro.
<b>Step over</b>	Runs a sub-macro and stops at the first action block in the main macro.
<b>Step into</b>	Stops at the first action block in a sub-macro.
<b>Load / Save / Save as</b>	Use <b>Load</b> to open an existing macro and <b>Save</b> to store the macro. <b>SaveAs</b> allows storing the same macro under a different name.

**Variables** By clicking the button the list of defined variables is displayed. New ones can be added by typing in name and value in the next free line. Variables are assigned to macros. For each new macro a new list of variables has to be generated.

The editor uses predefined action blocks. The action blocks for setting up the work flow of the macro are categorized according to their properties. The following categories are displayed on the left-hand side of the **Visual Macro Editor** control window.

- Acquisition
- Windows
- Load/Save
- Program Flow
- Process
- Hardware Control
- Sub Macro

- Click on one of the category selection buttons will display the available action blocks.

The action blocks can be positioned according to their function either in the **Program Flow** column or the **Data Flow** column. The color of the connection arrows of the action block defines the possibility to position the block in the program flow column or the data flow column(s).

- Click at the action block with the left mouse button (hold down the mouse button) and drag the mouse cursor into the flow column. Release the mouse button.
  - Action blocks for the program flow show red connection arrows ▼ as well as blue output arrows ▼ to connect to the data flow.
  - Action blocks for the data flow show blue connection arrows ▼ or green output arrows ▼ to connect back to the program flow.



It is not possible to drag an action block for program flow into the data flow or an action block for data flow into the program flow.

Action blocks can be removed using the **Delete** key of the keyboard.



A block is highlighted by clicking onto the block icon. To highlight more than one block drag the cursor over the block icons to be marked. A click on the right mouse button also allows the use of the functions **cut**, **copy**, **paste**, and **delete** to modify a macro.



Without using the **Image Display** action block in the data flow column, the result of the scan will not be displayed (see also description of **Scan** and **Time Series** action blocks).

The work flow is generated by connecting the action blocks using the mouse cursor.

- Click onto an output arrow with the left mouse button (hold down the mouse button) and drag the mouse cursor away.
  - A connecting line appears.
- Drag the line to the input arrow of another action block. Only arrows of the same color can be connected.

According to the action blocks a set of properties is listed which can be assigned with free or predefined values. These values can also be variables. A set of defined properties is assigned to one block only. If this block is used more than once the properties can be set differently.

Clicking the **Read Back** button will take over the current settings from the LSM main program for the displayed properties list.

- Mark the check boxes next to the parameters individually or, click **Use all** or **Use none** to check or uncheck all parameters with one click.
- For changing value click into the **Value** column.

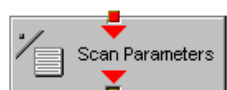
It is possible to define variables. A variable has a (property) name and a mathematical value. The **Assignment** action block can be used to assign a new value to a variable when inserted into the program flow using mathematical algorithms. The new value is then used for any subsequent calculation in the program flow where the variable is part of the calculation.

- Click the **Variable** button on the toolbar to display the variable list. Click once more to display the parameter list again.
- New variables can be added by typing in name and value in the next free line.

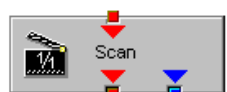
## Acquisition Action Blocks



Loads a Beam Path Configuration from the Configuration list or uses the current one that is set up in the main program. Use the pull down menu in the **Property/Value** list to select the Configuration that should be used for imaging. Changing between Single Track and Multi Track allows to choose between the different Configuration lists. Multi Track also lists tracks that are used for Emission Fingerprinting or Online Fingerprinting. Depending on the type of track different settings can be loaded optionally. If they are not loaded and the parameters list of the Scan Parameter block does not offer them as being editable, for example detector gain and offset, default values will be used. Read back will use the actual parameters of the main program.



The list of properties lists all parameters that can be adjusted for the scan procedure. The value for each parameter can be typed in individually. Clicking the **Read Back** button will take over all current settings from the main software. The check boxes next to the parameters can be marked individually or, clicking **Use all** or **Use none** they can be all checked or unchecked with one click. Only the parameters with a mark in the check box will be taken into account and adjusted when the Scan Parameter block is set in the program flow column. This allows to change only some of the parameters, for example the zoom, at a certain position within the program flow as for each block the check boxes can be set.



This block performs the scanning of the sample according to the settings chosen with Beam Path and Scan Parameters. It can be linked to the Data flow column. If no connection to the data flow is made (for example: Display Image) the result of the scan will not be displayed.



The parameters for bleaching the sample can be loaded from the list of bleach settings. Bleach settings are assembled and stored in the Bleach control window of the main software.



This block performs the bleaching of the sample according to the bleach settings that have been defined in the Bleach Parameters block.



The list of properties lists all parameters that can be adjusted for the acquisition of the time series. The value for each parameter can be typed in individually. Clicking the **Read Back** button will take over all current settings from the main software. The check boxes next to the parameters can be marked individually or, clicking **Use all** or **Use none** they can be all checked or unchecked with one click. Only the parameters with a mark in the check box will be taken into account and adjusted when the Time series block is used in the program flow column. This allows to change only some of the parameters at a certain position within the program flow, for example the trigger used, as for each Time series action block the check boxes can be set individually. Activating the Bleach parameter will bleach the sample according to the settings in the Bleach Parameters action block during the time series. The Time series action block can be connected to the data flow column.





This block performs the **Find** function according to the main software.



This action block sets the Z-position according to the focusing procedure set with the parameters available. The image generated displays the image or image series acquired to set the focus position. The following properties are available to define the auto focus function:

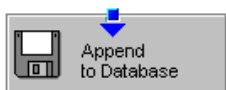
- Mode/Precise: Uses fast XYZ-scan(s); the scan parameters can be defined via Scan Parameters Action Block
- Mode/Fast: Uses fast XZ-scan(s)
- Method/Intensity: auto focus mode which searches for maximum intensity.
- Method/Contrast: auto focus mode which searches for maximum contrast.
- Range ( $\mu\text{m}$ ): The system will perform the auto focus search within that range.
- Offset ( $\mu\text{m}$ ): Defines the distance between the focus plane set by the auto focus function (e.g. coverslip reflex) and the focal plane for imaging.
- Assign position to: Chose a variable to which the new position of the focus plane set by the auto focus function (e.g. coverslip reflex) should be assigned.
- Assign offset to: Chose a variable to which the offset value should be assigned.

### Windows Action Blocks



This block has to be used if the actually scanned image(s) should be displayed. For each Image Display block a new image window is opened. If not saved, the image will be overwritten when the program flow is passing by again.

### Load / Save / Action Blocks



The image(s) are stored to an image database. The database taken is either the last one, that was open or the current open one. Clicking Read back updates the database to be used for storing the image(s). In addition some other parameters can be set according to the list of properties displayed.



Within the program flow already existing images can be loaded.

The database taken is either the last one, that was open or the current open one. Clicking Read back updates the database to be used to load the image(s) from. The images can be loaded by Index or by the name of the image.

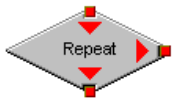


It is possible to export images choosing a certain file format from the pull down list that appears when highlighting the line File Format in the properties list. Specific parameters are assigned to each file format which can be set for the image export.

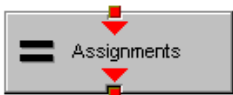


Images can be imported within the program flow. The image to be loaded can be selected by typing in the file path. The import of single images or an image series is possible. The images to be imported as series must have the same name and must be numbered in ascending order.

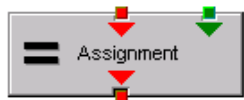
**Program Flow Action Blocks**



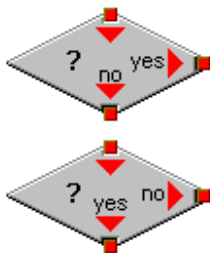
If any action(s) should be repeated a defined number of times, the Repeat block should be set into the loop of the program flow. The number of repeats is set in the properties list. The repeat is only performed using the connection of the right arrow. After finishing the repeats, the program flow connected to the arrow pointing down is performed.



Using this block in the program flow allows to assign new values to a selected variable using mathematical calculations which can be typed into the value line. All predefined variables can also be used for calculations. By passing this block the variable will be transiently changed and the new value can for example be used to assign different numbers to the image name or to increase the time delay during a time series acquisition.



This block assigns a new value to a variable which has to be selected from the drop down list. The value assigned results from a calculation taken from the statistics action block (see below).



The two blocks are used as decision makers. Depending on the value of a predefined variable, the program flow can be directed to one of the two possible directions. The expression of the variable's value which should be used to decide on the program flow is set in the properties list individually for each used decision making block. It takes the actual transient value of the variable into account.

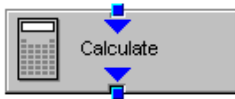


This block introduces a delay in the program flow. The delay time is started when the block is reached within the program flow.

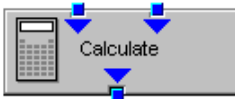


The block allows to assign a potential error message with a textbox and to decide on the Error where to direct the program flow. If no textbox is assigned the program flow will just continue according to the presence of an error message. Choosing a textbox requires the input of the user to continue with the program flow.

## Process Action Blocks



This block allows to do image calculation using the pixel intensities of the image. The operators for the formulas that can be used are described in the Ratio Dialogue within the Process Menu. The formulas set up in the Image calculation window can be copied and pasted into the properties list of the Visual Macro Editor.



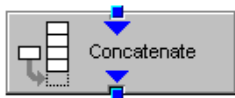
For calculations using two different sources the Calculate block with two possible inputs is used. The calculation starts when both inputs have arrived. Within program loops the next calculation will start when the inputs have arrived again in the same sequence as the first time. The operators for the formulas that can be used are described in the Ratio Dialogue within the Process Menu. The formulas set up in the Image calculation window can be copied and pasted into the properties list of the Visual Macro Editor.



The input image will be copied to a new image format that can be defined by changing the values of the listed properties.



Using this block enables to copy one image into another with the coordinates and settings put into the properties list. The destination image is connected to the left input; the source image is connected to the right input.



The subsequently arriving images will be concatenated according to the coordinate setting and treated as one image. A subsequent image display will be updated each time a new image arrives.



The second concatenate action block allows to concatenate images from two different acquisition blocks.



The block uses an input image (left input) and chooses a region within that image according to a mask (right input) that has been defined in the Mask action block (see below). Within that mask region of a selected channel, one of the following result values can be selected which is then assigned to the chosen variable in the corresponding assignment action block:

- Mean intensity: Equals mean intensity within mask region
- Standard deviation: Equals standard deviation of intensity distribution within mask region
- Number Pixels: Number of pixels that constitute the mask region
- Area ( $\mu\text{m} \times \mu\text{m}$ ): Area of mask region
- Minimum: Minimum intensity value found within mask region
- Maximum: Maximum intensity value found within mask region



This action block defines a mask (image region with defined properties) derived from an image window. The image can be a newly acquired or imported image. The properties of the mask based on the image data can be selected according to the following properties:

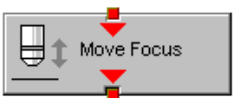
- Scan region(s): The mask is based on the scan region(s) defined in the **ScanControl**.
- Bleach region(s): The mask is based on bleached region(s) defined in the **BleachControl**.
- Image overlay: The mask is based on image overlay regions.
- ROI list entry: The mask is based on ROIs listed in Edit ROI control window.
- Interactive overlay: The mask is defined using overlay regions which are defined while the macro is executed.
- Interactive histogram: The mask is defined via histogram settings (min/max) interactively defined while the macro is executed. This works in conjunction with ROI selection (interactive or stored) as described above.
- Region index: Index of ROI (i.e. ROI number) if for example multiple bleach regions are active.
- Channel: Image channel which is used to derive the mask parameters from. This works in conjunction with interactive settings or loaded images.
- Intensity range: Defines the intensity range which is applied for mask definition.
- Lower threshold: Defines lower intensity threshold which is applied for mask definition. This threshold can be an absolute number or a floating point.
- Upper threshold: Defines upper intensity threshold which is applied for mask definition. This threshold can be an absolute number or a floating point.

### Hardware Control Action Blocks



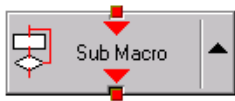
If a motorized stage is available the movement of the stage can be set up as part of the program flow. The stage can be moved to an absolute position which can also be taken from the actual stage position using the Read back function or typed in individually. Using a relative position will use the current stage position as relating position. The values have to be typed in.


It is also possible to choose the position from the **Mark and Find** position list which must have been set up in the stage control menu to be available.



The movement of the focus can be set up as part of the program flow. The focus can be moved to an absolute position which can also be taken from the actual focus position using the Read back function or typed in individually. Using a relative position will use the current focus position as relating position. The values have to be typed in. For large travel ranges which exceed half of the objectives working distance a warning can be displayed.

**Sub Macro Actions Blocks**



This block allows to set a sub macro within a main macro. The sup macro is opened by clicking onto the arrow in the block icon and closed clicking the  top right of the program flow column. The number of program flow in- and outputs as well as the number of program flow in- and outputs (= number of available connection arrows indicated on the block icon) can be defined for each sub macro. Multiple connections are therefore possible. The maximum of inputs and outputs for each flow is ten.

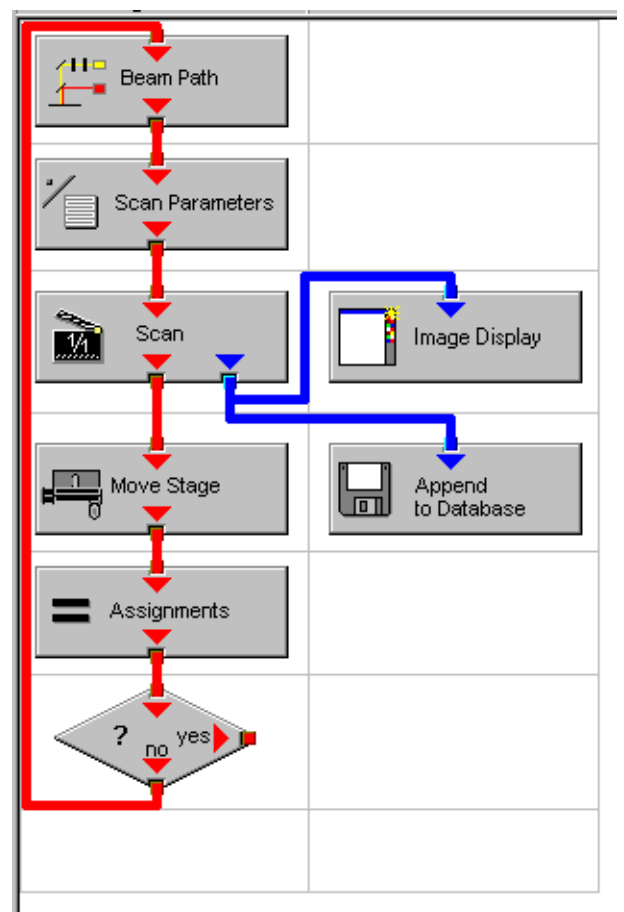


Using this block allows to integrate macros or macro procedures programmed with visual basic into a VME macro. The number of data flow inputs and outputs can be set to a maximum number of ten. The name of the macro indicates the name within the VME macro. The file name refers to the path and name of the VBA macro to be loaded into the VME macro. If no procedure available within the VBA macro is selected the whole VBA macro will be executed.

**1.8.3 Editing a Macro**

Following Example shows a macro which acquires five subsequent images and moves the stage 10 microns in X and Y following each image. The images are stored and numbered in ascending order.

- Arrange and connect the action blocks (Fig. 24).



**Fig. 24 Arrangement of action blocks**

Property	Value
Index	1

Fig. 25 Assignment of the variable

- Assign the value 1 to a variable named Index (Fig. 25).

Property	Value
Configuration Type	Single Track
Configuration	488 FITC
Load Speed	No
Load Bits per Sample	No
Load Pinhole Diameter	No
Load Gain and Offset	No

Fig. 26 Property list of the Beam Path action block

- Click the single action blocks and assign the values to the listed properties (beam path and scan parameters can be chosen according to the sample).

Use	Property	Value
<input checked="" type="checkbox"/>	LSM Pixels X	512
<input checked="" type="checkbox"/>	LSM Pixels Y	512
<input checked="" type="checkbox"/>	LSM Step Y	1
<input checked="" type="checkbox"/>	LSM Zoom	1.600000
<input checked="" type="checkbox"/>	LSM Rotation [°]	45.000000
<input checked="" type="checkbox"/>	LSM Offset X [µm]	0.000000
<input checked="" type="checkbox"/>	LSM Offset Y [µm]	0.000000
<input checked="" type="checkbox"/>	LSM Pixel Period [µs]	2.560000
<input checked="" type="checkbox"/>	Camera Mode	Mono 1300 x 1030
<input checked="" type="checkbox"/>	Camera Pixels X	1300
<input checked="" type="checkbox"/>	Camera Pixels Y	1030
<input checked="" type="checkbox"/>	Camera Offset X [Pixels]	0.000000
<input checked="" type="checkbox"/>	Camera Offset Y [Pixels]	0.000000
<input checked="" type="checkbox"/>	Pixels Z	1
<input checked="" type="checkbox"/>	Scaling Z [µm]	1.000000
<input checked="" type="checkbox"/>	Offset Z [µm]	0.000000
<input checked="" type="checkbox"/>	Bits per Sample	8 bit

Fig. 27 Property list of the Scan Parameter action block

Property	Value
Database	D:\Projekte\Release 3.5 RT\SW\Scripting\test 2.mdb
Name	"Cell culture" + Index
Description	
Notes	
Compress	Yes

Fig. 28 Property list of the Append to Database action block

- The database the images will be appended to is automatically displayed when highlighting the **Append to Database** action block. The last one used will be taken. To update this, open the desired database and use the **Read back** function. Type in a name for the images with or without quotation marks. All images will get the same name then. For numbering the images use quotation marks for the name and add the variable Index, which has been assigned with the value 1. This value or the transiently changed value of the variable will be added to the name of the image.

- The movement of the stage is defined highlighting the Move stage action block, choosing the Mode Relative Position in the Property list and typing in the value 10 for X and Y which then will move the stage 10 microns into X and Y direction starting with the current stage position on passing that action block.
- Now the first complete scan has been performed and the image is stored. For the next cycle the variable Index has to get another value to make sure the numbering of the images works. Using the **Assignments** action block, the Variable Index is chosen and the value assigned is **Index + 1**. Such the variable will get the transient value 2 for the next cycle, the value 3 for the over next cycle and so on.
- The number of repeats can be defined using a Decision block and using the value of the variable as base for the decision. By defining that if the Expression of the variable is larger than 5 and not connecting the output arrow marked with 'yes' to any other block, the macro will stop after the acquisition of five images. Alternatively one can use at this part of the program flow the Repeat block and define the number of repeats to be performed, which would be 5 in this case (The first scan is already counted!)

Property	Value
Mode	Relative Position
Distance X [µm]	10
Distance Y [µm]	10

**Fig. 29** Property list of the Move stage action block.

Property	Value
Index	Index + 1

**Fig. 30** Property list of the Assignments action block

Property	Value
Expression	Index > 5

**Fig. 31** Property list of the Decision action block

## 2 VBA Programming for LSM

### 2.1 VBA Programming

#### 2.1.1 Example Macro: Working with Files

I/O operations, file operations are implemented in VBA

Use of the implemented functions

#### Example:

```
Type CALIBRATION_PARAM
    lambda As Long
    angle As Double
    Translation As Double
End Type
```

```
Function DataFromCalibFile(strGetFile As String, calibArray() As CALIBRATION_PARAM) As Integer
```

```
    Dim hFile As Long
    Dim strLine As String
    Dim varData(1 To 3) As Variant
    Dim paramCnt As Integer
```

```
    paramCnt = -1
    'get the next free file handle
    hFile = FreeFile
    'check if file exist
    If (Len(Dir(strGetFile)) > 0) Then
        Open strGetFile For Input Access Read Shared As hFile
        If (Not EOF(hFile)) Then
            Line Input #hFile, strLine

            paramCnt = 0
            Do Until EOF(hFile)
                Input #hFile, varData(1), varData(2), varData(3)
                calibArray(paramCnt).lambda = varData(1)
                calibArray(paramCnt).angle = varData(2)
                calibArray(paramCnt).Translation = varData(3)
                paramCnt = paramCnt + 1
            Loop
        End If
        Close #hFile
    End If
```

```
    DataFromCalibFile = paramCnt
End Function Objektstruktur
```

Use of the API function for additional functionality



## 2.1.2 Example: Access to Windows API Function and external DLLs

- with Type Libraries

OLE

- with Declare Statements

API Text Viewer

searches WIN32API.TXT

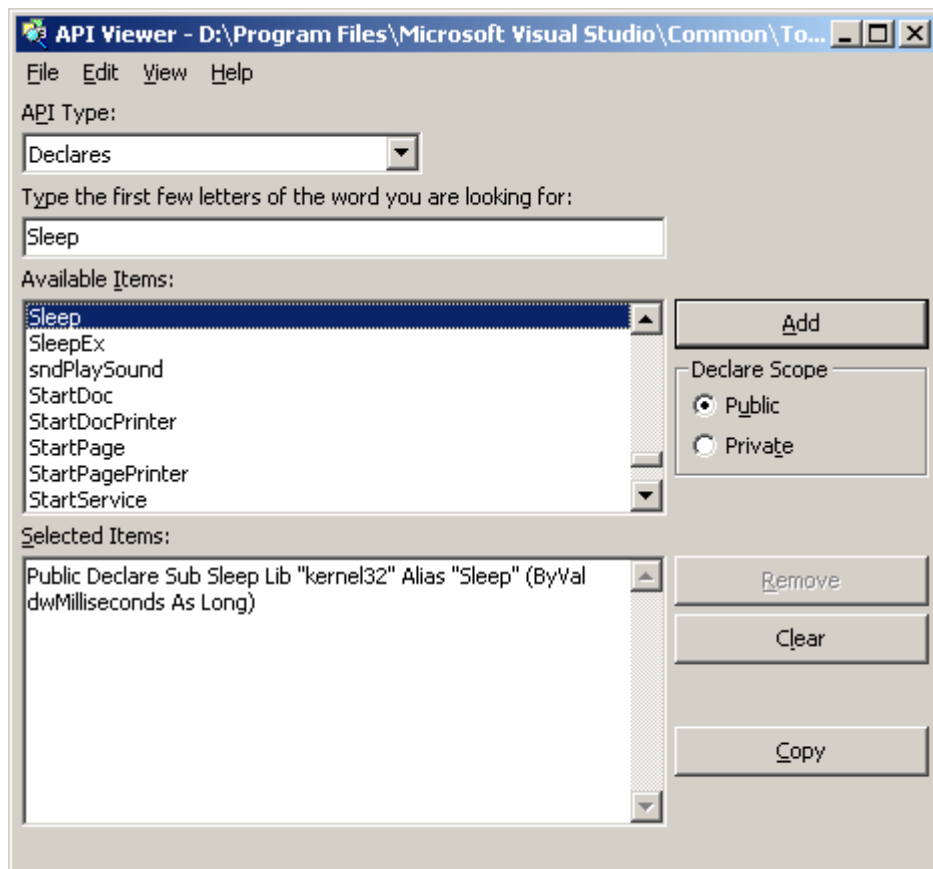


Fig. 32 API Viewer window

**Example:**

*Declare Sub Sleep Lib "kernel32" (ByVal dwMilliseconds As Long)*

*Declare Function RegOpenKeyEx \_  
Lib "advapi32.dll" Alias "RegOpenKeyExA" \_  
(ByVal hKey As Long, ByVal lpSubKey As String, \_  
ByVal ulOptions As Long, ByVal samDesired As Long, \_  
phkResult As Long) As Long*

*Public Declare Function RegCloseKey \_  
Lib "advapi32.dll" (ByVal hKey As Long) As Long*

*Public Declare Function RegQueryValueEx \_  
Lib "advapi32.dll" Alias "RegQueryValueExA" \_  
(ByVal hKey As Long, ByVal lpValueName As String, \_  
ByVal lpReserved As Long, lpType As Long, \_  
lpData As Any, lpcbData As Long) As Long ' Note that if you declare the lpData parameter  
as String, you must pass it By Value.*

*Public Const HKEY\_CLASSES\_ROOT = &H80000000*

*Public Const SYNCHRONIZE = &H100000*

*Public Const READ\_CONTROL = &H20000*

*Public Const STANDARD\_RIGHTS\_READ = (READ\_CONTROL)*

*Public Const KEY\_QUERY\_VALUE = &H1*

*Public Const KEY\_ENUMERATE\_SUB\_KEYS = &H8*

*Public Const KEY\_NOTIFY = &H10*

*Public Const KEY\_READ = ((STANDARD\_RIGHTS\_READ Or KEY\_QUERY\_VALUE Or  
KEY\_ENUMERATE\_SUB\_KEYS Or KEY\_NOTIFY) And (Not SYNCHRONIZE))*

*Public Const REG\_SZ = 1 ' Unicode null terminated string*

*Public Const ERROR\_SUCCESS = 0&*

---

```
Function FServerFromDescription(strName As String, strPath As String) As Boolean
    Dim lngResult As Long
    Dim strTmp As String
    Dim hKeyServer As Long
    Dim strBuffer As String
    Dim cb As Long
    Dim i As Integer

    FServerFromDescription = False

    strTmp = VBA.Space(255)
    strTmp = strName + "\CLSID"
    lngResult = RegOpenKeyEx(HKEY_CLASSES_ROOT, strTmp, 0&, KEY_READ, hKeyServer)

    If (Not lngResult = ERROR_SUCCESS) Then GoTo error_exit
    strBuffer = VBA.Space(255)
    cb = Len(strBuffer)

    lngResult = RegQueryValueEx(hKeyServer, "", 0&, REG_SZ, ByVal strBuffer, cb)
    If (Not lngResult = ERROR_SUCCESS) Then GoTo error_exit

    lngResult = RegCloseKey(hKeyServer)
    strTmp = VBA.Space(255)
    strTmp = "CLSID\" + Left(strBuffer, cb - 1) + "\LocalServer32"
    strBuffer = VBA.Space(255)
    cb = Len(strBuffer)
    lngResult = RegOpenKeyEx(HKEY_CLASSES_ROOT, strTmp, 0&, KEY_READ, hKeyServer)
    If (Not lngResult = ERROR_SUCCESS) Then GoTo error_exit

    lngResult = RegQueryValueEx(hKeyServer, "", 0&, REG_SZ, ByVal strBuffer, cb)
    If (Not lngResult = ERROR_SUCCESS) Then GoTo error_exit
    strPath = Left(strBuffer, cb - 1)
```

***IngResult = RegCloseKey(hKeyServer)***

*hKeyServer = 0*

*i = Len(strPath)*

*Do Until (i = 0)*

*If (VBA.Mid(strPath, i, 1) = "\") Then*

*strPath = Left(strPath, i - 1)*

*FServerFromDescription = True*

*Exit Do*

*End If*

*i = i - 1*

*Loop*

*error\_exit:*

*If (Not hKeyServer = 0) Then IngResult = RegCloseKey(hKeyServer)*

*hKeyServer = 0*

*End Function*

## 2.2 Programming for LSM

### 2.2.1 Object structure

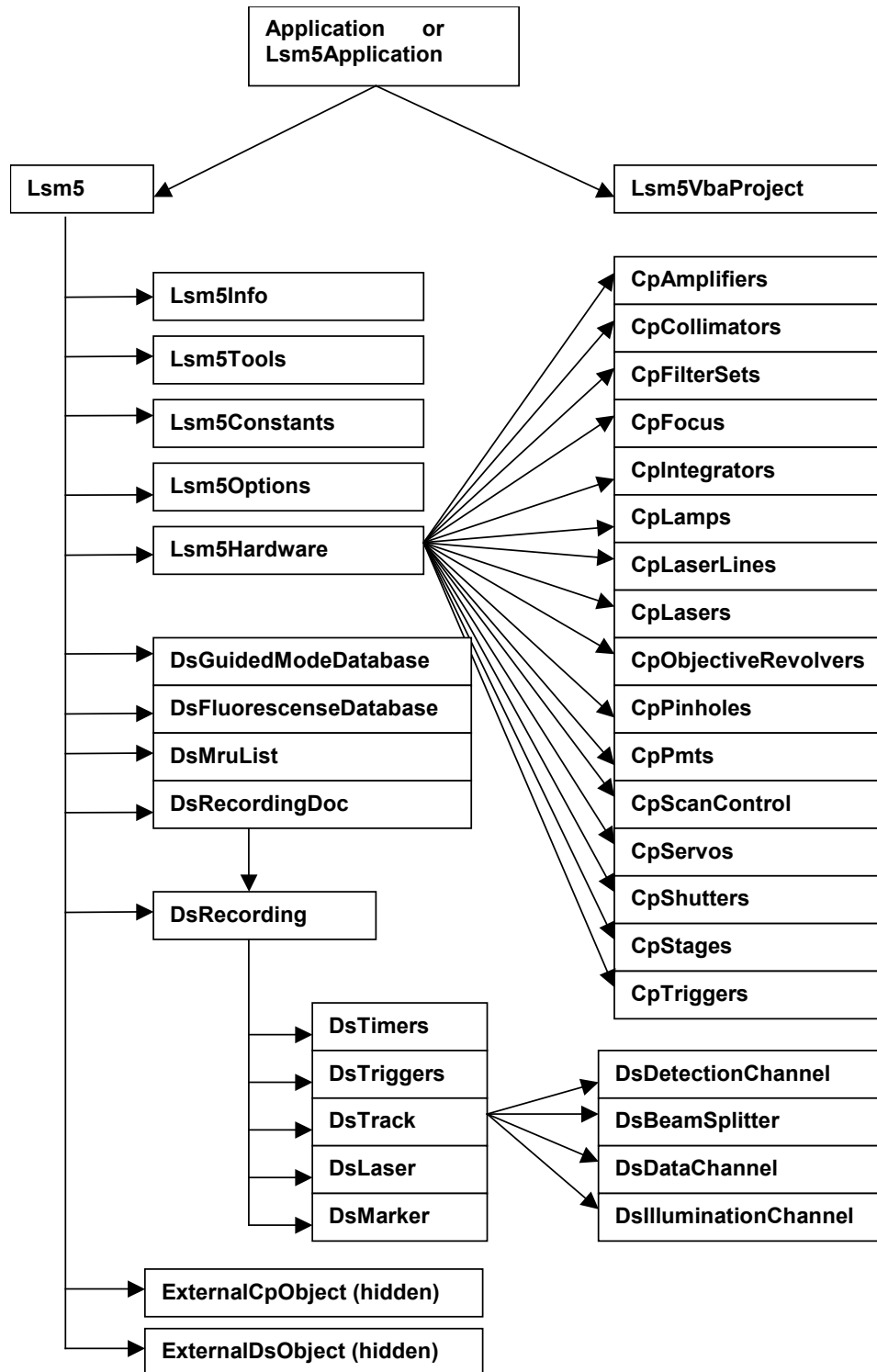


Fig. 33 Object structure

To search the object structure, use the object browser.

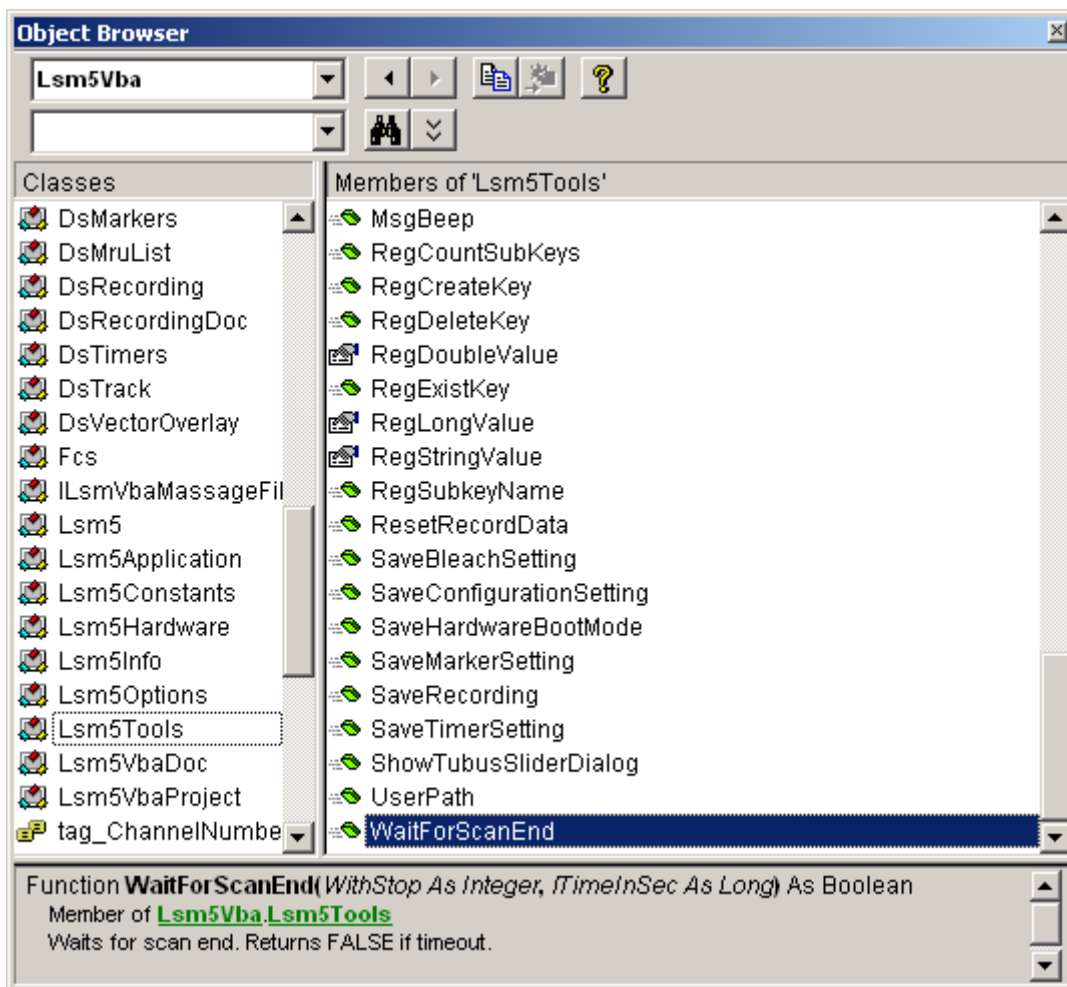


Fig. 34 Object Browser window

## 2.2.2 Differences in access to LSM Hardware with LSM 7 Hardware Object / DsRecording Object

Object Browser Project / Library LsmVba auswählen und Objekt Struktur untersuchen

- Access with DsRecording Object

Status maintains at Scan Start

```
Public Sub CopyRecording(Destination As DsRecording, Source As DsRecording)
    Dim TS As DsTrack
    Dim TD As DsTrack
    Dim DataS As DsDataChannel
    Dim DataD As DsDataChannel
    Dim DetS As DsDetectionChannel
    Dim DetD As DsDetectionChannel
    Dim IIS As DsIlluminationChannel
    Dim IID As DsIlluminationChannel
    Dim BS As DsBeamSplitter
    Dim BD As DsBeamSplitter
    Dim IT As Long
    Dim II As Long
    Dim success As Integer

    Destination.Copy Source
    Destination.Objective = Source.Objective
    For IT = 0 To Destination.TrackCount - 1

        Set TS = Source.TrackObjectByIndex(IT, success)
        Set TD = Destination.TrackObjectByIndex(IT, success)

        TD.Collimator1Position = TS.Collimator1Position
        TD.Collimator2Position = TS.Collimator2Position

        For II = 0 To TD.DataChannelCount - 1
            Set DataS = TS.DataChannelObjectByIndex(II, success)
            Set DataD = TD.DataChannelObjectByIndex(II, success)
            DataD.ColorRef = DataS.ColorRef
        Next II
    
```

```
For II = 0 To TD.DetectionChannelCount - 1
    Set DetS = TS.DetectionChannelObjectByIndex(II, success)
    Set DetD = TD.DetectionChannelObjectByIndex(II, success)
    DetD.Filter1 = DetS.Filter1
    DetD.Filter2 = DetS.Filter2
    DetD.DetectorGain = DetS.DetectorGain
    DetD.AmplifierGain = DetS.AmplifierGain
    DetD.AmplifierOffset = DetS.AmplifierOffset
    DetD.PinholeDiameter = DetS.PinholeDiameter
Next II
```

```
For II = 0 To TD.IlluminationChannelCount - 1
    Set IIS = TS.IlluminationObjectByIndex(II, success)
    Set IID = TD.IlluminationObjectByIndex(II, success)
    IID.Acquire = IIS.Acquire
    ID.Power = IIS.Power
    IID.DetectionChannelName = IIS.DetectionChannelName
Next II
```

```
For II = 0 To TD.BeamSplitterCount - 1
    Set BS = TS.BeamSplitterObjectByIndex(II, success)
    Set BD = TD.BeamSplitterObjectByIndex(II, success)
    BD.Filter = BS.Filter
Next II
```

```
Next IT
End Sub
```



---

Status will be updated at Scan Start to current recording parameters

```
Private Sub FillPinholeList()  
    Dim count As Long  
    Dim i As Long  
    Dim ObjPinholes As CpPinholes  
    Dim Servos As Object  
    Dim ServoX As Object  
    Dim ServoY As Object  
    Dim success As Boolean  
  
    CmbPinhole.Clear  
  
    Set Servos = Lsm5.ExternalCpObject.pHardwareObjects.pServos  
    Set ObjPinholes = Lsm5.Hardware.CpPinholes  
    count = ObjPinholes.count  
    For i = 0 To count - 1  
        success = ObjPinholes.Select(i)  
        If (success) Then  
            Set ServoX = Servos.pltem(ObjPinholes.Name + "X")  
            Set ServoY = Servos.pltem(ObjPinholes.Name + "Y")  
            If ((Not ServoX Is Nothing) And (Not ServoY Is Nothing)) Then  
                CmbPinhole.AddItem ObjPinholes.Name  
            End If  
        End If  
    Next i  
    If (count) Then CmbPinhole.ListIndex = 0    ' select first pinhole  
  
    Set ObjPinholes = Nothing  
    Set Servos = Nothing  
    Set ServoX = Nothing  
    Set ServoY = Nothing  
End Sub
```

- How do I find the desired Object / Method?

Open Tool / References

Browse CP.dll search, open

Choose Object Browser Project / Library DS or CP

Examine Object Structure

**Example:**

```
Function GetLaserKind(WaveLength As String, kind As Integer)
```

```
    Dim count As Long
```

```
    Dim CpObject As Object
```

```
    Dim Lines As Object
```

```
    Dim i As Long
```

```
    Dim Success As Integer
```

```
    Dim WaveLenghtOfIndex As Long
```

```
    Dim Attenuation As Double
```

```
    Dim Enable As Integer
```

```
    Dim Name As String
```

```
    GetLaserValueMax = False
```

```
    Set CpObject = Lsm5.ExternalCpObject()
```

```
    Set Lines = CpObject.pHardwareObjects.pLaserLines
```

```
    count = Lines.lCount
```

```
    For i = 0 To (count - 1)
```

```
        Success = Lines.bLineInfo(i, WaveLenghtOfIndex, Attenuation, Enable, Name)
```

```
        If (Success And (WaveLenghtOfIndex = WaveLenght)) Then
```

```
            kind = Lines.AttenuatorType(WaveLenght)
```

```
            Set Lines = Nothing
```

```
            Set CpObject = Nothing
```

```
            Exit Function
```

```
        End If
```

```
    Next i
```

```
    Set Lines = Nothing
```

```
    Set CpObject = Nothing
```

```
End Function
```

### 2.2.3 Access to scanned pictures

**Example:**

```
Function ChannelIndexFromChannelName(ChannelName As String, ChannelIndex As Long)
    Dim num As Long, num1 As Long, num2 As Long
    Dim i As Long
    Dim Success As Integer
    Dim DetectionChannel As DsDetectionChannel
    Dim LsmInfo As Lsm5Info
    Dim Recording As DsRecording

    ChannelIndexFromChannelName = False

    Set Recording = Lsm5.DsRecording
    Set LsmInfo = Lsm5.Info
    If (LsmInfo.NumberOfPmtsInSystem(num, num1, num2) = True) Then
        For i = 0 To (num + num1 - 1)
            Set DetectionChannel = Recording.DetectionChannelOfActiveOrder(i, Success)
            If Success Then
                If (StrComp(DetectionChannel.Name, ChannelName) = 0) Then
                    ChannelIndex = i
                    ChannelIndexFromChannelName = True
                    GoTo exit_function
                End If
            End If
        Next i
    End If

exit_function:
    Set LsmInfo = Nothing
    Set Recording = Nothing
End Function
```

```
Function GetAveragePixel(ScanDoc As DsRecordingDoc, ChannelName As String, mean As Double)
    Dim line As Variant
    Dim x As Long, xmax As Long
    Dim y As Long, ymax As Long
    Dim spl As Long
    Dim bpp As Long
    Dim Sum As Double
    Dim Channel As Long

    GetAverageScanLineParams = False

    If (ChannelIndexFromChannelName(ChannelName, Channel) = False) Then
        Exit Function
    End If

    'check for valid doc
    If (ScanDoc Is Nothing) Then Exit Function
    Sum = 0
    xmax = ScanDoc.Recording.SamplesPerLine
    ymax = ScanDoc.Recording.LinesPerFrame
    For y = 0 To ymax - 1
        line = ScanDoc.ScanLine(Channel, 0, 0, 0, spl, bpp)

        For x = 0 To spl - 1
            Sum = Sum + line(x)
        Next x
    Next y
    mean = Sum / (xmax*ymax)

    GetAverageScanLineParams = True
End Function
```

## 2.2.4 Backup Recording

```
.  
. .  
  
Dim Recording As DsRecording  
Dim BackupRecording As DsRecording  
Dim ScanDoc As DsRecordingDoc  
  
Set Recording = Lsm5.DsRecording  
'create a backup recording  
Set BackupRecording = Lsm5.CreateBackupRecording  
Success = BackupRecording.Copy(Recording)  
  
Recording.ScanMode = "Line"  
Recording.FramesPerStack = 1  
Recording.StacksPerRecord = 1  
Recording.ScanDirection = eSingleForeward  
Recording.SpecialScanMode = "NoSpecialScanMode"  
Recording.TimeSeries = False  
Recording.ZoomX = ZOOM_X  
Recording.ROTATION = ROTATION  
Recording.Sample0X = OFFSET_X  
Recording.Sample0Y = OFFSET_Y  
Recording.SamplesPerLine = IMAGE_SIZE  
Recording.StartScanTime = 8  
  
Success = SetChannelDetection(ChannelName, 1, AmpOffset, 100)  
Set ScanDoc = Lsm5.StartContinuousScan  
  
. . .  
  
Lsm5.StopScan  
'restore the backup recording  
Recording.Copy BackupRecording  
Set Recording = Nothing  
Set BackupRecording = Nothing
```

### 2.2.5 Events

- What are Events good for?
- Mouse Events
- Scan state events
- Hardware Events

***Private Sub Lsm5VbaDoc\_SystemEvent(ByVal EventNr As Long, ByVal Param As Variant)***

*Dim x As Long*

*Dim y As Long*

*Dim z As Long*

*Dim t As Long*

*Dim c As Long*

*Dim tmp As Long*

*Dim pt As POINT*

*Dim dsDoc As DsRecordingDoc*

*'get the active Recording Document*

*Set dsDoc = Lsm5.DsRecordingActiveDocObject*

*If (dsDoc Is Nothing) Then Exit Sub*

*If (Not dsDoc.IsValid) Then Exit Sub*

*If (EventNr = elmageWindowLeftButtonDownEvent) Then*

*If dsDoc.GetCurrentMousePosition(c, t, z, y, x) <> 0 Then*

*' do something*

*.*

*.*

*.*

*End If*

*End If*

*End Sub*

### 3 ZEN VBA-GUI Interface

#### 3.1 Introduction

The ZEN VBA-GUI interface allows you to program ZEN with the same comfort and ease as using the mouse to control the GUI. All capabilities of the ZEN system are accessible via an intuitive syntax.

The main idea behind the ZEN VBA-GUI interface is to make ZEN commands available in the same way as they are in the GUI. The image shows how this principle works: To set the acquisition frame size, one has to

1. Select the **Acquisition** tab
2. Go to the **Acquisition** mode box
3. Set **Frame Size X** and **Y**

In the VBA-GUI interface, this translates to "Acquisition.AcquisitionMode.FrameSizeX" and "FrameSizeY".

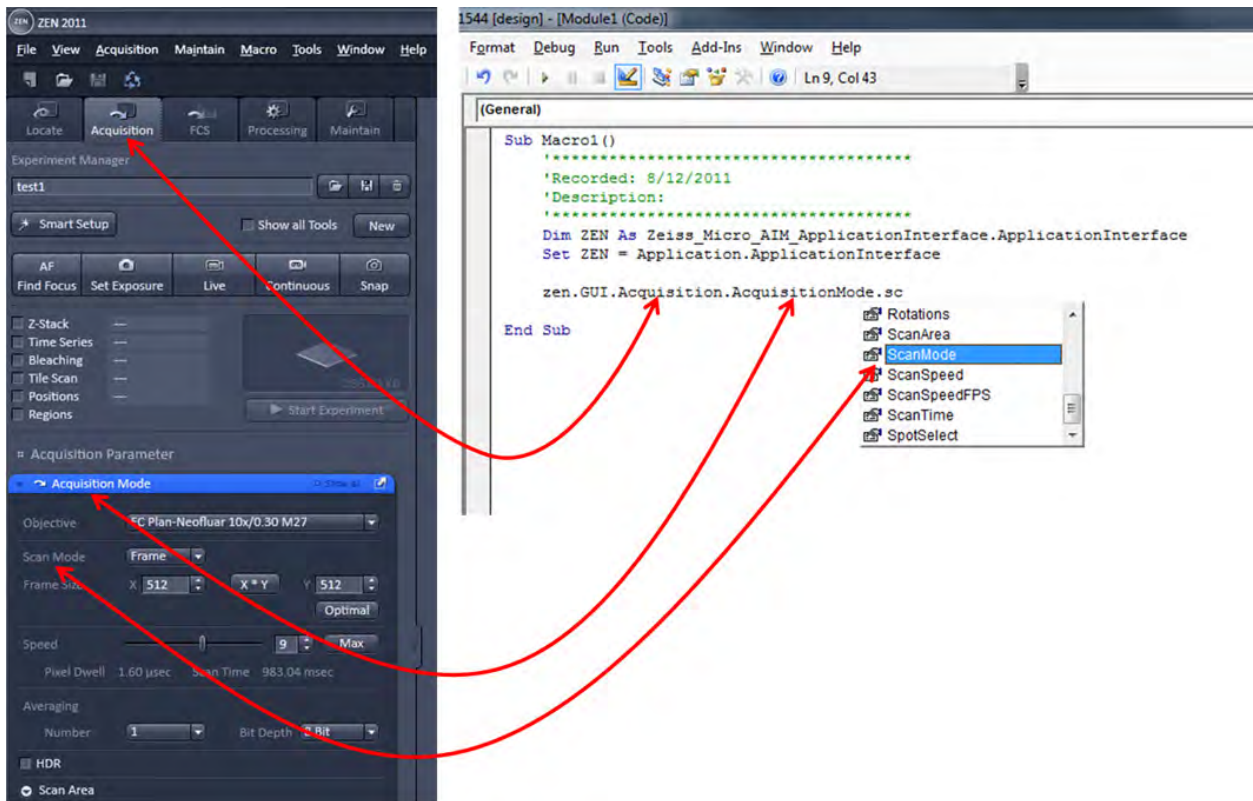


Fig. 35 ZEN VBA-GUI Interface

The easiest way to access the VBA-GUI interface is to start with a recorded macro. This contains a "ZEN" object, which has a "GUI" property. "ZEN.GUI" is the entry point to the VBA-GUI interface.

### 3.2 Structure of a VBA-GUI command

There are three different kinds of actions that can be performed in the ZEN GUI: Setting a value (either a number or a string, e.g. the frame size or configuration name), selecting from a list of options (e.g. the scan mode), and executing a command (e.g. Snap). To reflect this, different kinds of commands are available in the VBA-GUI interface.

All commands have a (Boolean, read-only) "IsEnabled" property that can be used to check whether the command is supported by the current system configuration. Also, commands have a (Boolean, read/write) "AsyncMode" property, that determines whether this command is executed asynchronously (i.e. program flow continues without waiting for the command to finish) or synchronously. The default is synchronous execution.

A command corresponding to a value in the GUI offers a "Value" property, which can be used to read or write the corresponding value. For numeric values, the command also offers (read-only) "Minimum" and "Maximum" properties.

A command corresponding to a range of options (i.e. a combo bvgbox or drop-down box) offers a selection using the "ByIndex" or the "ByName" properties. A list of available options (as name strings) can be queried from the "Items" property, and these name strings can be used in the "ByName" property.

A command corresponding to a button (e.g. Snap) offers two methods: "CanExecute" can be used to check whether the command can currently be executed, and "Execute" causes the specified action to happen.

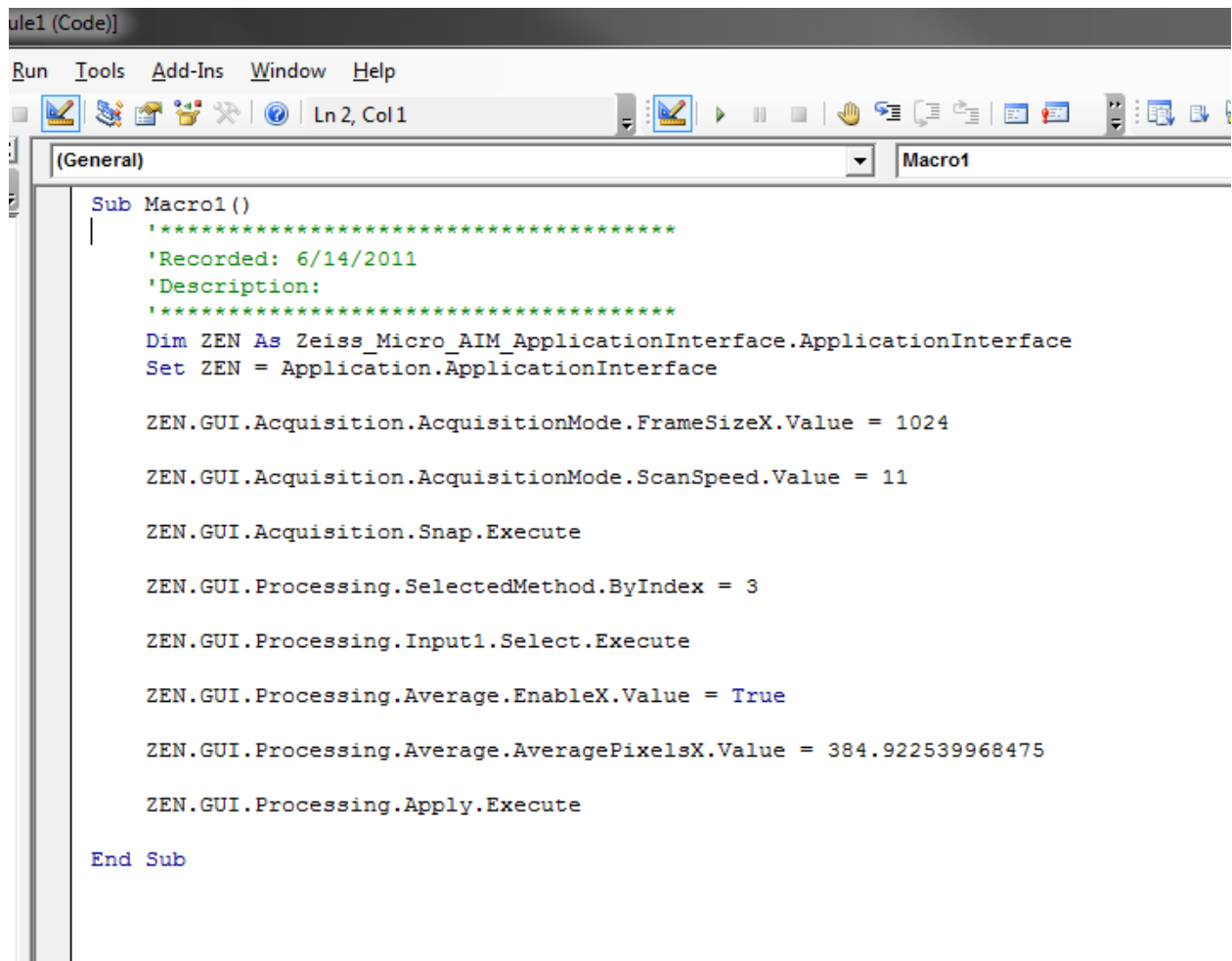
### 3.3 Recording a macro

When recording a macro, each click in the GUI is translated into a macro command in the editor. The image below shows an example in which the following steps are recorded:

- Set the frame size in X to 1024
- Set the scan speed to 11 (values are rounded)
- Take a Snap
- Select the processing function "Average"
- Select the currently active image as Input1
- Enable averaging in X-direction
- Set the averaging window in X-direction to a value of 385
- Start the processing function

The recorded macro can then be edited and executed just like a hand-written VBA program.





```
ule1 (Code)
Run Tools Add-Ins Window Help
Ln 2, Col 1
[General] Macro1
Sub Macro1 ()
    '*****
    'Recorded: 6/14/2011
    'Description:
    '*****
    Dim ZEN As Zeiss_Micro_AIM_ApplicationInterface.ApplicationInterface
    Set ZEN = Application.ApplicationInterface

    ZEN.GUI.Acquisition.AcquisitionMode.FrameSizeX.Value = 1024

    ZEN.GUI.Acquisition.AcquisitionMode.ScanSpeed.Value = 11

    ZEN.GUI.Acquisition.Snap.Execute

    ZEN.GUI.Processing.SelectedMethod.ByIndex = 3

    ZEN.GUI.Processing.Input1.Select.Execute

    ZEN.GUI.Processing.Average.EnableX.Value = True

    ZEN.GUI.Processing.Average.AveragePixelsX.Value = 384.922539968475

    ZEN.GUI.Processing.Apply.Execute

End Sub
```

Fig. 36 ZEN VBA-GUI Interface - Editor

### 3.4 Selecting and managing image documents

Image document handling in the VBA-GUI interface happens via the "GUI.Document" command. From the list of open documents, images can be selected by index and by name, they can be closed and their acquisition parameters can be re-used. Also, a DsRecordingDoc pointer to the image object can be obtained directly from here.

### 3.5 Technical note

The ZEN VBA-GUI interface is a new component for the 2011 release of ZEN (black edition only). It is not backwards compatible with earlier releases of ZEN, which means that macros using this interface cannot run on earlier ZEN systems. However, macros using the conventional ZEN VBA commands can still be written and run on ZEN 2011. Thus, as long as no command in your macro begins with "ZEN.GUI", it is compatible to earlier releases.

Additionally, the VBA commands for ZEN black edition (including the VBA-GUI interface) are not compatible to ZEN blue edition.

### 3.6 COM interface

For users with more advanced needs, the full range of ZEN VBA commands is also available via COM, by connecting a client program to a running ZEN system. There are two COM objects available, for the conventional VBA commands and for the VBA-GUI interface. The COM object for conventional ZEN VBA commands is called "Lsm5Vba.Application", and the one for the VBA-GUI interface is called "Zeiss.Micro.AIM.ApplicationInterface".

For example to connect a MATLAB session to ZEN, start MATLAB first, and then type

```
>> ZENVBA = actxGetRunningServer('Lsm5Vba.Application')
>>ZEN = actxGetRunningServer('Zeiss.Micro.AIM.ApplicationInterface.ApplicationInterface')
```

To start a scan, two commands are now available

```
>> ZENVBA.Lsm5.StartScan
>> ZEN.GUI.Acquisition.Snap.Execute
```

### 3.7 Glossary

- VBA: Visual Basic for Applications is an integrated programming environment that comes embedded into ZEN. It allows to automate routine operations by recording macros, and to write application programs that can use most features of the ZEN software.
- Macro: A program written in the VBA environment is usually called a macro.
- GUI: Graphical User Interface

# CHAPTER 5 TOOLS, ADDITIONAL SOFTWARE

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# 1 3D for LSM

## 1.1 Overview and Explanations

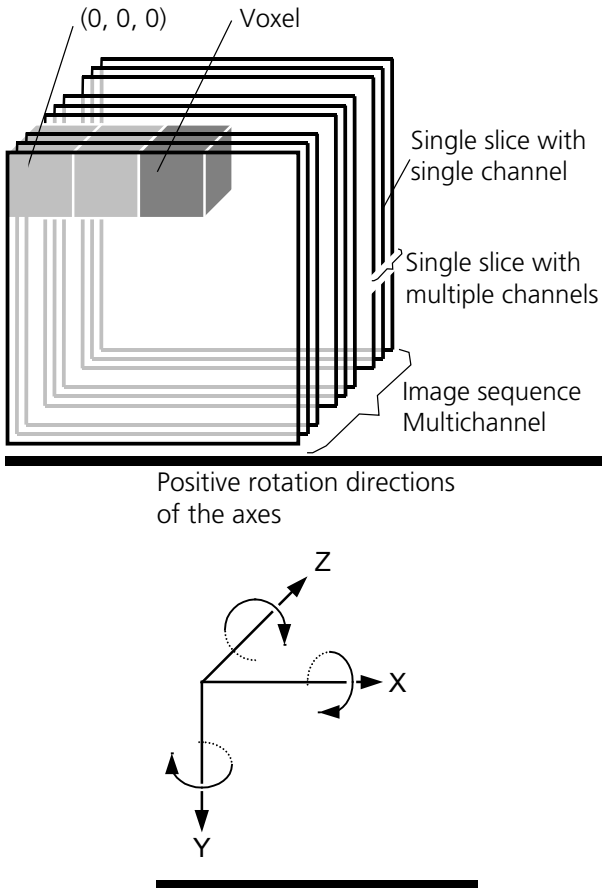


Fig. 1

- 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.

### 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.

## 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.

## 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.

## 1.2 User Interface

### 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**.

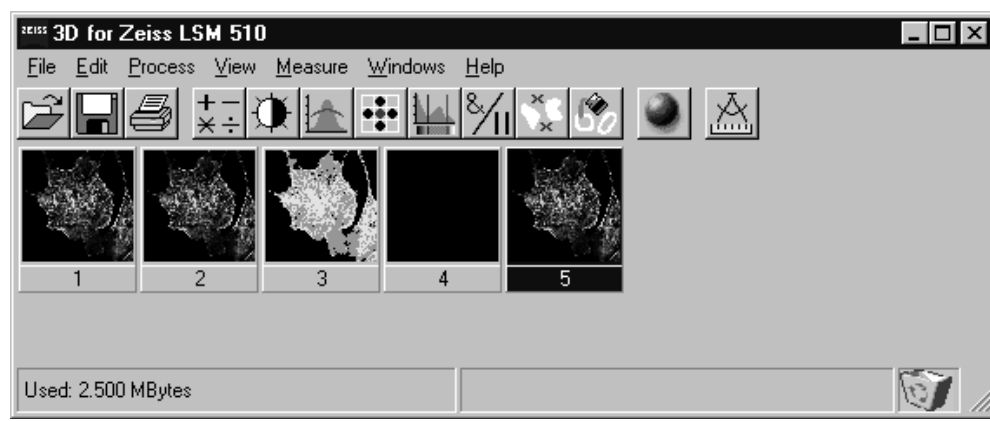
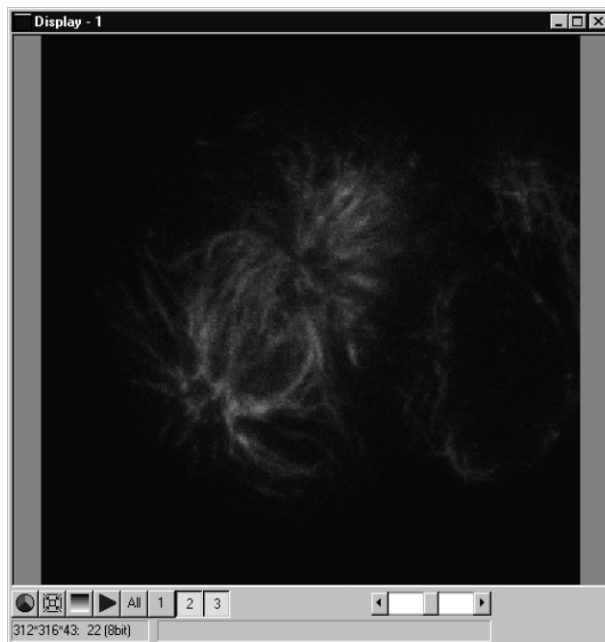


Fig. 2

**Tool bar**              This menu shows all image processing functions.

**Display window** This window is used to display image sequences.



**Fig. 3** Display window

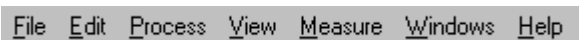
### Dialog boxes

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.

### 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.

### 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.

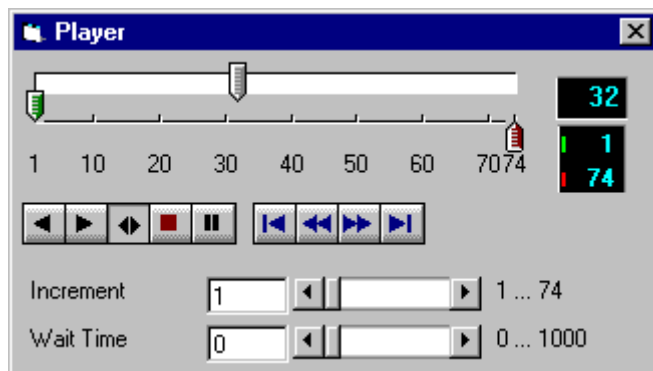


Fig. 4

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.

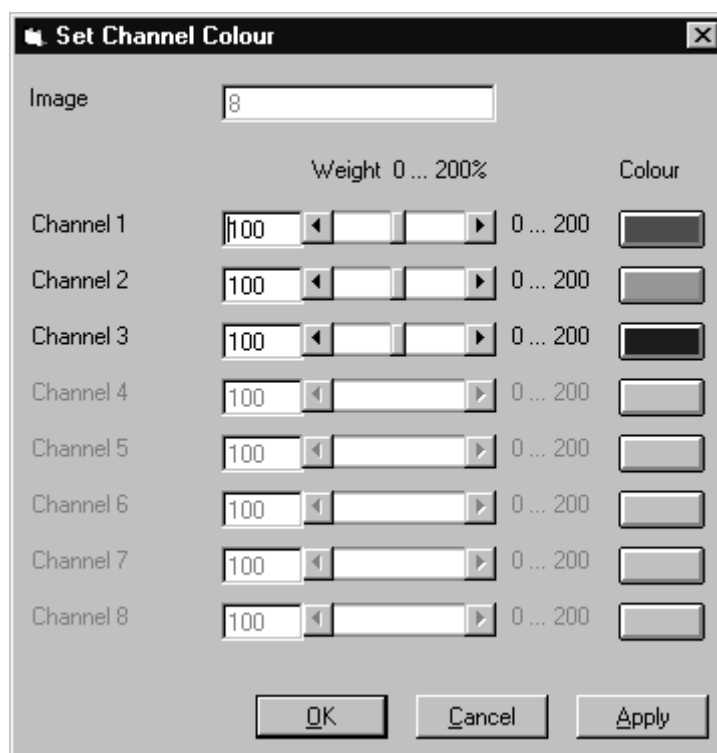


Fig. 5

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:

<b>Image</b>	Image sequence to edit
<b>Weight</b>	Colour weighting for each channel
<b>Colour</b>	Base colour for each channel

## 1.3 Functions

### 1.3.1 Functions in the File Menu

#### Open Image

This function reads a Zeiss LSM (\*.lsm), Zeiss LSM TIFF (\*.000.tif) or Carl Zeiss Vision (\*.img) image sequence from a disk or network drive.

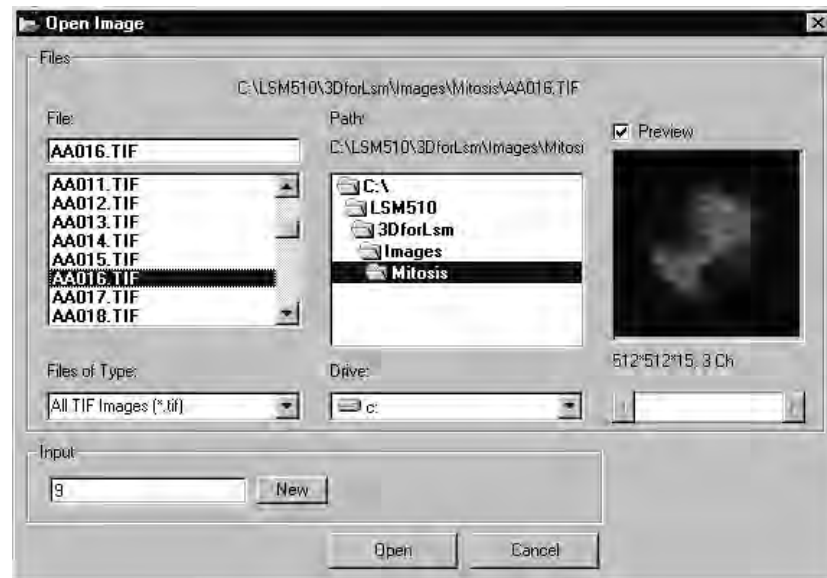


Fig. 6

The individual files of a Zeiss TIFF image sequence are read and saved as an image sequence in image memory. In addition, the image properties are read out of the TIFF files and allocated to the image sequence **Input**.

The directories of the current drive are listed in the **Directories** list box. Use the **Drives** list box to choose a different drive.

In case of choosing the TIFF-format in the **Files of Type** box, three number characters are always expected before the dot in the filename extension. The first number must be 000 at the end of the filename. From a complete sequence only this file is listed in the dialog, if "LSM TIF Images (\*.000.tif)" is selected in the **Files of Type** box. To view all TIFF files "All TIF Images (\*.tif)" in the **Files of Type** box must be selected. This selection enables to start with a different file than with the very first (named \*.000.tif) at the end of the filenames three number digits.

Currently the Carl Zeiss Vision file format "KE Images (\*.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.

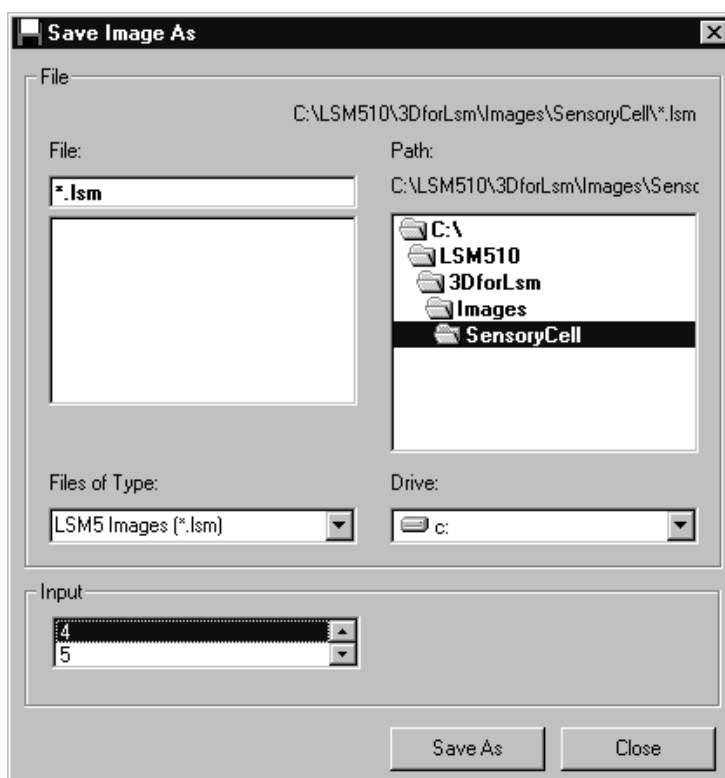
The window incorporates the usual file selection controls. The bottom half displays a selection of the image properties that are stored in the image sequence.

*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. 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. 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**

### 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:*

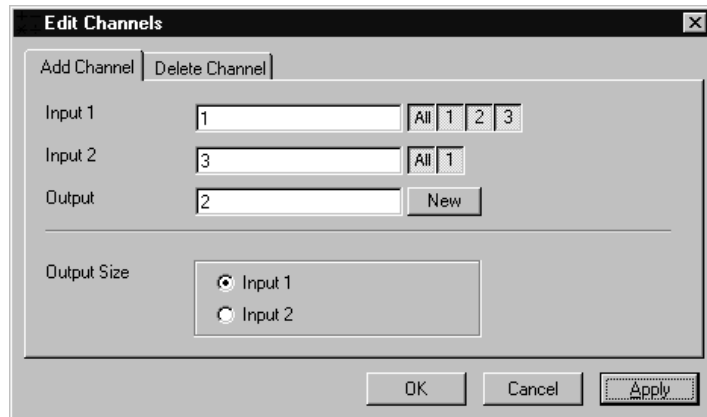
**None**



## 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. 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:

<b>Input 1</b>	First input image sequence
<b>Input 2</b>	Second input image sequence
<b>Output</b>	Output image sequence
<b>Output size</b>	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.

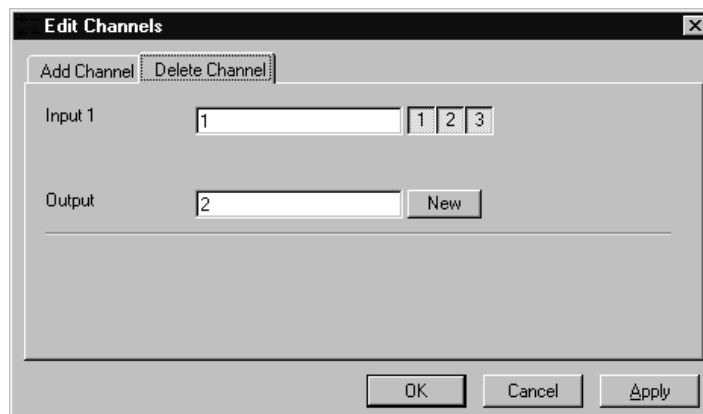


Fig. 10

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:*

<b>Input 1</b>	Input image sequence
<b>Output</b>	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**

### 1.3.3 Functions in the Process Menu

#### Arithmetics - Add

This function adds two image sequences.

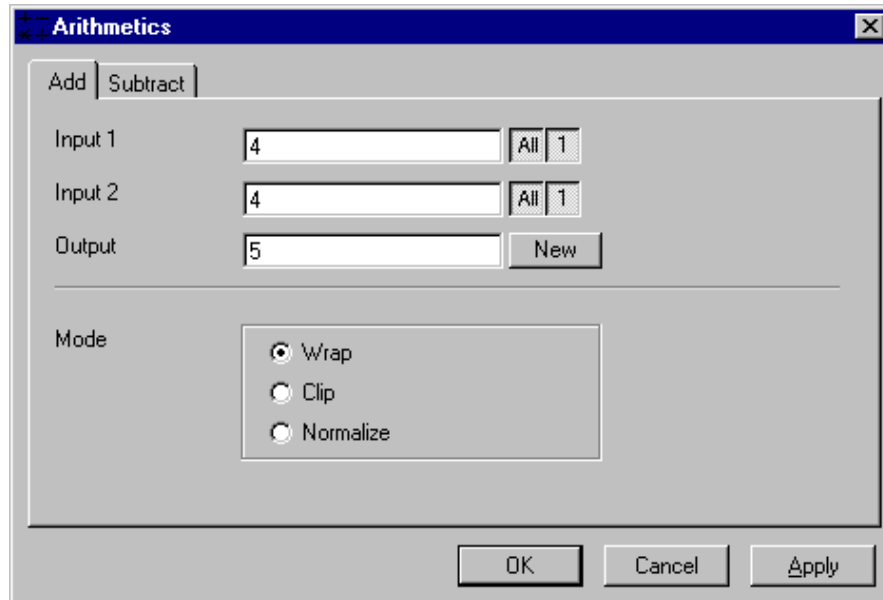


Fig. 11

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:

- |                |                                       |
|----------------|---------------------------------------|
| <b>Input 1</b> | First input image sequence            |
| <b>Input 2</b> | Second input image sequence           |
| <b>Output</b>  | Output image sequence                 |
| <b>Mode</b>    | 1 - Wrap<br>2 - Clip<br>3 - Normalize |

## Arithmetics - Subtract

This function subtracts two image sequences.

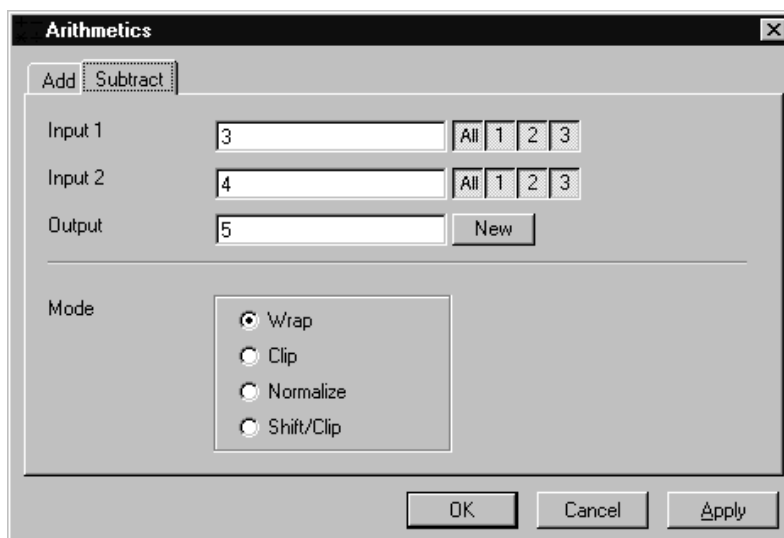


Fig. 12

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:

- |                |   |
|----------------|---|
| <b>Input 1</b> | First input image sequence                              |
| <b>Input 2</b> | Second input image sequence                             |
| <b>Output</b>  | Output image sequence                                   |
| <b>Mode</b>    | 1 - Wrap<br>2 - Clip<br>3 - Normalize<br>4 - Shift/Clip |

## Contrast - Interactive

This function allows interactive changes of the contrast of an image sequence.

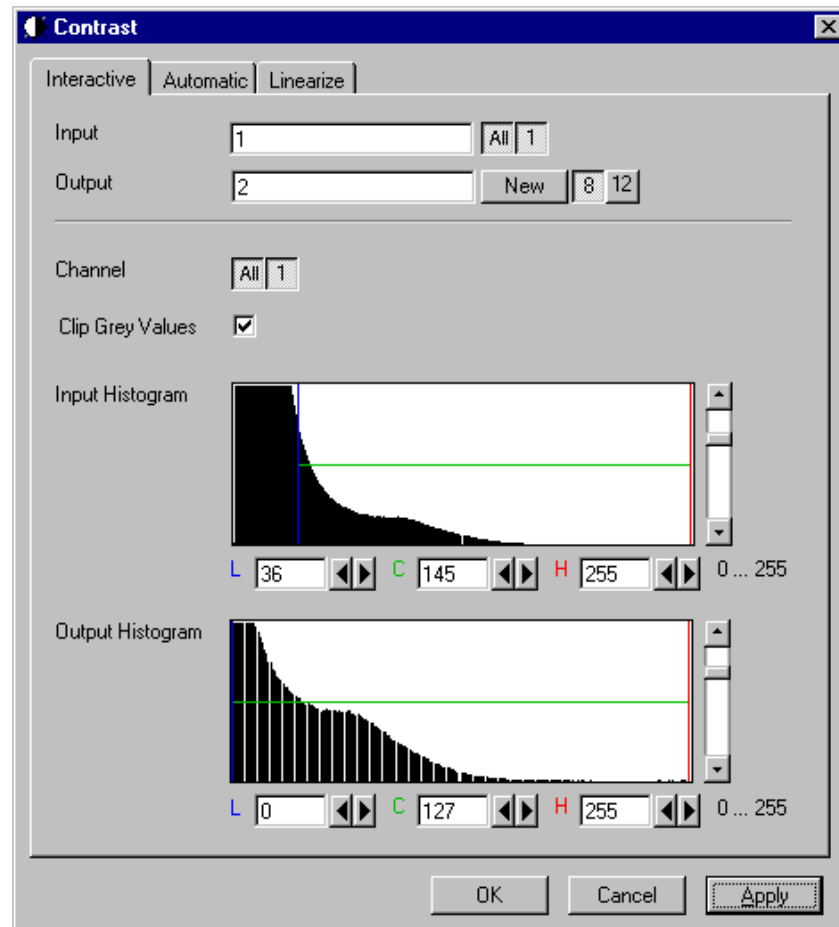


Fig. 13

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:*

<b>Input</b>	Input image sequence
<b>Output</b>	Output image sequence
<b>Channel</b>	Selection of the channel numbers for the <b>Output</b> image after contrast enhancement
<b>Clip Grey Values</b>	Clipping of grey values to the <b>Low (L)</b> and <b>High (H)</b> output grey values boundaries
<b>Input L</b>	Lower boundary of grey value range <b>Input</b>
<b>Input C</b>	Center of grey value range <b>Input</b>
<b>Input H</b>	Upper boundary of grey value range <b>Input</b>
<b>Output L</b>	Lower boundary of grey value range <b>Output</b>
<b>Output C</b>	Center of grey value range <b>Output</b>
<b>Output H</b>	Upper boundary of grey value range <b>Output</b>

## Contrast - Automatic

This function scales the grey values of an image sequence to the maximum possible range.

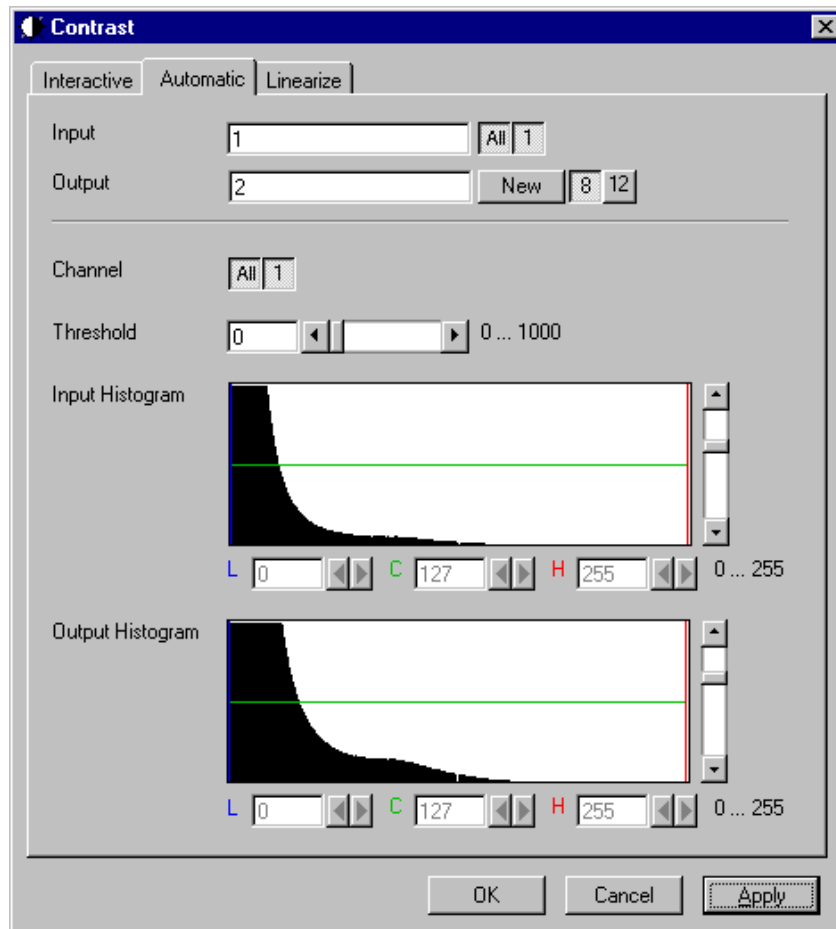


Fig. 14

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:*

<b>Input</b>	Input image sequence
<b>Output</b>	Output image sequence
<b>Threshold</b>	Exclusion value - 0...1000
<b>Input L</b>	Lower boundary of grey value range <b>Input</b>
<b>Input C</b>	Center of grey value range <b>Input</b>
<b>Input H</b>	Upper boundary of grey value range <b>Input</b>
<b>Output L</b>	Lower boundary of grey value range <b>Output</b>
<b>Output C</b>	Center of grey value range <b>Output</b>
<b>Output H</b>	Upper boundary of grey value range <b>Output</b>



## Contrast – Linearize

This function scales a range of grey values of an image sequence to equal area fractions in the histogram.

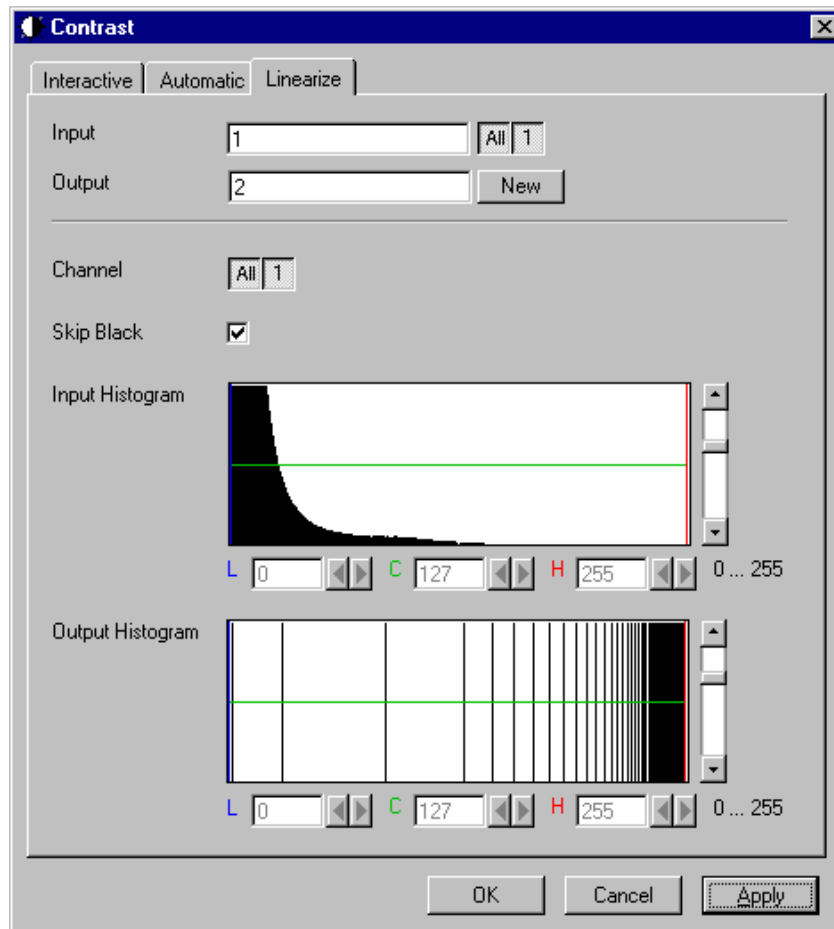


Fig. 15

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**.

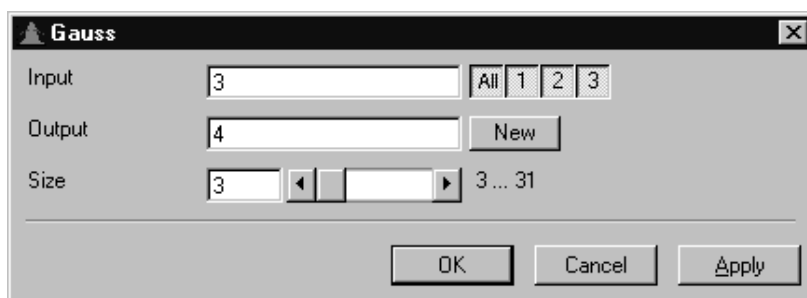
The **Output** histogram shows the resulting histogram. The horizontal axis represents the grey values from 0 to 255. The vertical axis represents the pixel count. The vertical scale of the histogram is set using the scroll bar. The units are percentages of the grey value distribution maximum. This setting has no influence to the function.

*Parameters:*

<b>Image</b>	Input image sequence
<b>Output</b>	Output image sequence
<b>SkipBlack</b>	0 - Grey value black is ignored 1 - Grey value black is taken into account
<b>Input L</b>	Lower boundary of grey value range Input
<b>Input C</b>	Center of grey value range Input
<b>Input H</b>	Upper boundary of grey value range Input
<b>Output L</b>	Lower boundary of grey value range Output
<b>Output C</b>	Center of grey value range Output
<b>Output H</b>	Upper boundary of grey value range Output

**Smooth (Gauss)**

This function performs a Gauss filter.



**Fig. 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:*

<b>Input</b>	Input image sequence
<b>Output</b>	Output image sequence
<b>Size</b>	Filter size (3...31, only odd numbers)

## Morphology

The following four functions perform basic operations of mathematical morphology on image sequences.

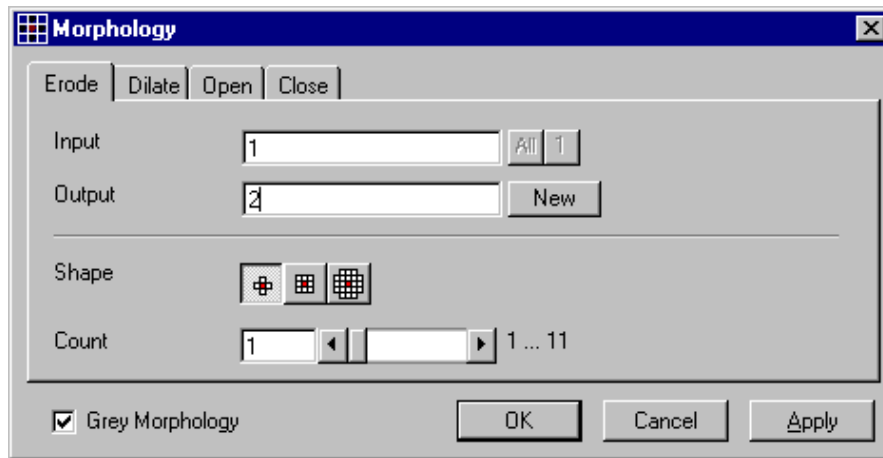


Fig. 17

As generalization of the morphology of two-dimensional images to three dimensions the structural elements are small volumina.

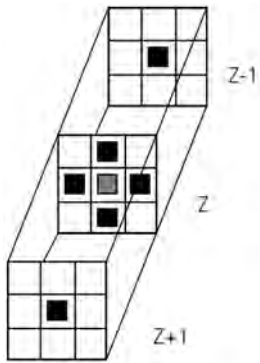
### Literature

- 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

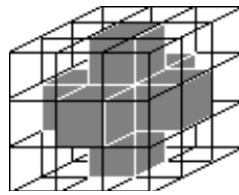
The input image sequence is analyzed voxel by voxel with a selected shape (**Shape**). The voxel to be analyzed is always the central voxel of the shape. The shape type determines which neighboring voxels are used to compute the resulting voxel.

The following structural elements are available for all morphological operations. They represent approximated spheres with an increasing radius.

Sequential image:

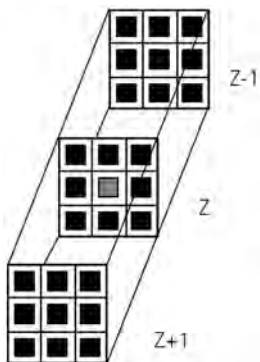


Volume view:

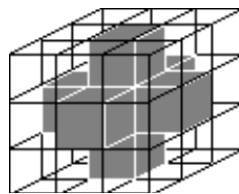


Cross shape

Sequential image:

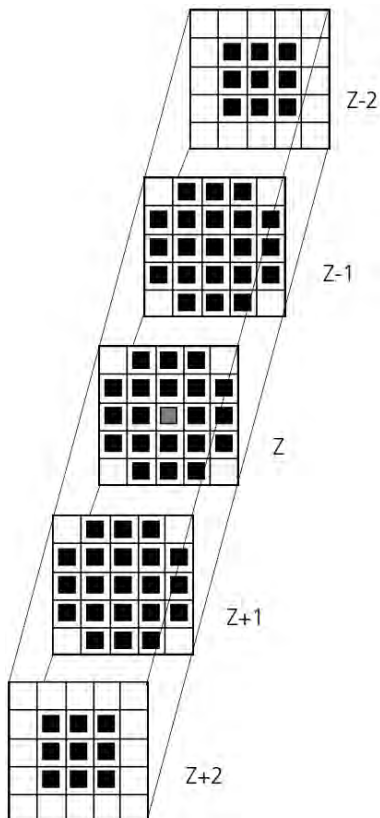


Volume view:

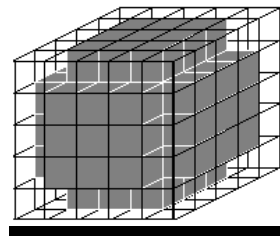


Cross shape

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** tickbox is activated, erosion sets the grey value of the central voxel to the minimum of all neighboring voxels affected by the structural element; dilation sets the grey value of the central voxel to the maximum.

If the **Grey Morphology** tickbox is not activated, the neighboring voxels are only distinguished by grey value 0 and non-0. For erosion the central voxel is set to 0 if any of the neighbors is 0. It is set to 255 (4095) if any neighbor is not 0. For dilation the central voxel is set to 255 (4095) if any of the neighbors is not 0. It is set to 0 if all neighbors are 0.

Erosion reduces the size of bright regions, separates thin connections between them, and makes small regions disappear. Dilation, on the other hand, makes bright regions of the image grow in size, fills gaps, and smoothes small contour details.

The result of erosion and dilation is called opening. On the one hand, this maintains to some extent the original size of the regions while not losing the smoothing effect of erosion on the image. This name stands for the operation of reducing convex bulges in the contour of the region. Thin connections between regions are eliminated, broken borders between regions are connected, and small regions disappear.

The opposite operation (first dilation, then erosion) is called closing. Concave bulges in the contours of regions are filled in; connections are formed between adjacent regions.

The following example illustrates the operations "Open" and "Close" in two dimensions:

Open = Erosion + Dilation

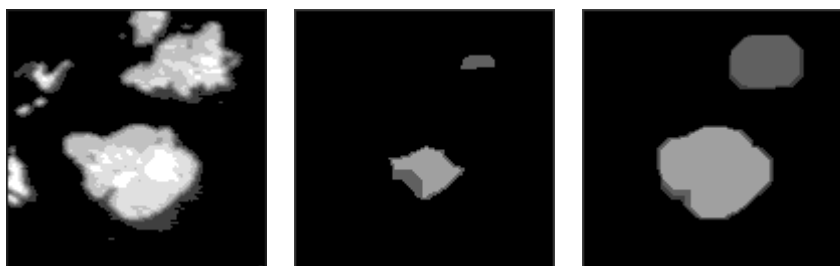


Fig. 18

Close = Dilation + Erosion

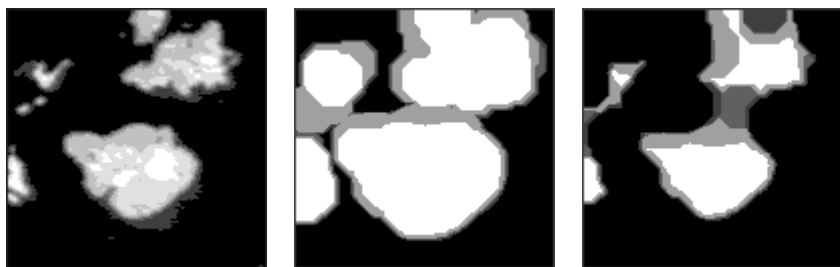


Fig. 19

The "cube cross" shape was used for the operations shown.

## Morphology - Erode

This function erodes structures in an image sequence.

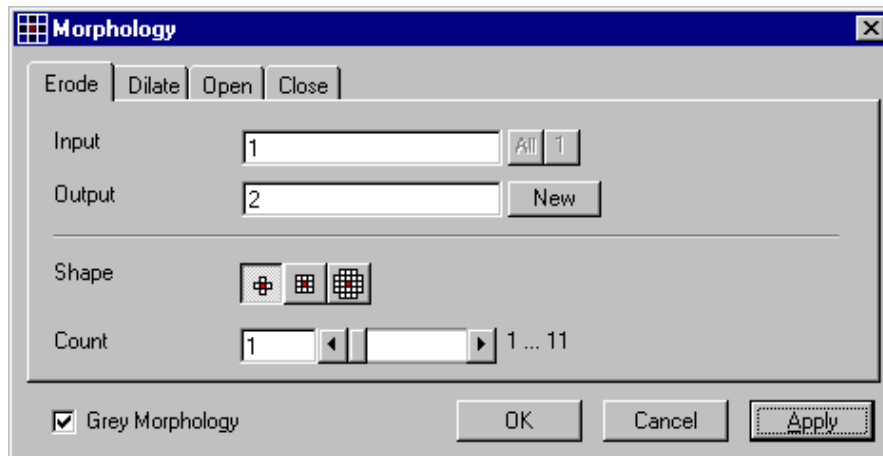


Fig. 20

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:

<b>Input</b>	Input image sequence
<b>Output</b>	Resulting image sequence
<b>Shape</b>	Shape used 1 - cross 2 - cube 3 - cube cross
<b>Count</b>	Number of recursive operations
<b>Grey Morphology</b>	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.

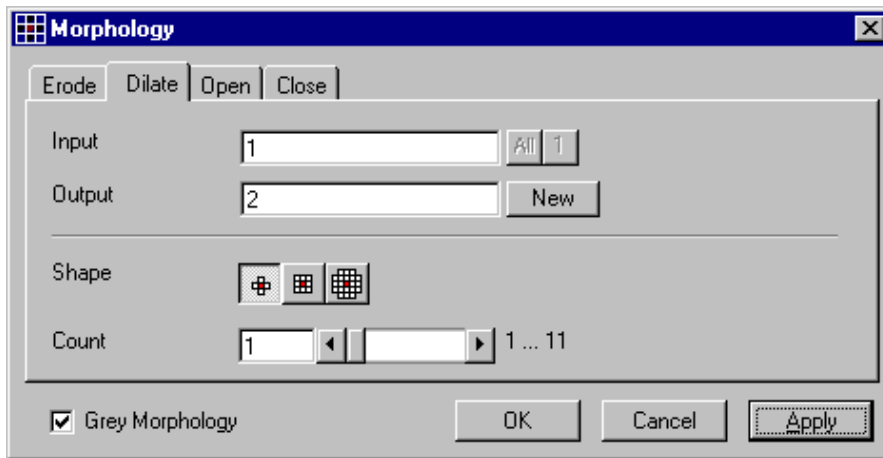


Fig. 21

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:

<b>Input</b>	Input image sequence
<b>Output</b>	Resulting image sequence
<b>Shape</b>	Shape used 1 - cross 2 - cube 3 - cube cross
<b>Count</b>	Number of recursive operations
<b>Grey Morphology</b>	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.

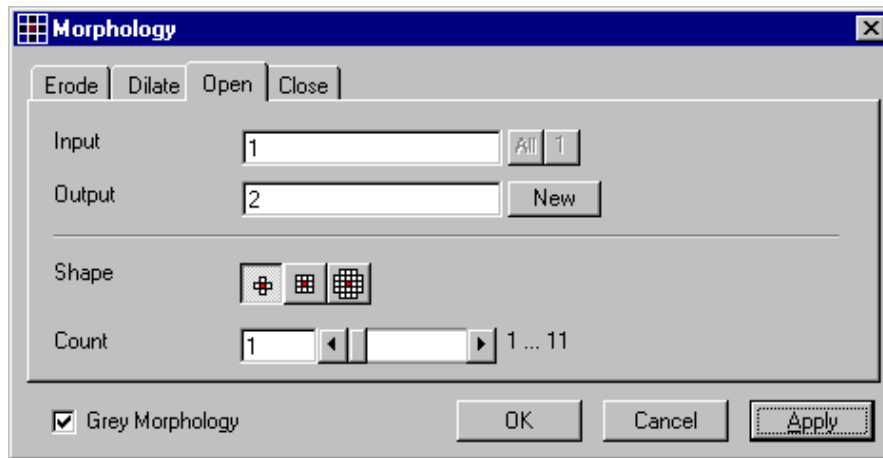


Fig. 22

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:

<b>Input</b>	Input image sequence
<b>Output</b>	Resulting image sequence
<b>Shape</b>	Shape used 1 - cross 2 - cube 3 - cube cross
<b>Count</b>	Number of recursive operations
<b>Grey Morphology</b>	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.

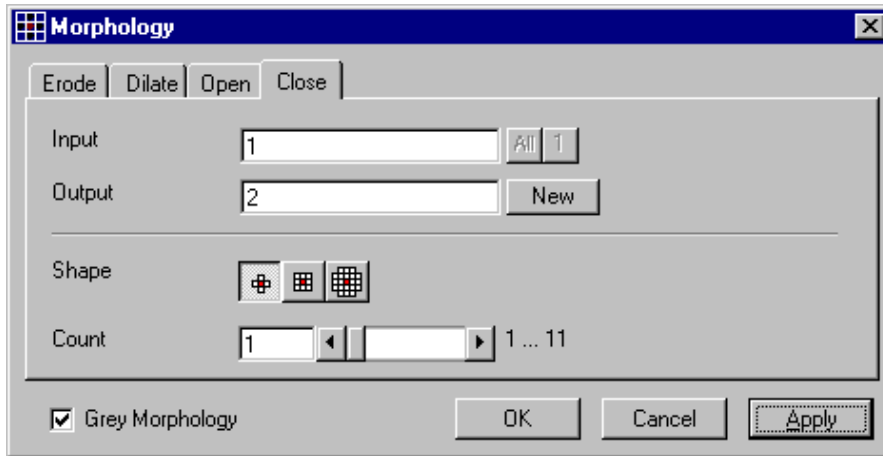


Fig. 23

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:

<b>Input</b>	Input image sequence
<b>Output</b>	Resulting image sequence
<b>Shape</b>	Shape used 1 - cross 2 - cube 3 - cube cross
<b>Count</b>	Number of recursive operations
<b>Grey Morphology</b>	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.

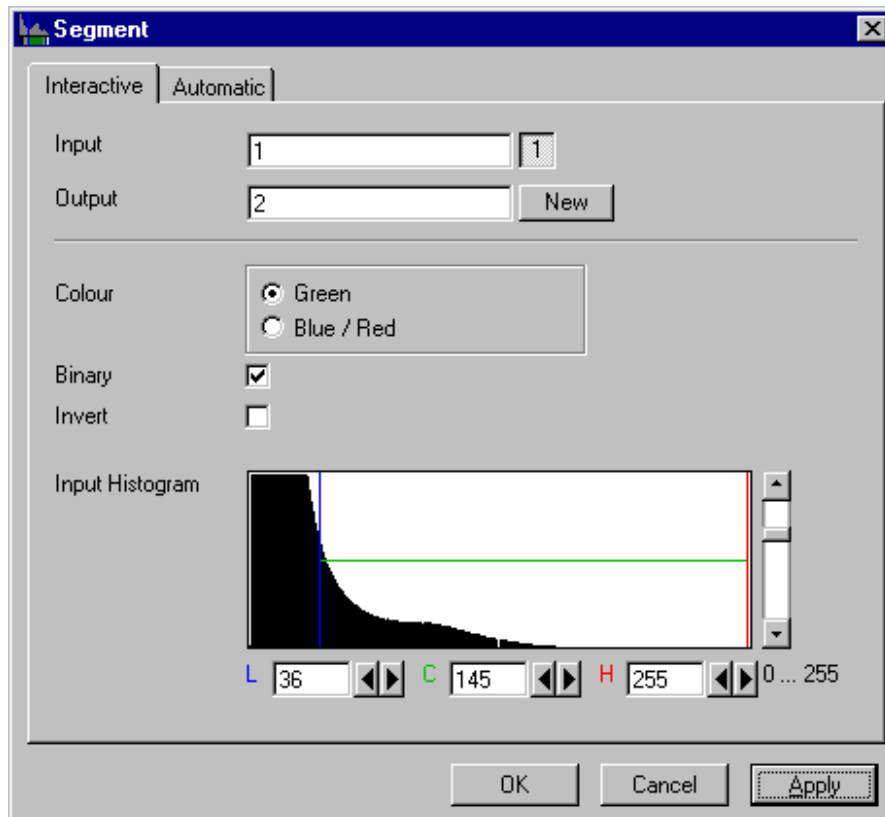


Fig. 24

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.

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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:*

<b>Input</b>	Input image sequence
<b>Output</b>	Resulting image sequence
<b>Colour</b>	Green - Selected interval is displayed in green
<b>Blue/Red</b>	Grey values below the selected interval are displayed in blue, grey values above in red
<b>Binary</b>	0 - Selected voxels retain the original grey value 1 - Selected voxels are set to grey value 255, the rest to grey value 0
<b>Invert</b>	0 - Grey values inside the selected interval are segmented 1 - Grey values outside the selected interval are segmented
<b>L</b>	Low grey value threshold
<b>C</b>	Center of threshold interval
<b>H</b>	High grey value threshold

## Segment - Automatic

The function carries out an automatic grey value segmentation by means of thresholding.

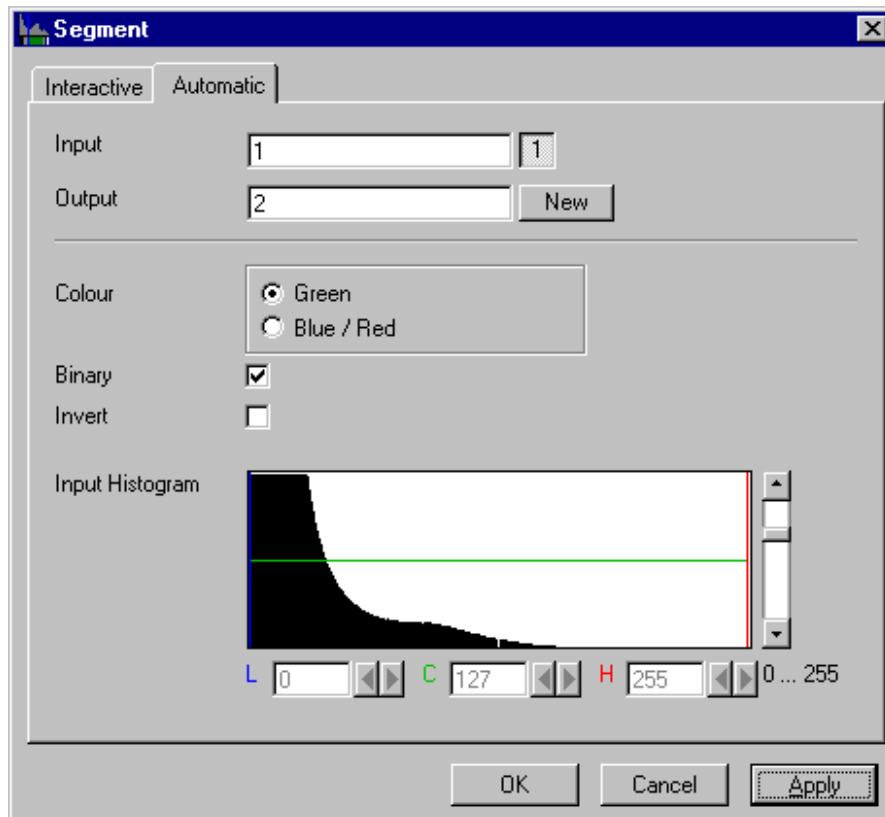


Fig. 25

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:*

<b>Input</b>	Input image sequence
<b>Output</b>	Resulting image sequence
<b>Colour</b>	Green - Selected interval is displayed in green Blue/Red - Grey values below the selected interval are displayed in blue, grey values above in red
<b>Binary</b>	0 - Selected voxels retain the original grey value 1 - Selected voxels are set to grey value 255 (4095), the rest to grey value 0
<b>Invert</b>	0 - Grey values inside the selected interval are segmented 1 - Grey values outside the selected interval are segmented
<b>L</b>	Low grey value threshold
<b>C</b>	Center of threshold interval
<b>H</b>	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**.

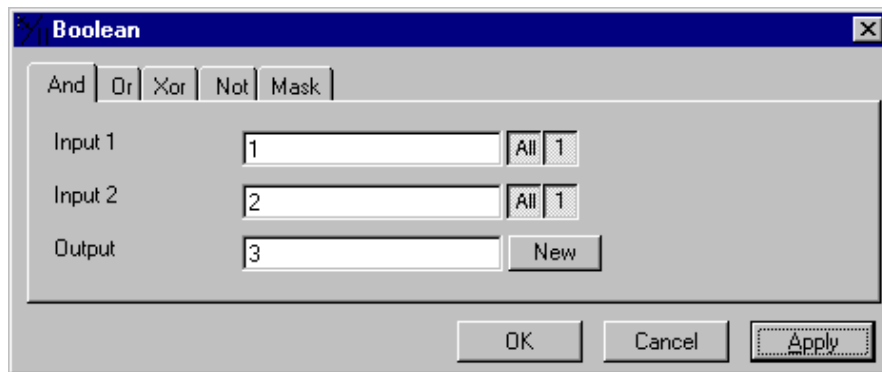


Fig. 26

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:*

<b>Input 1</b>	First input image sequence
<b>Input 2</b>	Second input image sequence
<b>Output</b>	Resulting image sequence

## Boolean - Or

This function carries out a bit-by-bit **Or** calculation for the images **Input 1** and **Input 2**.

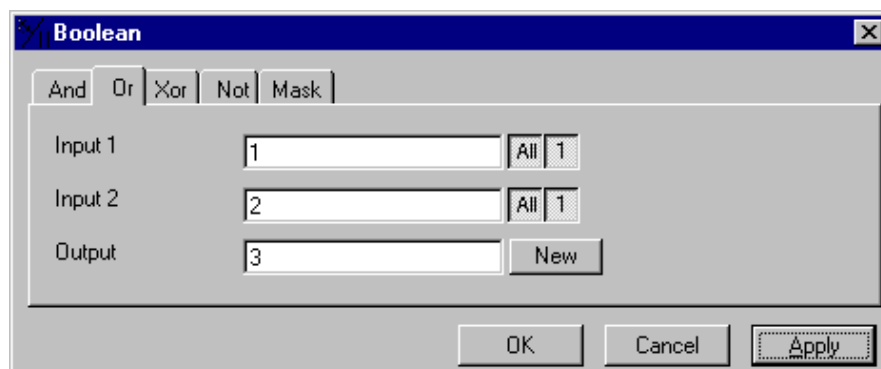


Fig. 27

The **Or** tab sheet of the **Boolean** dialog window must be selected.

This function can be used to combine binary masks or regions.

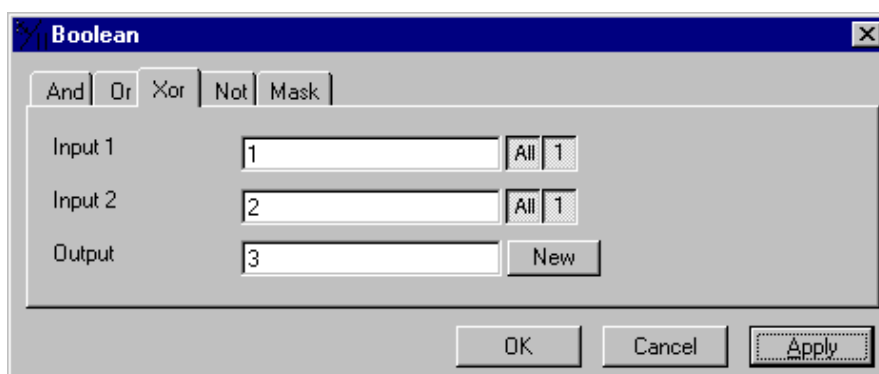
If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

*Parameters:*

<b>Input 1</b>	First input image sequence
<b>Input 2</b>	Second input image sequence
<b>Output</b>	Resulting image sequence

### Boolean - Xor

This function carries out a bit-by-bit **Xor** calculation for the images **Input 1** and **Input 2**.



**Fig. 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:*

<b>Input 1</b>	First input image sequence
<b>Input 2</b>	Second input image sequence
<b>Output</b>	Resulting image sequence



## Boolean - Not

This function carries out a bit-by-bit negation of an image.

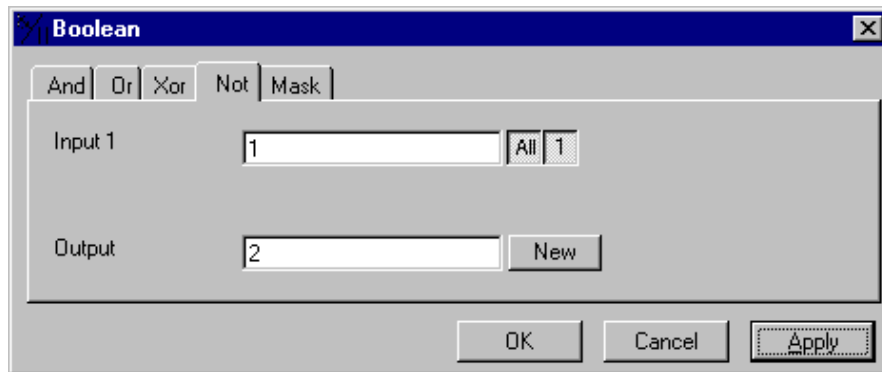


Fig. 29

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:*

<b>Input</b>	Input image sequence
<b>Output</b>	Resulting image sequence

## Boolean - Mask

This function masks a grey value image sequence.

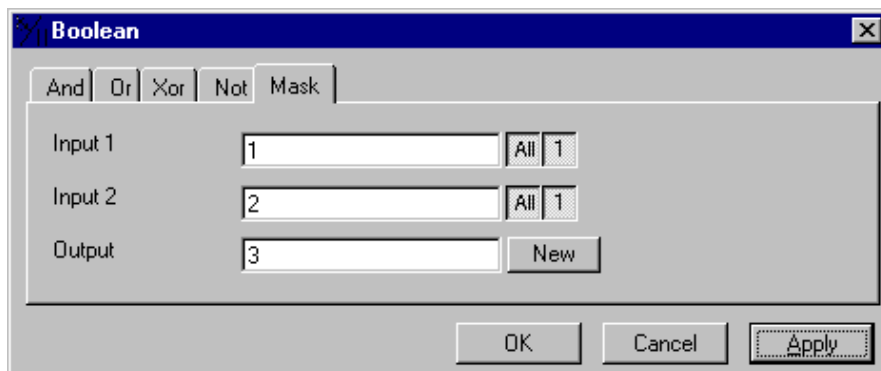


Fig. 30

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:

<b>Input 1</b>	First input image sequence
<b>Input 2</b>	Second input image sequence
<b>Output</b>	Resulting image sequence

## Scrap

This function deletes or selects objects in a specified size range.

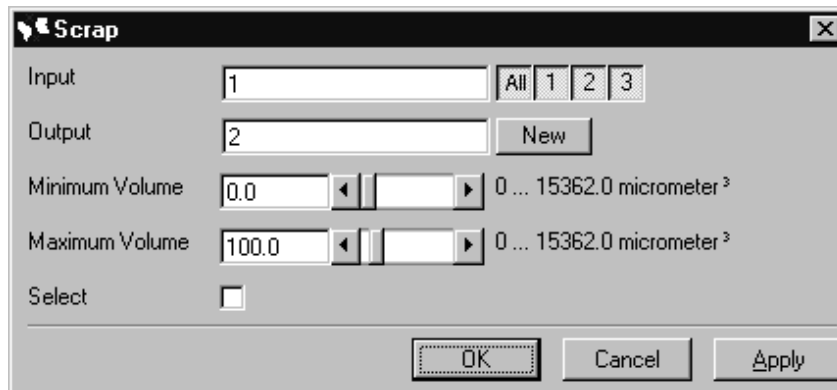


Fig. 31

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:

<b>Input</b>	Input image sequence
<b>Output</b>	Output image sequence
<b>MinVolume</b>	Minimum object size
<b>MaxVolume</b>	Maximum object size
<b>Select</b>	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. 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:

<b>Input</b>	Input image sequence
<b>Output</b>	Output image sequence

### 1.3.4 Functions in the View Menu

#### Render - Surface

This function displays an image sequence according to the **gradient shading method**.

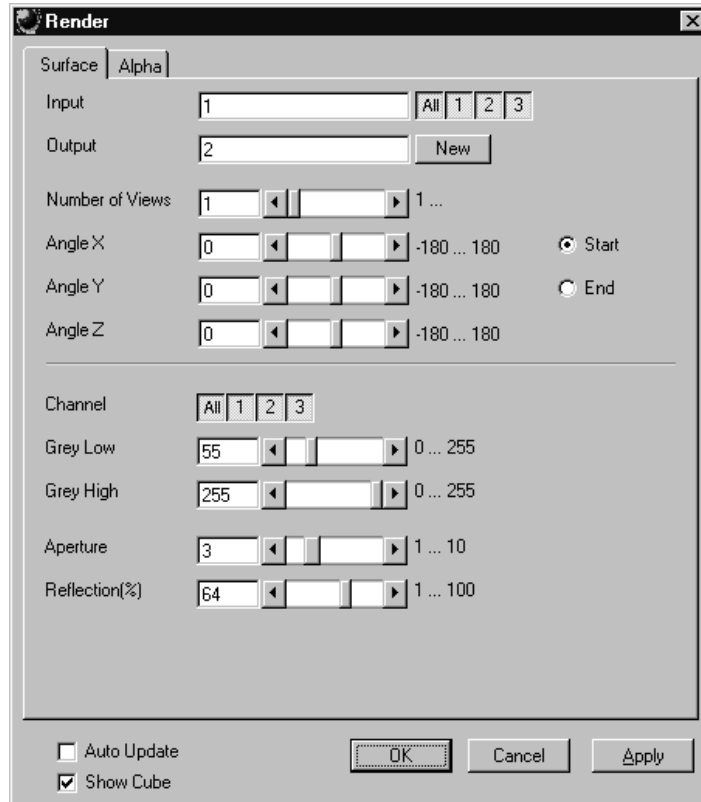


Fig. 33

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:*

<b>Input</b>	Input image sequence
<b>Output</b>	Resulting image sequence
<b>Number of Views</b>	Number of reconstructions to be calculated
<b>Angle X</b>	Angle of rotation on the X-axis, start position
<b>Angle Y</b>	Angle of rotation on the Y-axis, start position
<b>Angle Z</b>	Angle of rotation on the Z-axis, start position
<b>Channel</b>	All - The following parameters are valid for all channels X - The following parameters are valid for the selected channel only
<b>Grey Low</b>	Low grey value threshold of the region to be displayed

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<b>Grey High</b>	High grey value threshold of the region to be displayed
<b>Aperture</b>	Measure of the extent of the highlights
<b>Reflection</b>	Weight of the diffuse brightness components in comparison to the highlights
<b>Auto Update</b>	0 - Function execution is performed on <b>OK</b> or <b>Apply</b> 1 - Function execution for the current angle is performed on any parameter change
<b>Show Cube</b>	0 - The wire frame cube is not shown 1 - The wire frame cube is shown in the <b>Display window</b>

### 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**.

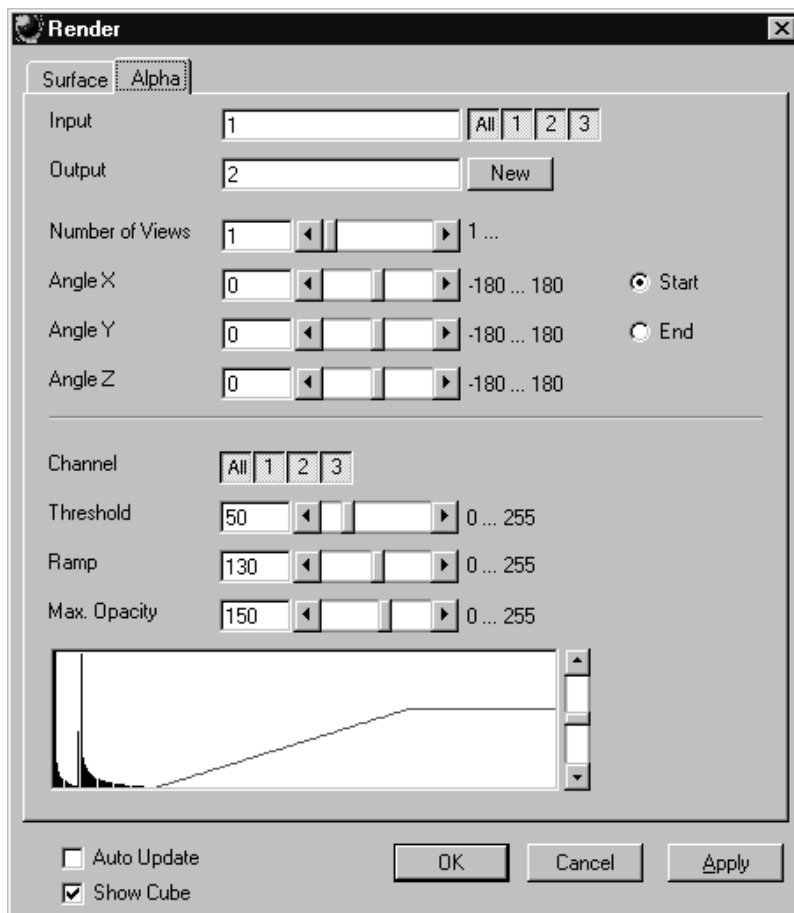


Fig. 34

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.



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## 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:*

<b>Input</b>	Input image sequence
<b>Output</b>	Resulting image sequence
<b>Number of Views</b>	Number of reconstructions to be calculated
<b>Angle X</b>	Angle of rotation on the X-axis, start position
<b>Angle Y</b>	Angle of rotation on the Y-axis, start position
<b>Angle Z</b>	Angle of rotation on the Z-axis, start position
<b>Channel</b>	All - The following parameters are valid for all channels X - The following parameters are valid for the selected channel only
<b>Threshold</b>	Grey value where the opacity starts rising
<b>Ramp</b>	Length of the opacity slope
<b>Max. Opacity</b>	Maximum opacity value
<b>Opacity Table</b>	Maximum opacity value
<b>Auto Update</b>	0 - Function execution is performed on OK or Apply 1 - Function execution is performed on any parameter change
<b>Show Cube</b>	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.

### 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:

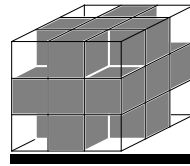
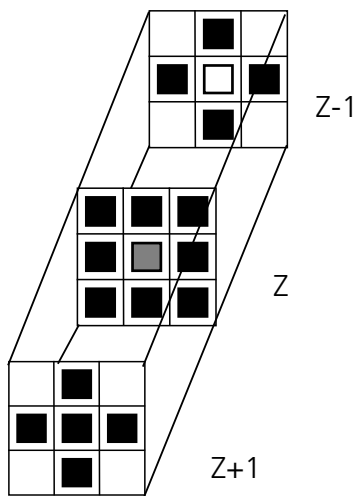


Fig. 35

## Measurement Process

The measurement process consists of three steps: region definition, checking of the validity of the regions, and feature calculation.

Region definition:

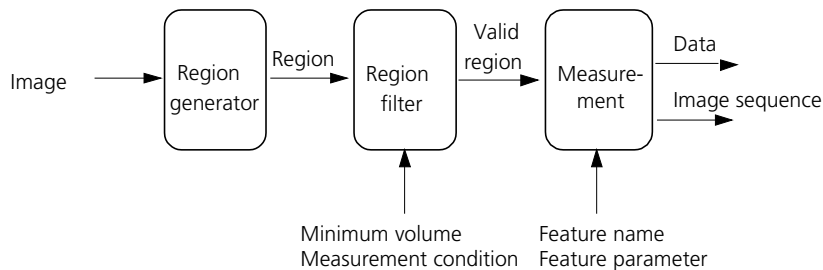
- Automatically from the mask image

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. 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.

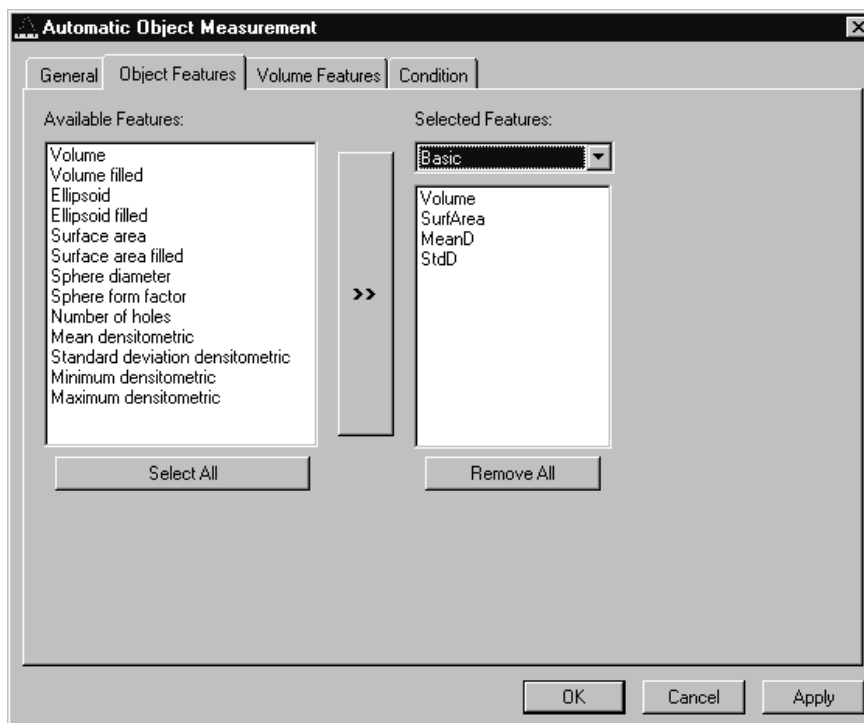


Fig. 37

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:*

<b>Available Features</b>	List of available object features
<b>Selected Features</b>	List of selected object features
<b>Select All</b>	Select all available object features for measurement
<b>Remove All</b>	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.

<b>Group Name</b>	<b>Name</b>	<b>Description</b>
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.

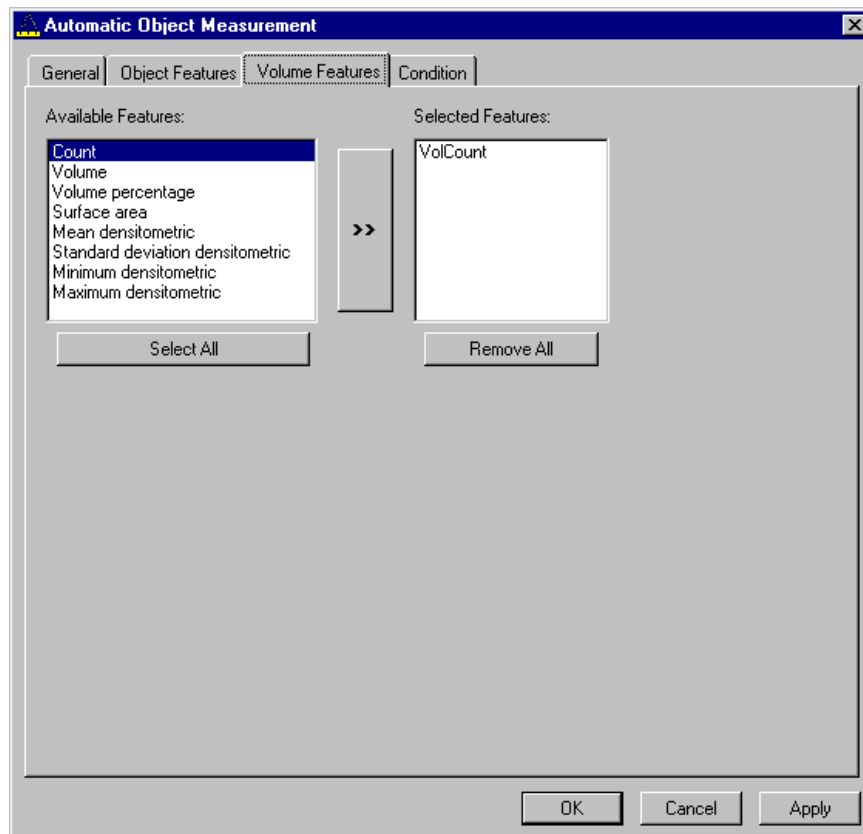


Fig. 38

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:*

<b>Available Features</b>	List of available object features
<b>Selected Features</b>	List of selected object features
<b>Select All</b>	Select all available object features for measurement
<b>Remove All</b>	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.

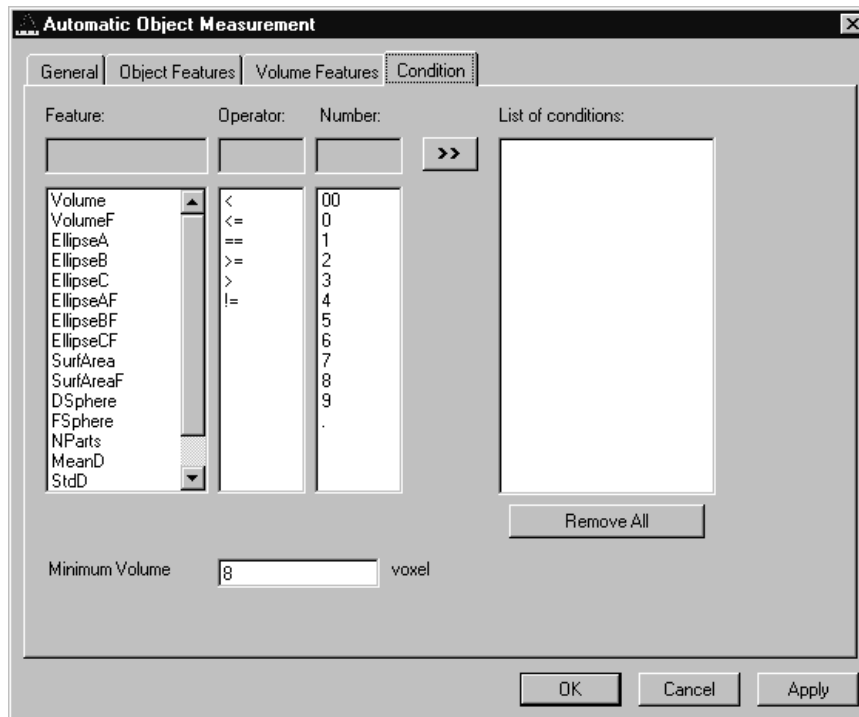


Fig. 39

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:*

<b>Feature</b>	List of available object features
<b>Operator</b>	List of available condition operators
<b>Number</b>	List of numbers to compose the value
<b>List of conditions</b>	Defined condition list
<b>Remove All</b>	Remove all entries from the <b>List of conditions</b>
<b>Minimum Volume</b>	Minimum object volume in voxel

## Automatic Object Measurement - General

This function carries out an automatic measurement and labeling.

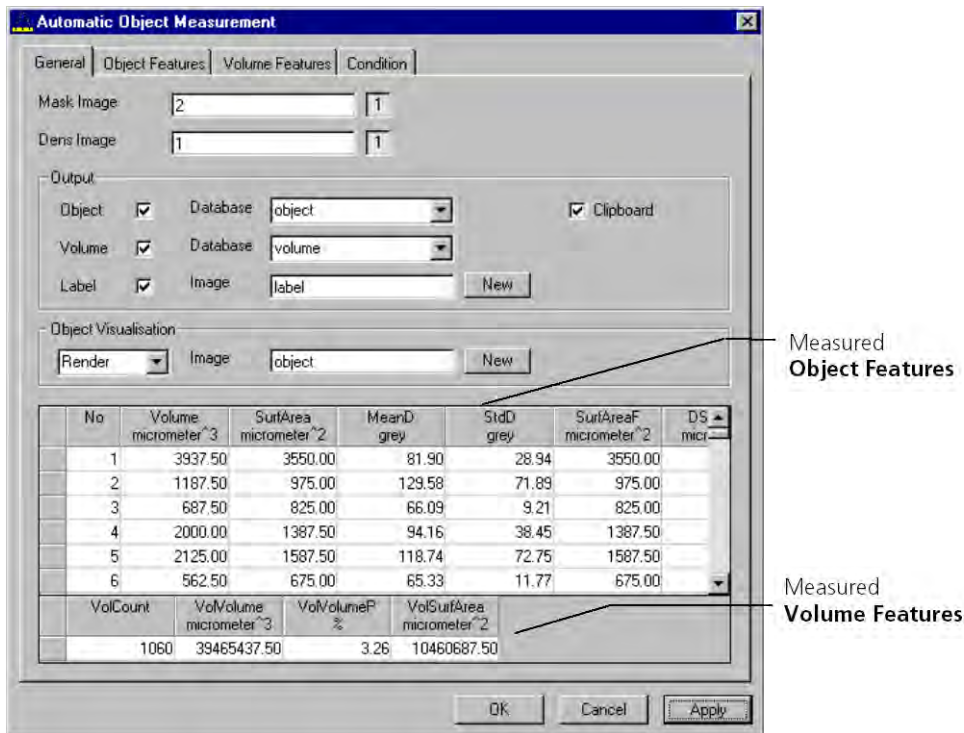


Fig. 40

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:*

<b>Mask Image</b>	Single channel mask image sequence that defines the objects
<b>Dens Image</b>	Image sequence for densitometric measurement and property source
<b>Object</b>	Stores measurement values of objects, including database filename
<b>Volume</b>	Stores volume measurement values of objects, including database filename
<b>Label</b>	Generates an image sequence with all objects labelled in different pseudo colours
<b>Clipboard</b>	Measurement values are automatically written to the clipboard

## 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).

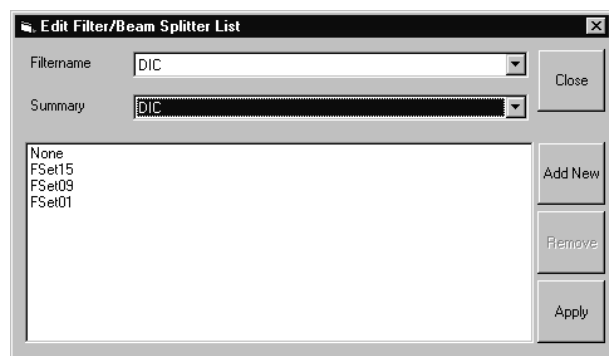


Fig. 41 Edit Filter/Beam Splitter List window

### 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 **Filtername** list box to open it.
- Select the filter set which shall be included in the list.
- Click on the **Apply** button.

The selected filter set is included and displayed in the list (below the **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. 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.

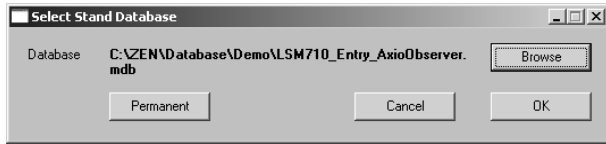


Fig. 43 Select Stand Database window

- 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.

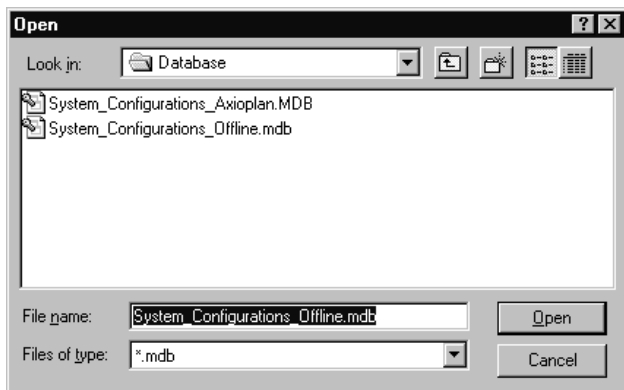


Fig. 44 Open window

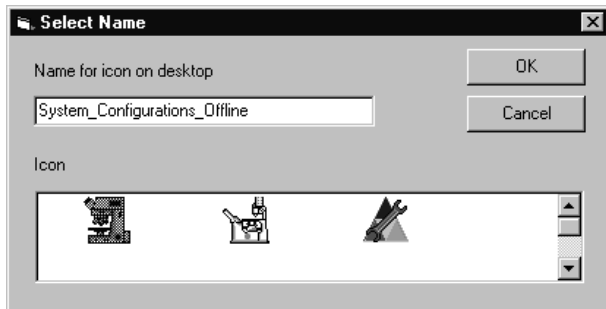


Fig. 45 Select Name window

### 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:

- Click on the **Browse** button to activate the new database.
  - The **Open** window appears on the screen.
- Select the directory where the new database is stored.
- Click on the name of the database (file extension: **\*.mdb**) and then on the **Open** button.
  - The **Open** window is closed and the name of the new database appears in the **Database** box.
- Click on the **Permanent** button. The **Select Name** window appears.
- Select the relevant stand icon from the **Icon** list box and click on **OK**. The **Select Name** window is closed and the desktop icon is updated.
- Then click on the **OK** button in the **Select Stand Database** window to accept the new settings and to close the window. (Clicking on **Cancel** will cancel the procedure.)
  - After the next restart of the ZEN software, the new database will be automatically loaded.



## 4 Maintenance Tool

With a calibration objective and correct system configuration, the maintenance tool allows convenient self adjustment of the LSM 710 and LSM 780 systems. The optical beampath, relative pinhole position and scanner adjustment can be set and checked automatically on the LSM 710 and LSM 780 systems.

### 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 and at the TFT display.
- Calibration objectives with other samples than shown in Fig. 46 shall not be used. The cap with the calibration sample can be obtained separately under SAP-No. 420639-9900-700.

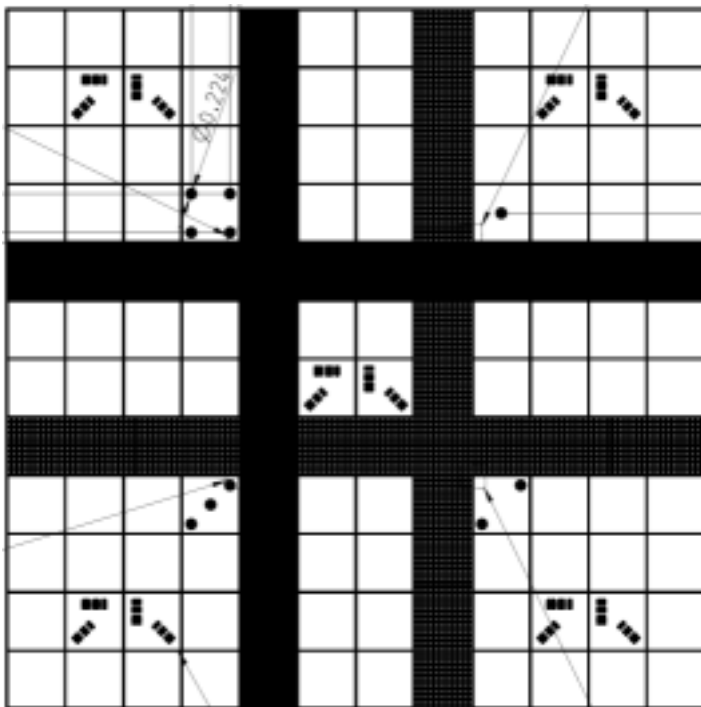


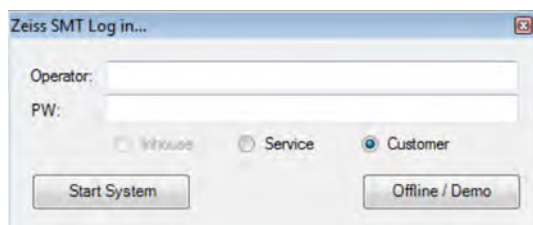
Fig. 46 Calibration objective

## 4.2 Starting the Maintenance Tool

Before starting the maintenance tool, the system hardware must be fully activated.

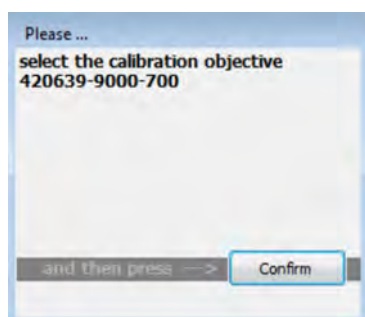
With ZEN 2011, check the following settings:

- 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
- If all these settings are correct, close ZEN 2011 and open the Maintenance tool.
- After start, choose "Log in... Customer" (type-in any name, password is = LSMUserAdmin).



## 4.3 General Operation of the Maintenance Tool


The Maintenance tool can only start, if the calibration objective is entered as "APO Calibration LSM". If this is not the case, the calibration objective has to be selected first.



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 green is indicated, the task has been fully accepted.
- 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.

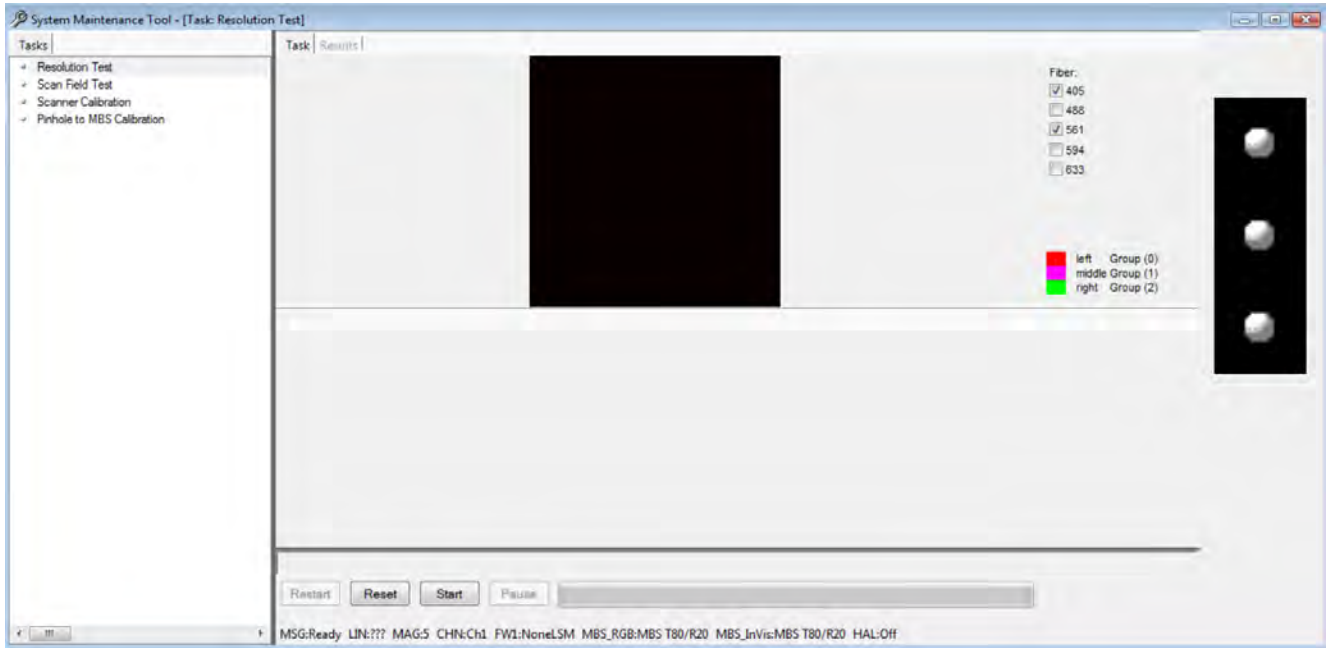
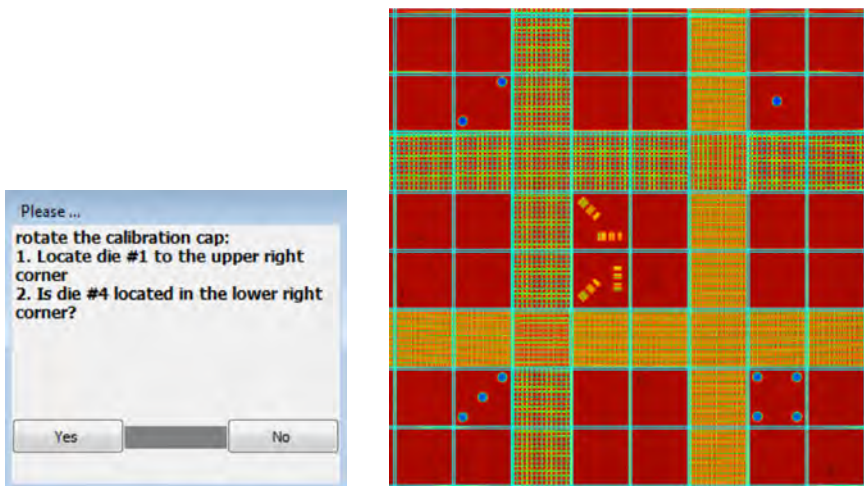


Fig. 47 Maintenance Tool main window

### Resolution test

=> Test tool: calibration objective

Rotate the calibration cap of the objective until #1 is located in the upper right corner and #4 located in the lower right corner. Then confirm this position.



Check the resolution of the fine structures on the calibration tool. If the resolution test is not successful, this points to a wrong position of the collimator and / or the pinhole.

**Scan field test:**

=> Test tool: calibration objective

Rotate the calibration cap of the objective until lines are arranged horizontally and confirm this position.

The scan field test checks the scanner linearity and runs much faster than the scanner calibration.

**Scanner calibration**

=> Test tool: calibration objective

Rotate the calibration cap of the objective until lines are arranged horizontally and confirm this position.

- The scanner calibration requires a properly focused optics (see section 4.2).



There is no longer a difference between electrical and optical scanner calibration (as it was with earlier LSM systems).

**Pinhole to MBS calibration:**

Allows to automatically adjust the optimal beampath between the MBS, the scanning mirrors and the master pinhole on all LSM 710 and LSM 780 systems. On LSM 710 and LSM 780 systems with additional 405, 440 or IR (NLO) lasers, make sure the respective lasers are checked.

=> Test tool: calibration objective

- KÖHLER illumination for transmitted light must be properly set

The calibration data will be included in the file "... \ bin \ PH1\_6.pos".

# CHAPTER 6 ANNEX

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## 1 Software and Hardware Options

This section describes optional software and hardware configurations. Depending on your configuration, the content of dialogue and function may differ.

### 1.1 Software

The following software packages for ZEN 2011 are available:

- Software "ZEN Module Physiology"
- Software "ZEN Module Topo"
- Software "ZEN Module Visual Basic Maxro, VBA "
- Software "ZEN module 3D Analysis"
- Software "ZEN Module Experiment Designer"
- Software "ZEN Modul 3D VisArt"
- Software "ZEN Modul 3D Deconvolution, DCV" (Available in ZEN 2011 (blue edition))
- Software "ZEN Modul Tiles & Positions"
- Software "ZEN Module FRAP Efficiency Analysis"
- Software "ZEN Module FRET plus Macro"
- Software "ZEN Module Visual Macro Editor, VME"
- Software "ZEN Module Image Correlation Spectroscopy RICS"

If your configuration does not include any of the ZEN Modules "Physiology", "FRAP Efficiency Analysis" or "FRET plus Macro", the following functions are not available:

- Mean of ROI view type and analysis functions

If your configuration does not include the "ZEN Module Physiology", the following functions are not available:

- Mean of ROI scan button in Time Series Control
- Ion Concentration analysis in the Process tab

If your configuration does not include the "ZEN Module FRAP Efficiency Analysis", the following functions are not available:

- Kinetics analysis view type.

If your configuration does not include the "ZEN Module FRET plus Macro", the following functions are not available:

- **FRET** Macro button in the **Macro** Menu

If your configuration does not include the "ZEN Module Visual Macro Editor, VM", the following functions are not available:

- **VME** button in the **Macro** Menu
- If your configuration does not include the "ZEN Module Top" , the following functions are not available:
- **Topo** view type in the **Image Display** document.

If your configuration does not include the "ZEN Module Visual Basic Macro, VBA", the following functions are not available:

- **New, Save** and **Save as** buttons in the **Macro Control** window
- **Edit, Step, Delete, Editor** buttons in the **Macro Control** window

If your configuration does not include the "ZEN module 3D Analysis", the following separate application is not available:

- **3D for LSM**

If your configuration does not include the "ZEN Module Experiment Designer", the following function is not available:

- Macro: "Advanced Time Series"

If your configuration does not include the "ZEN Modul 3D VisArt", the following functions are only available in a basic, stripped down version.

- **3D** view type in the **Image Display** document

If your configuration does not include the "ZEN Modul 3D Deconvolution, DCV", the following function is not available in ZEN 2011 (blue edition)

- **DCV** button in the **Process** menu

If your configuration does not include the "ZEN Modul Tiles & Positions", the following function is not available:

- Macro: "**StitchArt plus**" and **Stitch** function in **Processing**

If your configuration does not include the "ZEN Module Image Correlation Spectroscopy RICS", the following function is not available:

- **Correlation** function in **Processing**

## 1.2 Hardware

Depending on whether the following hardware components are available or not, the content of the screens may differ:

- Piezo objective focusing device / Z-Piezo stage
- X-Y scanning stage (different types available)
- Axio Observer.Z1 / Axio Imager.Z2 / Axio Examiner.Z1
- Depending on the configuration the scan head equipment may differ in filters, beam splitters and the number of photomultiplier
- Transmitted-light PMT
- Non Descanned Detectors (NDD)
- Monitor diode

If your configuration does not include the Piezo objective focusing device or the Z-Piezo stage, the following functions are not available:

- **Hyperfine Z Sectioning** in the **Z Stack** tool in the left tool area
- **Fine Focus** parameters in the **Stage and Focus Control** window

If your configuration does not include a X-Y scanning stage, the following functions are not available:

- **Stage** tool, **Positions** tool and **Tile Scan** tool in the left tool area

Depending on the used microscope stand: Axio Imager.Z2, Axio Imager.M2, Axio Examiner or Axio Observer.Z1, the following dialogue and available functions may differ:

- Context and accessibility of the **Locate** tab.

If your configuration does not include scan head QUASAR, monitor diode, transmitted light PMT and/or NDD, the following functions may differ:

- Context and accessibility of the **Light Path** tool

If your configuration does not include an AxioCam, the following functions are not available:

- **Camera** in the **Light Path** tool, **Channel** tool

If your configuration does include the LSM *DuoScan* or a second LSM LSM 7 MP scanner the content of the **Bleaching** tool varies.



## 2 Courses on "How to Operate the System in an Optimized Way"

Carl Zeiss is offering training courses on how to operate the system in an optimized way.

- Courses are held in our application center in Jena, Germany.
- Courses are held in English or German language, respectively.

Check out:

**[www.zeiss.de/lsm](http://www.zeiss.de/lsm)**

for the latest dates and ask your Zeiss representative for a quotation on courses.

## 3 Beam path Configuration Guide for Fluorescence Microscopy with the LSM 710 and LSM 780 Systems

### 3.1 Optical elements in the Configuration Control window

All wavelength values given in Nanometer [nm].

#### 3.1.1 Main Dichroic Beam Splitter (MBS)

- A **MBS XXX/YYY** deflects the indicated laser lines onto the specimen and allows the emitted fluorescent light to pass through.  
Example: MBS 458/514, MBS 488/543/633
- A **MBS XXX+** is a special type of a main dichroic used for IR multiphoton excitation. The **MBS 690+** for example, deflects laser light with a wavelength longer as 690 nm onto the specimen and allows fluorescent emission light in the visible range below 660 nm to pass through.  
Example: **MBS 690+**
- A **MBS XXX/YYY+** is a combination of a MBS XXX and MBS YYY+ used for simultaneous IR multi photon and single photon excitation.  
Example: MBS 405/710+.

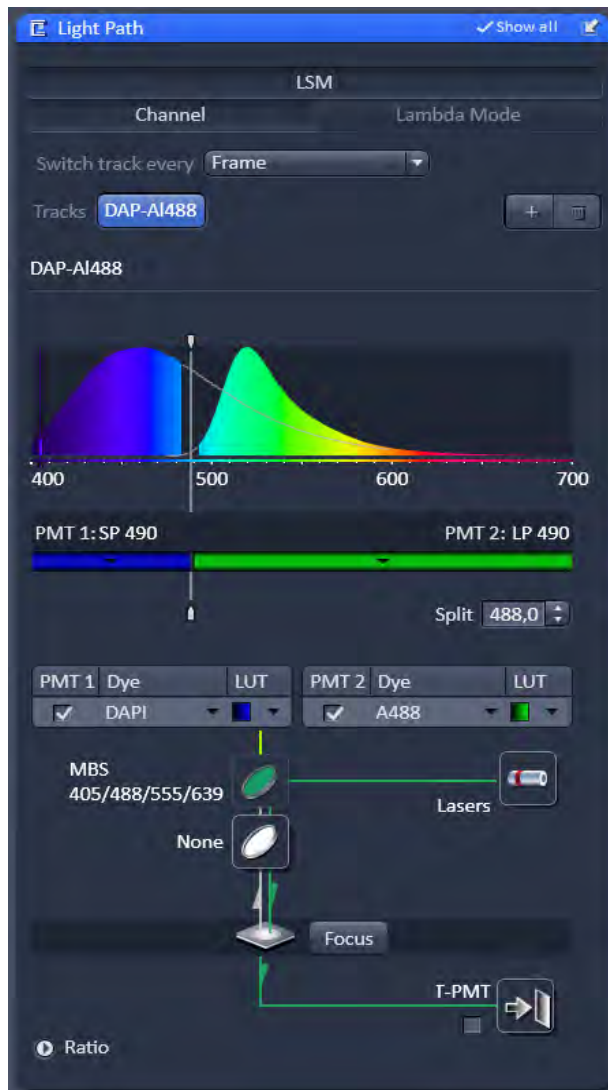


Fig. 1 Light Path tool

### 3.2 Setup of Tracks Using Single Detectors (channel mode)

#### a) One track only – simultaneous acquisition of channels

- Switch on the suitable lasers for excitation of the dyes in the specimen in the **Laser** tool. For the Argon laser set the tube current of the laser to a value of app. 50%. Example: for Alexa 488 and CY 3 switch on **Argon** (blue excitation) and **HeNe 543 or DPSS 561** (green excitation).
- Activate the appropriate laser lines in the **Channels** tool by checking the box of the laser line and set the transmission for each active line using the slider next to the indicated laser wavelength.  
Example: Select 488 to 2% and 543 to 50%
- Select a main dichroic beamsplitter (MBS) which deflects the selected laser lines to the specimen.  
Example: MBS 488/543
- Select the proper emission bands in front of the channels and activate channels.  
Example: select 500 - 545 nm in front of Ch1 and 554 - 660 nm in front of ChS1 (or Ch2).
- The active detection bands should not include any of the active laser lines but this will not interfere with imaging using LSM 7 system types.

#### Additional hints:

- Do not forget to turn on the corresponding lasers and detectors.
- Assign appropriate colors to these activated channels.  
Example: Ch1 - green (for Alexa 488 emission, Ch2 (or ChS1) - red (for CY3 emission)

#### b) Multiple tracks – sequential acquisition of channels

Multi-tracking is the method of choice for multi-fluorescence imaging. It allows avoiding artifacts from emission crosstalk that can occur in simultaneous excitation and detection of multiple dyes. To sequentially acquire multiple channels, laser lines are switched very fast and channels are recorded quasi-simultaneously.

The configuration of multiple tracks follows the same rules described above for a single track. The main difference is that each track is configured to excite and detected only one fluorescent dye to prevent cross talk (or two dyes with non overlapping emission spectra).

- Create a track for Alexa 488.
- Add a new track by clicking the **+** button next to Tracks in the **Light Path** tool.
- Create a second track for Cy 3.
- By selecting the dyes from the pulldown menu, you can see the emission spectra to set the matching detection bands.

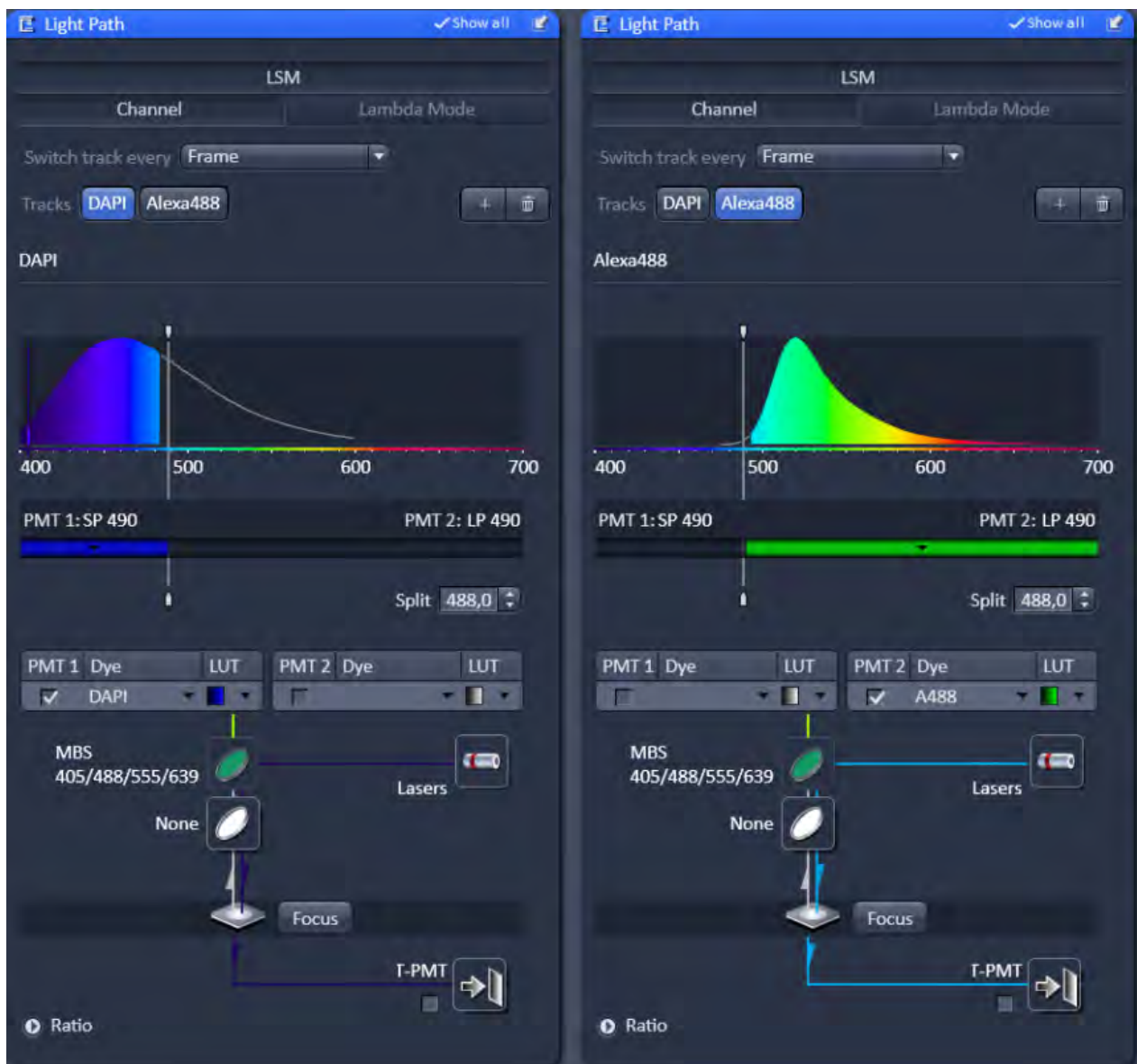


Fig. 2 Light Path tool showing the tool alternating tracks for image

### Line and Frame Mode of Multitracking

- Settings can be used for **Line** or **Frame** wise multi-tracking acquisition.
- In Line mode the lines are scanned in turns for all tracks with the corresponding laser lines turned on exclusively. Preferred for living samples with moving objects. Acquisition time can be reduced using bidirectional scan mode.
- In Frame mode, the whole frames are scanned in turns for all tracks with the corresponding laser lines turned on exclusively. This mode can be advantageous for dyes that tend to bleach and need time to recover.
- There are parameters that can be changed quickly in a line wise acquisition: Digital Gain, Digital Offset, Laser Line Attenuation,
- Any other changes of track settings of the selected tracks, e. g. different filters, dichroics or Detector Gain settings, need a bit more time to be changed and therefore can be changed only when **Frame** mode is used.
- There is a fast Frame mode, which requires identical settings of these parameters.

### 3.3 Beam path Configuration for Multi Photon Excitation

- The beam path configuration for multi photon excitation follows the same rules as described for a single and multi track configuration (see above, paragraph 0)
- Use a MBS 690+ or MBS 760+ as main dichroic beam splitter to deflect the IR excitation light (690 or 760 -1050 nm) onto the specimen.
- On the detection side, use an additional band pass filter in front of the NDD to prevent a bleed through of IR excitation light to the detector.

### 3.4 Acquisition conditions for RICS

For RICS the pixel size should be at least 5 times smaller than the PSF of the objective, so appropriate zooms have to be employed.

For the C-Apo 40 x objective with a beam radial extension of approximately 0.25  $\mu\text{m}$  zoom factors of 10-20 will guarantee a sufficient size ratio. In addition, scanning speed should not be too high or slow.

As a rule of thumb for solution studies speeds 7-8, for measurements in cytodol and nucleus of 5-6 and for membrabe studies of 3-4 will be appropriate.

Expression levels should not be too high. A maximum of 1  $\mu\text{M}$  concentration can be tolerated.

## 4 Recommendations for Excitation Laser Lines and Emission Filters of Dyes

Dye	Laser line/MBS	Emission bands
DAPI	405	> 385/420, max. at 461
EBFP	405	> 385/420, max. at 447
Hoechst	405	> 385/420, max. at 440
Fluoro-Gold	405 or 458	> 420/475, max. at 536
ECFP	405 or 458	> 420/475, max. at 501
Lucifer Yellow	458	> 475, max. at 536
EGFP	477 or 488	> 505, max. at 507/516
FM 1-43™	477 or 488	> 505, max. at 598
Alexa Fluor 488™	488	> 505, max. at 520
Calcium Green	488	> 505, max. at 531
Cy2™	488	> 505, max. at 508
DiO (DiOC18(3))	488	> 505, max. at 508
Fluo-3	488	> 505, max. at 520
Fluorescein (FITC)	488	> 505, max. at 520
Cy3™	514/532	> 530, max. at 566
EYFP	488 or 514/532	> 530, max. at 535
Oregon Green	514/532	> 530, max. at 535
SYTOX Green	514/532	> 530, max. at 536
FM 4-46	514 or 532/543	> 560, max. at 640
Alexa Fluor 546™	532/543	> 560, max. at 572
Calcium Orange	532/543	> 560, max. at 575
Dil (DiI18(3))	532/543	> 560, max. at 565
DsRed	532/543 or 561	> 560, max. at 583
Tetramethylrhodamine (TRITC)	532/543 or 561	> 560, max. at 576
Rhodamine B	532/543	> 560/585, max. at 625
Texas Red™	561 or 594	> 570/600, max. at 620
Alexa Fluor 633™	633/635	> 650, max. at 654
Cy5™	633/635	> 650, max. at 666

## 5 Changing the Filter Wheel in the LSM 710 and LSM 780 Systems

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 and LSM 780 systems proceed as follows:

- Loosen the four fastening screws (Fig. 3/1).
- Remove the cover (Fig. 3/2) of the scanning module.
- Turn the filter wheel (Fig. 4/1) 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.

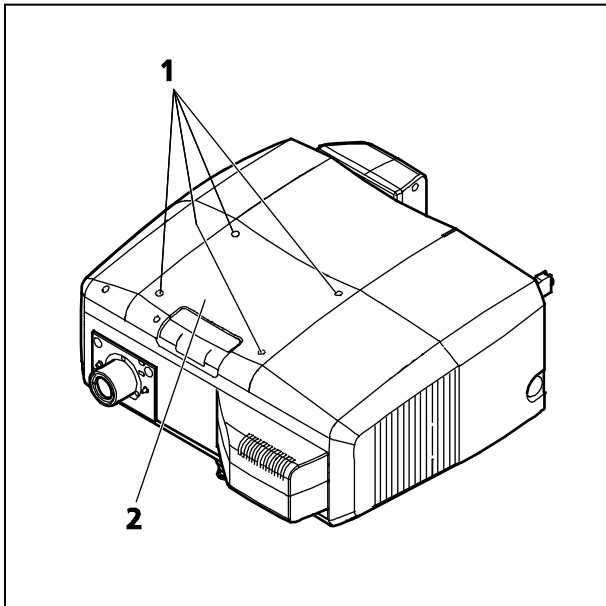


Fig. 3 Removing the cover

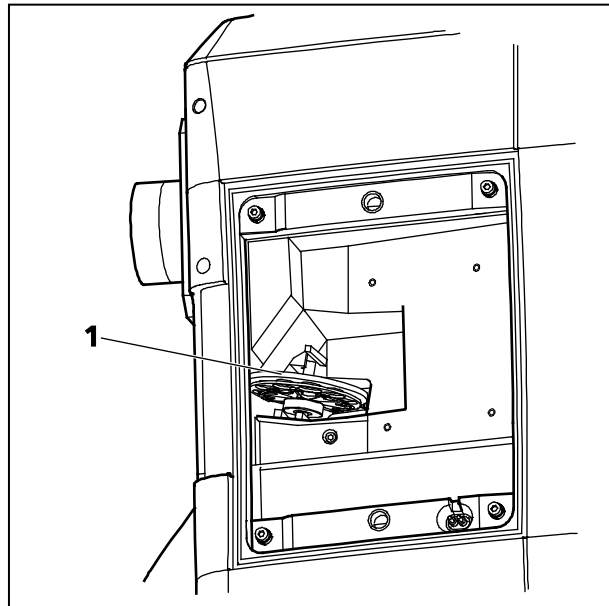


Fig. 4 LSM 710 / LSM 780 filter wheel

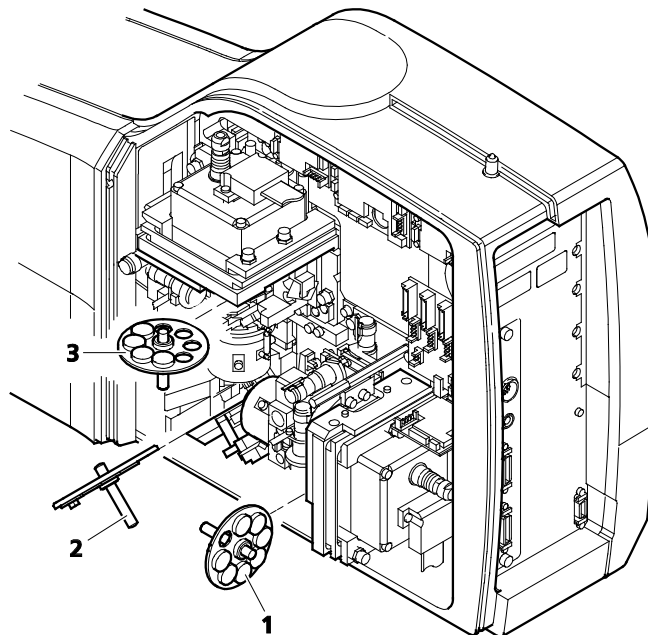
## 6 Changing Filters in the LSM 7 *LIVE* Scanning Module

For optimum investigation of specimens it is useful to employ filter wheels permitting the motor-controlled change between different filters for narrow-band or broad-band detection depending on the wavelength. The number of filters is limited by the capacity of the filter wheel. The change of the filter wheel as a whole involves complete readjustment.

The filter can be exchanged as a whole. Please ask your local LSM specialist to do this exchange in order to maintain your warranty.

### Filter-wheel change

- Pull the filter-wheels carefully out of the appropriate guide well, see Fig. 5:
  - **1** – filter-wheel 1 for emission filters (EM 1)
  - **2** – filter-wheel for secondary dichroic beam splitters (NFT)
  - **3** – filter-wheel 1 for emission filters (EM 2)
- Use the filter exchange tool carefully and applying as little force as possible to pull the filter-wheel.
- Do not touch the filters.





**Fig. 5** Change-over of the filter-wheels

- Change filter-wheel to suit the application. Make sure that the filter-wheel is correctly fitted in the mount.
- Enter the designation of this particular filters into the System Software database.

## 7 Mounting and Dismounting the LSM 710 / LSM 780 Scan Head

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

-  Be aware that the scan head weights up to 28 kg.
-  Please see Chapter I for safety relevant cables and connections.

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

- Loosen the three fastening screws of the LSM 710 / LSM 780 (Fig. 6/1 and Fig. 7/1).
- Slowly pull the scanning module (Fig. 7/2) away from the microscope port or the tube (Fig. 7/3).
- 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 / LSM 780 (Fig. 6/1 and Fig. 7/1).

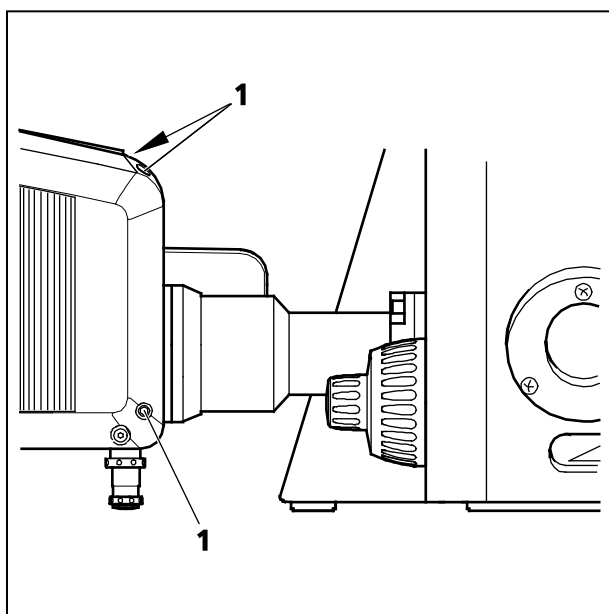


Fig. 6 Port connection between LSM and Axio Observer.Z1

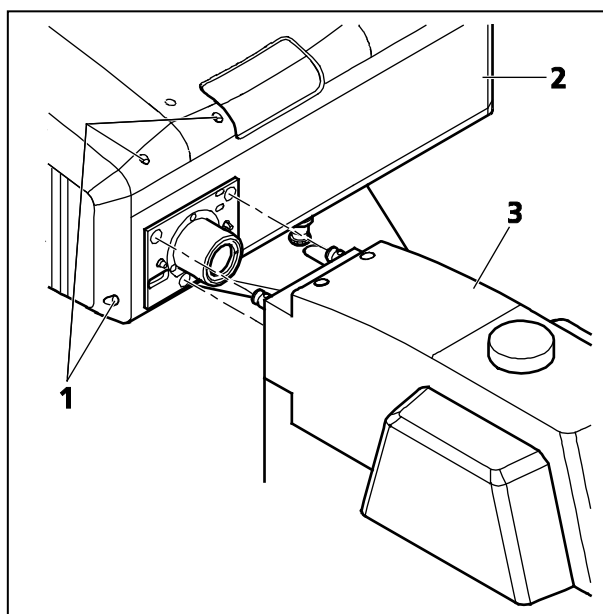


Fig. 7 Port connection between LSM and Axio Examiner or Axio Imager.Z2



## 8 Detaching / Attaching the LSM 7 LIVE Scanning Module from / to Microscope Stands

Tool needed: 3 mm Allen key



The user can remove the Scanning Module from one microscope and attach it to another within a few minutes. Described below is the change-over from an Axio Examiner to an Axio Observer.Z1 in sideport configuration.



**Before** the change-over, **shut down** the system as described in Chapter III in order to avoid damage to the system and loss of data.

- Loosen the three screws (8/1) at the Scanning Module (8/2) fitted to the Axio Examiner.
- Cautiously pull Scanning Module off the Axio Examiner stand.
- Attach Scanning Module to the left sideport of the Axio Observer.Z1, minding the guide pins (8/5), and secure it with the three screws (8/1).



As the Scanning Module is heavy, weighing about 19.5 kg, it is easier if the changeover is carried out by two persons.

- Pull off covering caps (8/3) from the CAN-BUS and RS232 interface ports at the rear of the Axio Observer.Z1, remove the two cables 457411-9011 (CAN-BUS) and 457411-9012 (RS232) from the Axio Examiner, plug them into the Axio Observer.Z1 and secure them there.
- Switch the LSM 7 *DUO* on.
- Click on the **Stand select** icon to update the system database with the new database of the Axio Observer.Z1 microscope.
- Restart the ZEN software.



Please see Chapter I for safety relevant cables and connections.



For optimal performance, an optical realignment of the LSM 7 *LIVE* scanhead is mandatory. Use the **Kollimatic** macro for this procedure, as described in Chapter III.

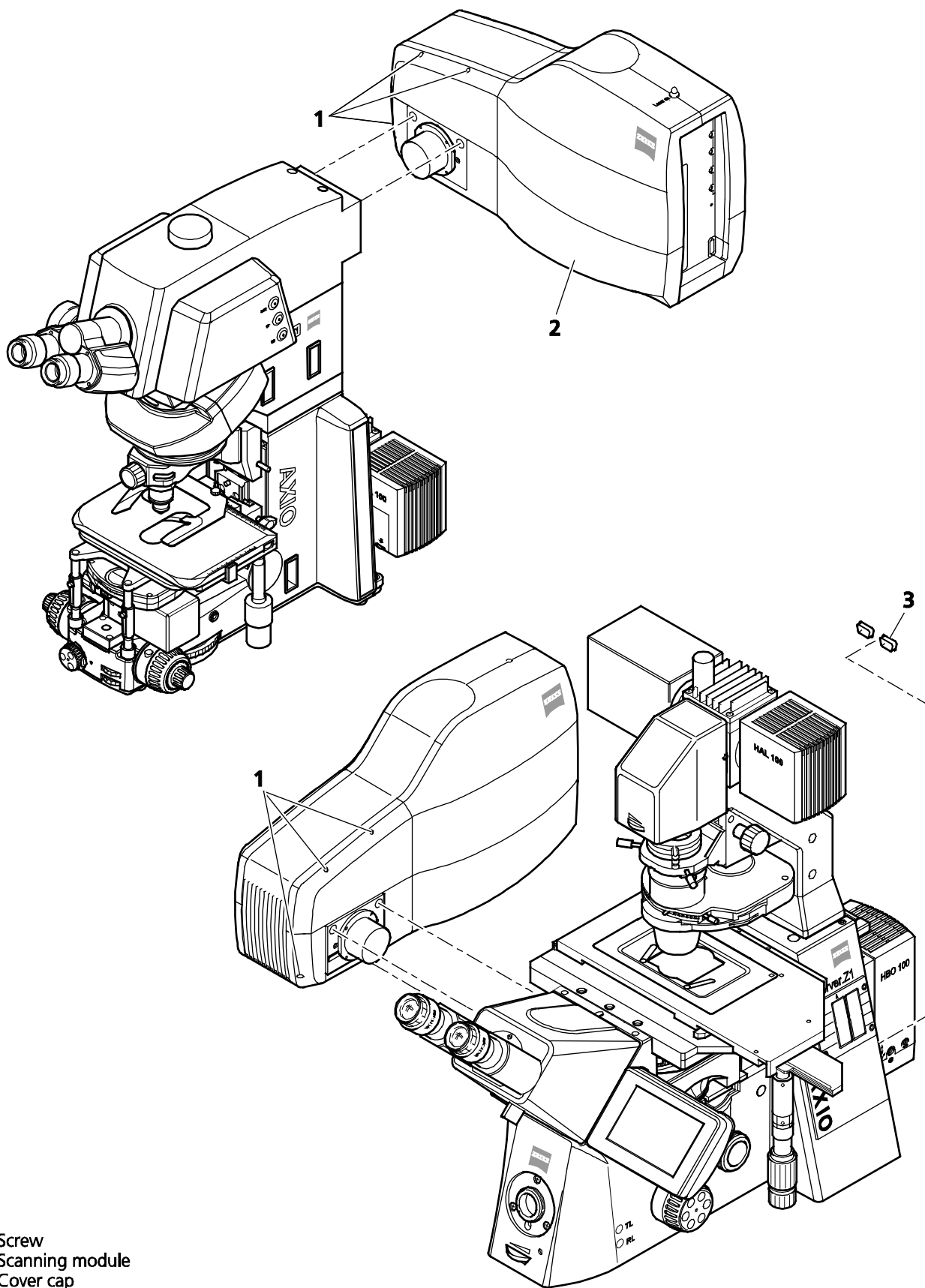


Fig. 8 Change-over of the Scanning Module

## 9 Changing Filters in the scanning module of the of the LSM 700

For optimum investigation of specimens it is useful to employ filter wheels permitting the motor-controlled change between different filters for narrow-band or broad-band detection depending on the wavelength. The number of filters is limited by the capacity of the filter wheel. The change of the filter wheel as a whole involves complete readjustment by trained Zeiss service personnel.

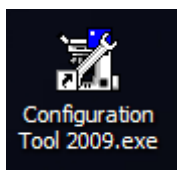
The filter wheels of channel 1 (vertical filter wheel) and 2 (horizontal filter wheel) of the Scanning Module have 7 positions in which a filter, including its mount, can be changed in a reproducible position without requiring readjustment.

### Removing the cover from the scanning module

- Close the software and **switch off the instrument. Make sure the instrument is disconnected from electricity and the laser rack is switched off using the power switch on the back of the rack!**
- Unscrew the 4 hex socket screws on the customer side of the scanning module. The screws are not secured against falling out!
- Pull cover cap off the Scanning Module.
- Unscrew the 4 screws to open the black cover.

### Filter change

- Turn the filter wheel required into a position where the filter to be changed is accessible.
- Use the filter tool carefully and applying as little force as possible to pull the filter mount with the filter out of the guide well.
- Change filter to suit the application. Make sure that the filter is correctly fitted in the mount.
- **Enter the designation of this particular filter into the System Software database by using the Configuration Tool.**



The link is on the desktop, if this is not the case – the program can be found in C:\ZEN\HWT\

## **10 The Use of the Piezo Fine Focusing Stage**

### **10.1 General Description**

The Piezo fine focusing stage is a compact attachment for the Axio Imager.Z2, Axioskop 2 FS MOT and Axio Observer.Z1 microscope stages, which allows the particularly fast and high-precision fine focusing of the object. The piezo stage permits fine focusing over a range of up to 250  $\mu\text{m}$  (200  $\mu\text{m}$  guaranteed), with the smallest step width being less than 10 nm, reproducibility better than 40 nm, and the maximum speed amounting to 60 Hz. The stage allows the use of specimens with a weight of less than 100 g.

The piezo stage is not used if manual coarse focusing is performed. To position the objective in relation to the optical Z-axis, the standard XY-microscope stage is used.

The piezo stage features a mount for standard object carriers of 76 mm x 26 mm x 1 mm and a milled-out receptacle for  $\varnothing$  36 mm x 1 mm Petri dishes.

### **10.2 Applications of the Piezo Fine Focusing Stage**

- High-precision fine focusing and translation of the object along the optical axis.
- Fast and high-precision mounting of one-dimensional Z-line sections.
- Fast and high-precision mounting of two-dimensional R-Z-longitudinal sections.
- Fast and high-precision mounting of XY-Z-Stacks for the three-dimensional reconstruction of the object.
- Exact measurement of Point-Spread-Functions for deconvolution.

### **10.3 Additional Information on the Operation**

The piezo fine focusing stage is a high-precision, sensitive accessory for the LSM systems from Carl Zeiss and must therefore be treated carefully.

High mechanical stress, such as the use of specimens weighing more than 100 g or the application of pressure or knocks on the movable stage tongue, can result in damage and therefore in failure of the stage function.

To be able to fully utilize the outstanding precision attainable with the fine focusing stage, anything which could interfere with its operation, especially mechanical knocks and impact of the LSM system components, should be avoided. We would recommend you to always use the actively vibration-damped table.

The specifications of the stage are obtained only after a warm-up phase of approx. 30 minutes. Furthermore, the installation conditions for the LSM system must be observed.

The maximum reproducibility (better than 40 nm) for moving to an absolute position in Z is achieved by always moving to the required position from below.

---

Fine focusing is performed mechanically via an inclined position of the stage tongue. Therefore, the lifting range Z at the location of the image field depends on the position of the piezo stage in relation to the optical axis. This means: if the user shifts the object on the microscope stage to the right via the piezo stage, the lift will be different from the one in the zero position of the stage (max. 250  $\mu\text{m}$ ) and also from the one after a shift of the stage to the left.

If the LSM system is equipped with a motorized scanning stage, this shift is read back to  $\Delta x$  and the lift is calibrated automatically if the zero position of the piezo stage has been matched to the zero position of the scanning stage via an initialization run. For this, activate the **Stage** button of the **Acquire** toolbar. Then position the scanning stage in such a way that the optical axis of the microscope corresponds to the zero position of the piezo stage, i.e. to the center of the specimen holder in the stage tongue. Then perform initialization by pressing the **piezo Null** button. This step must be repeated after every new start of the system. Also see the notes on the operation of the motorized scanning stages.

If the system is equipped with a manual microscope stage, the user has the option of performing the calibration by entering the  $\Delta x$  shift in mm via the **Calibration** slider.

The shift is read off from the microscope stages. In the case of the manual Axio Imager stage,  $\Delta x$  can be read directly from the scale adhered to the front of the stage. In the case of the manual Axio Observer.Z1 stage, a scale is located on the right of the knob, where the 45 mm  $\Delta x$  shift relative to the zero position of the microscope stage can be read off. The  $\Delta x$  value is positive for both stages if shift from the zero position is made to the right and negative if the shift is made to the left.

On account of the inclined position of the stage tongue, the object is also shifted laterally during the fine focusing motion. This lateral shift is negligibly small if, as recommended by us, specimen carriers with thickness 1.0 mm are used exclusively. Otherwise, the marked lateral shift of the object during fine focusing can result in image distortion. For the same reason, Petri dishes without fixation ring must be used exclusively.

The nosepiece of the Axio Observer.Z1 stand is moved to the load position prior to switching off the LSM system and the piezo stage is then moved to the lowest position to avoid damage of the objective or object by a possible collision. The user must refocus after start-up of the system. Before an objective change in the Axio Observer.Z1 or the Axio Imager.Z2 the nosepiece and the microscope stage must be moved to the load position by the user, and then back to the work position to prevent the objectives from hitting the piezo components. This is performed automatically if the objectives are changed menu-controlled via the relevant buttons of the ZEN program.

## 11 Piezo Objective Focusing Device

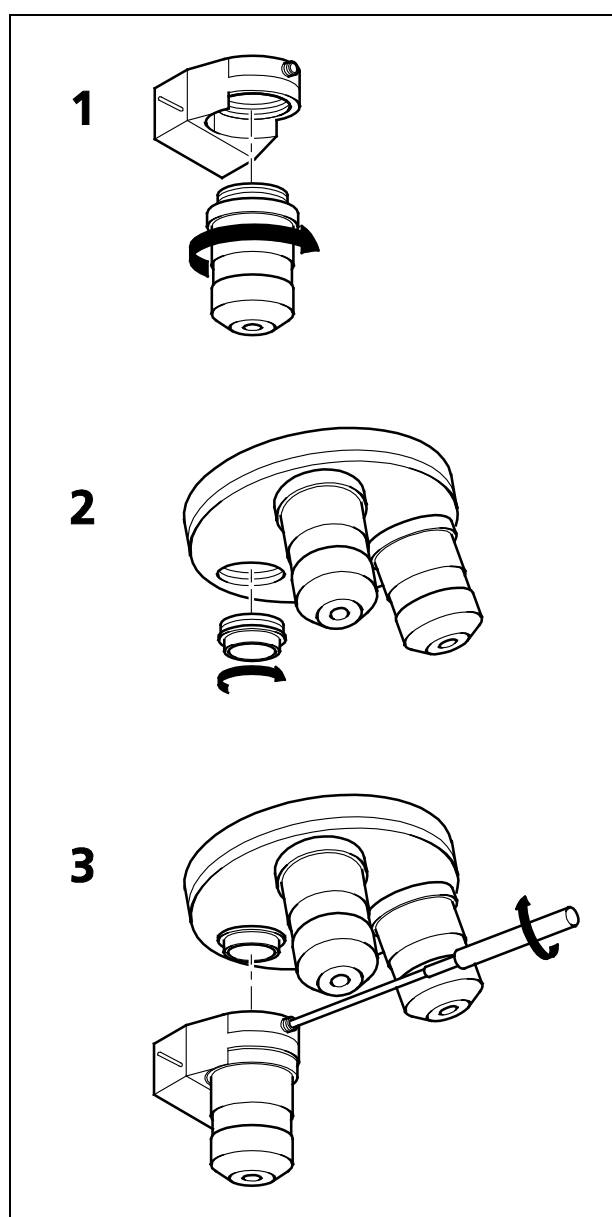
For Axio Imager.Z2, Axio Imager.M2 and Axio Observer.Z1

Range:  $\geq 200 \mu\text{m}$

Minimum step size: 15 nm

Speed:

		Piezo objective focussing device
Slices	Step size [ $\mu\text{m}$ ]	xz-lines / s
20	0.5	460



**Fig. 9** Installation of the Piezo Objective Focusing Device

Objectives:

- W0.8/M27
- Modified Achromplan 40x / 0.8 W with reduced length to compensate for piezo height

Installation:

- Screw in your microscope objective into Piezo Objective Focusing Device (see Fig. 9/1).
- Screw the thread-ring into your microscope (see Fig. 9/2).
- Easy clamp the Piezo Objective Focusing Device on the thread-ring (see Fig. 9/3).

## 12 Z-Piezo Focussing Insert for Scanning Stages

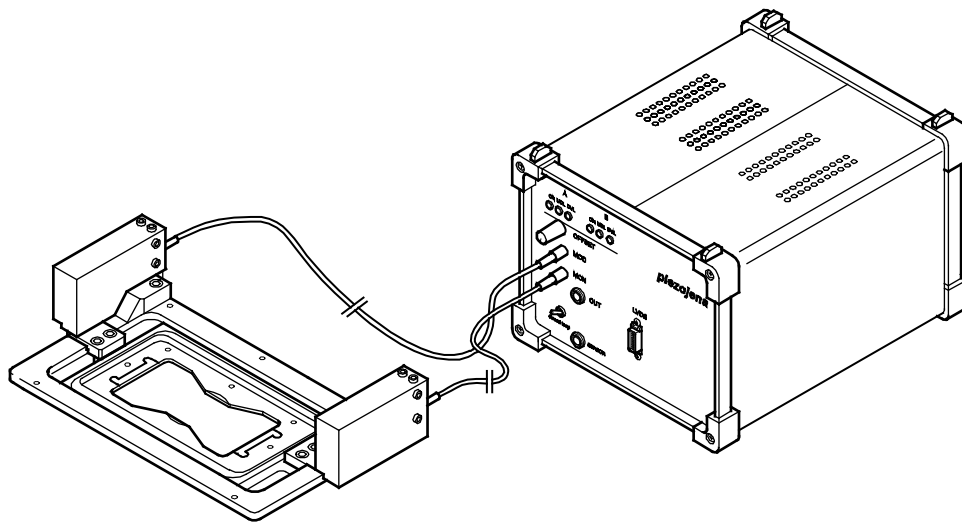
For Axio Imager.Z2, Axio Imager.M2 and Axio Observer.Z1

Range:  $\geq 200 \mu\text{m}$

Minimum step size: 5 nm

Speed:

		Piezo objective focussing device
Slices	Step size [ $\mu\text{m}$ ]	xz-lines / s
20	0.5	290



**Fig. 10** Z-Piezo focusing insert for scanning stages

The Z-Piezo stage can only be mounted onto scanning stages. The insert is used instead of the standard inserts. The Z-Piezo insert needs to be screwed onto the scanning stage to ensure maximum fixation to the stage. It can hold classic objective slides and 35 mm Petri dishes.

## 13 Specifications of Trigger-Interface

The LSM 710, LSM 780, LSM 710 NLO, LSM 780 NLO and LSM 7 *MP* provide two ways to control external devices or be controlled via external devices.

### Synchronisation signals:

This interface is provided directly by the electronics of the system and cannot be influenced via the user interface. These signals are always present according to their specification. By connecting to the user interface connector these signals can be used to control external devices or synchronise external devices, like for example pulse generators, with the LSM. The detailed specification of these signals is described below.

### Trigger signals:

The second interface is accessible via the software user interface in the tools **Time Series** and **Bleaching** and can be used to accept or provide signals for the following actions:

### Hardware Interface:

- User Port Adapter on the back of the Real Time Electronics in the System Electronic Rack
- Connector 2x 15-point high density D-Type plug, 1x coax with outer shield (Triax Lemo S. A. Serie 00, EPL.00.650NLN)

### Number of accessible interface signals:

- 4 x signal IN (asynchronous; able to generate an interrupt)
- 8 x signal OUT (synchronous)  
The label synchronous refers to the data stream PCIe2LVDS.  
The label asynchronous refers to the data stream PCIe2LVDS.
- 1x pixel clock

### Connectors:

Pixelclock = Coax: Triax Lemo S. A. Serie 00, EPL.00.650NLN

Pin	Signal name	Description
Signal	PCLK_Out	PixelClock Output
Signal shield	GND	Digital ground
Outer shield	Shield	Chassis ground

The pixel clock is exactly symmetrical to the pixel. For example if the pixel rate is 10 kHz the high-level and the low level both have a time of 50 microseconds. The width of the TTL pulse therefore is  $1/(2 \times \text{pixelrate})$ .



**15-point high density D-Type plug: Plug "A"**

Pin	Signal name	Description	Software interface
1	SyncOUT0 synch output	Synchronous output	Trigger 1 out
2	SyncOUT1	Synchronous output	Trigger 2 out
3	SyncOUT2	Synchronous output	Trigger 3 out
4	SyncOUT3	Synchronous output	Trigger 4 out
5	GND	Digital ground	-
6	AsyncOUT0 asynch output	Asynchronous output	-
7	AsyncOUT1	Asynchronous output	-
8	AsyncOUT2	Asynchronous output	-
9	+5 V	Va	-
10	GND	Digital ground	-
11	AsyncIN0 input	input	Trigger 1 in
12	AsyncIN1	input	Trigger 2 in
13	AsyncIN2	input	Trigger 3 in
14	AsyncIN3	input	Trigger 4 in
15	Connector detector*	Input / Connector detector	-

**15-point high density D-Type plug: Plug "B"**

Pin	Signal name	Description	Hardware output only
1	SyncOUT4 ** (stack)	Synchronous output	Sync. signal: Stack out
2	SyncOUT5 out Grabbing	Synchronous output	Sync. signal: Grabbing
3	SyncOUT outline **	Synchronous output	Sync. signal: Line out
4	SyncOUT outframe **	Synchronous output	Sync. signal: Frame out
5	GND	Digital ground	-
6	SyncOUT6	Synchronous output	-
7	SyncOUT7	Synchronous output	-
8	AsyncOUT6	Asynchronous output	-
9	+5 V	Asynchronous output	-
10	GND	Va	-
11	AsyncIN4 input	Digital ground	-
12	AsyncIN5	Asynchronous input	-
13	AsyncIN6	Asynchronous input	-
14	AsyncIN7	Asynchronous input	-
15	Connector detector*	Input / Connector detector	-

\*) If the pin is connected to ground, the software recognizes that at least one connector is connected to the UserPort2.

\*\*) The position of the two switches on the UserPortAdapter inside the RT-PC must switch thrown towards enclosure = default position.

### Timing of the synchronization signals Stack out, Line out and Frame out

These signals are directly generated by the hardware and cannot be influenced by the software. They are also not seen by the user software and cannot be used to generate any markers within the image acquisition. They are highly precise tools to control external hardware. The signals are precise to the pixel and depending on the chosen pixel time the time between the signal and the start of the image acquisition varies.

The Line OUT signal is set 44 pixels before the first image pixel of each line.

The Frame OUT signal is set 42 pixels before the first image pixel of each frame.

The Stack OUT signal is set 40 pixels before the first image pixel of each Z-stack.

The signal itself is identical to the signal of the pixelclock but is only set for one pixel.

The Grabbing signal OUT is set to high-level with the start of data grabbing and set to low-level with the stop of data grabbing for each line.



The Stack OUT signal is provided at the beginning of each Z-Stack of the Z-Stack is acquired with a Z-Piezo device (Z- Piezo stage frame or objective piezo device)

### Type/Voltage Range:

- TTL signal level 3.3 V, CMOS low power consumption
- 5.0 V tolerant input/output for interfacing with 5 V logic

### Load:

- Output: < 50 mA (internal serial 68 ohm resistor)
- Input: 4.7 kOhm input impedance (internal 4.7 kOhm pullup to 3.3 V)

### Trigger pulse description:

Signal output:

- Low level < 0.4 V, high level > 2.4 V (I = 10 mA)
- Slew rate 10 ns/V
- Reaction time trigger OUT 8 msec typical

Signal input:

- Low level <0.5 ... 0.8 V, high level >2.0 ... 5.5 V
- Falling edge force interrupt
- Pulse width to detect signal > 50 ns
- Reaction time trigger IN 16 msec typical

### Caution:

- Never apply more than 5 V or negative voltages to avoid any damage.
- In and outputs are not galvanically decoupled.
- Therefore proper measures for galvanic decoupling of external devices have to be taken (opto-coupler etc.).

### User interface box for synchronisation and trigger signals (optional)

The interface box provides connections to the trigger IN and OUT functions accessible in the software user interface, the synchronization signals and the pixel clock. External devices can be connected to the LSM via standard BNC cables that are plugged into the user interface box. The box itself is delivered with the necessary cables to be connected to the user interface on the realtime electronics.

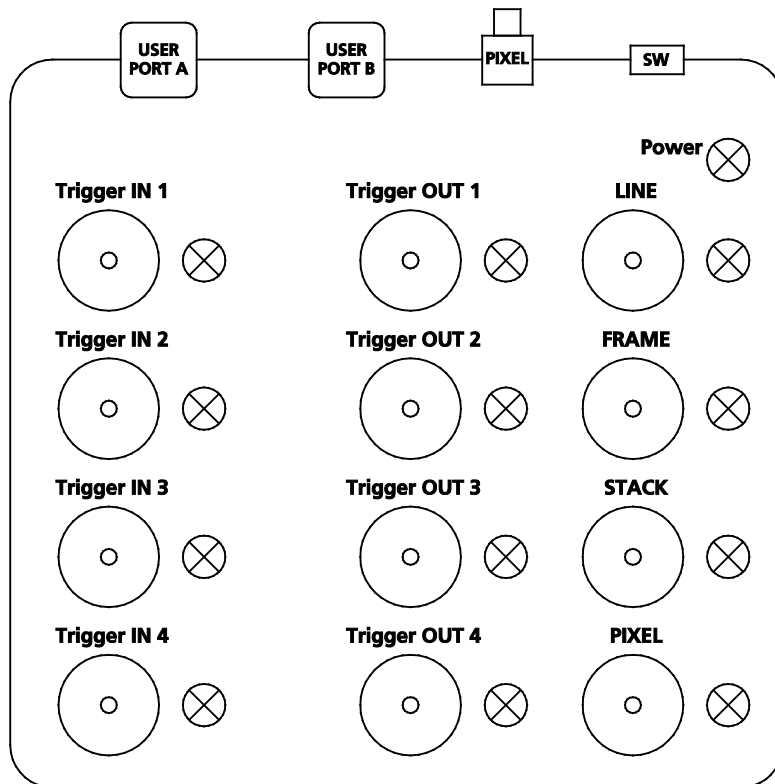


Fig. 11 Panel of the User Interface box

#### Trigger pulse description:

The pulses are identical to the pulses described for the user interface on the realtime electronics.

The LED lights signal the functionality of the individual plugs. The LED lights can be switched on or off with the switch **SW**. A LED blinks if a signal changes with a frequency of more than 2 Hz.

For a positive signal shorter than 500 ms, the LED is on for 500 ms. For a negative signal shorter than 500 ms, the signal is switched off for 500 ms.

The LED follows the level of the signals if these are longer than 500 ms. The LEDs react on pulses which are longer than 30 ns and have a frequency of less than 30 MHz.

## 14 AxioCam High Resolution Digital Cameras

### 14.1 High Resolution Microscopy Camera AxioCam HRm Rev.3 FireWire

Cat. No	426511-0000-000, incl. digital interface and cable		
High Range Monochrome			
Number of Pixels:	1388 (H) x 1040 (V) = 1.4 Mega pixel		
Chip size:	8.9 mm x 6.7 mm, equivalent to 2/3"		
Spectral range:	With BK-7 protection glass up to 1000 nm, with IR barrier filter BG40 limited to about 350 nm to 700 nm		
Selectable Resolution by Binning or Microscanning			
H	x	V	Acquisition Time (s) @ 20 ms exposure
1388	x	1040	0.2 (5 images / s)
2776	x	2080	0.8 (1.25 images / s)
4164	x	3120	1.7
Dynamic Range:	Better than 2000 : 1 @ 8 e readout noise		
Integration Time:	1 ms to several minutes		
Cooling:	Single stage Peltier cooling		
Optical Interface:	C-Mount		
Size:	about 11 cm x 8 cm x 6.5 cm (2.3" x 3.2" x 2.6")		
Registration:	CE, cUL		
Power Supply:	12 V DC, 1 A, 230 V/110 V, autodetecting		

### 14.2 High Resolution Microscopy Camera AxioCam HRc Rev.3 FireWire

Cat. No	426510-0000-000, incl. digital interface and cable		
High Range Color			
Number of Pixels:	1388 (H) x 1040 (V) = 1.4 Mega pixel		
Chip size:	8.9 mm x 6.7 mm equivalent to 2/3"		
Spectral range:	Limited by IR barrier filter BG40, about 400 nm to 700 nm		
Selectable Resolution by Binning or Microscanning			
H	x	V	Acquisition Time (s) @ 20 ms exposure
1388	x	1040	0.2 (Color interpolation)
1388	x	1040	0.8 (full resolution for color channels)
2776	x	2080	2.9 (full resolution for color channels)
4164	x	3120	6.5 (full resolution for color channels)
4164	x	3120	1.6 (Fast Scan)
Dynamic Range:	Typical 2000 : 1 @ 9 e readout noise		

---

Integration Time:	1 ms to several minutes
Cooling:	One stage Peltier cooling
Optical Interface:	C-Mount
Size:	about 11 cm x 8 cm x 6.5 cm (2.3" x 3.2" x 2.6")
Registration:	CE, cUL
Power Supply:	12 V DC, 1 A, 230 V/110 V, autodetecting

### 14.3 AxioCam MRm FireWire

Number of Pixels:	1388 (H) x 1040 (V) = 1.4 Mega pixel
Chip size:	8.9 mm x 6.7 mm, equivalent to 2/3"
Spectral range:	about 350 nm to 1000 nm, BK 7 protective cover glass

Resolutions with Binning:

<b>H</b>	<b>x</b>	<b>V</b>	
276	x	208	(Binning 5 x 5, RGB)
346	x	260	(Binning 4 x 4, B/W)
462	x	346	(Binning 3 x 3, RGB)
694	x	520	(Binning 2 x 2, B/W)
1388	x	1040	(Single exposure)

Live image frame rates:

<b>Max. frame rate</b>	<b>Mode</b>	<b>H</b>	<b>x</b>	<b>V</b>	<b>Binning</b>
11 frames/s	slow	1388		1040	1
26 frames/s	medium	460		344	3
38 frames/s	fast	276		208	5



Above frame rates are supported by the camera electronics at 20 ms exposure time, computer hardware, operating system and application software may influence the frame rates.

Digitization:	12 Bit / 24.57 MHz
Dynamic Range:	typical > 2200 : 1
Integration Time:	1 ms up to 20 s
Cooling:	single stage Peltier-cooling
Optical Interface:	C-mount adapter
Size / Weight:	about 11 cm x 8 cm x 4.5 cm / 370 g
Registration:	CE, cUL
Power Supply:	12 V DC, 350 mA (no external power supply required)

## 14.4 Microscope camera ports and camera port adapters for the AxioCam

### In order to mount a Camera onto the AxioObserver.Z1, the following parts are needed

To use the front port of the microscope:

**Adapter Video V200 C 2/3" 0.63x** at front port Axio Observer.Z1 Cat. No 000000-1071-171

To use the side port left on the microscope:

Sideport 60N 3 Positions. Cat. No's. 425151, 425154, 425155.

and

**Camera Adapter 60N C 2/3" 0.63x** Cat. No. 426113-0000-000

### In order to mount a Camera onto the Axio Imager M2, Z2 the following parts are needed

Camera path deflection on the left side, interface 60N Cat. No. 425103-0000-000

Path deflecting mirror 100% for camera deflection, 34x46x4 mm Cat. No. 425110-9110-000

and

**Camera Adapter 60N C 2/3" 0.63x** Cat. No 426113-0000-000

### In order to mount a Camera onto the Axio Examiner the following parts are needed

**Camera Adapter 60N C 2/3" 0.63x** Cat. No 426113-0000-000



**No other cameras are supported by the LSM Software!**

## **LSM 700**

### **Laser Scanning Microscope**



**LSM Software ZEN 2011**

September 2011



We make it visible.





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## Introduction

This LSM 700 Quick Guide describes the basic operation of the LSM 700 Laser Scanning Microscope with the ZEN 2011 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).

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 700 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

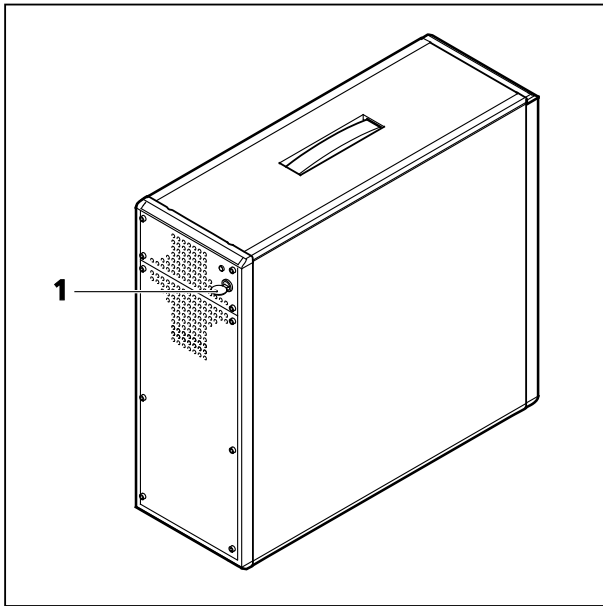


Fig. 1 Electronics and laser module

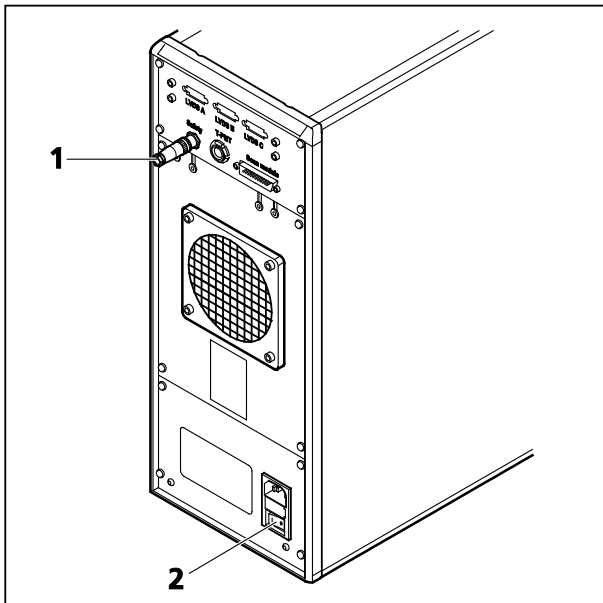


Fig. 2 Electronics and laser module

### Switching on the LSM 700 system

- Turn on the LSM 700 system via the switch-operated multipoint connectors (power strips 1 and 2) on the system. This switches on all system components except attached scanning stages, the microscope and its illumination (HBO 100, XBO 75 or HXP 120).
- If using the Axio Observer microscope stand, push the power button at the left hand side first to switch on the microscope.
- Turn the key switch (Fig. 1/1) of the electronics and laser module to the right to start the laser. The yellow LED in return turns on. If this is not the case, check the power switch (Fig. 2/2) on the back of the laser module.
- On the back of the system there is a plastic screw (Fig. 2/1). This is a laser interlock which can be used for coupling the laser module e.g. to doors. If this screw is **unscrewed** the lasers will **not** turn on!

### Switching on the HXP 120 or the HBO 100 or the XBO 75 lamps

- Switch on the main switch of the HXP 120 / HBO 100 / XBO 75 lamps for reflected light illumination via the power supply as described in the respective operating manual.

### Switching on the scanning stage

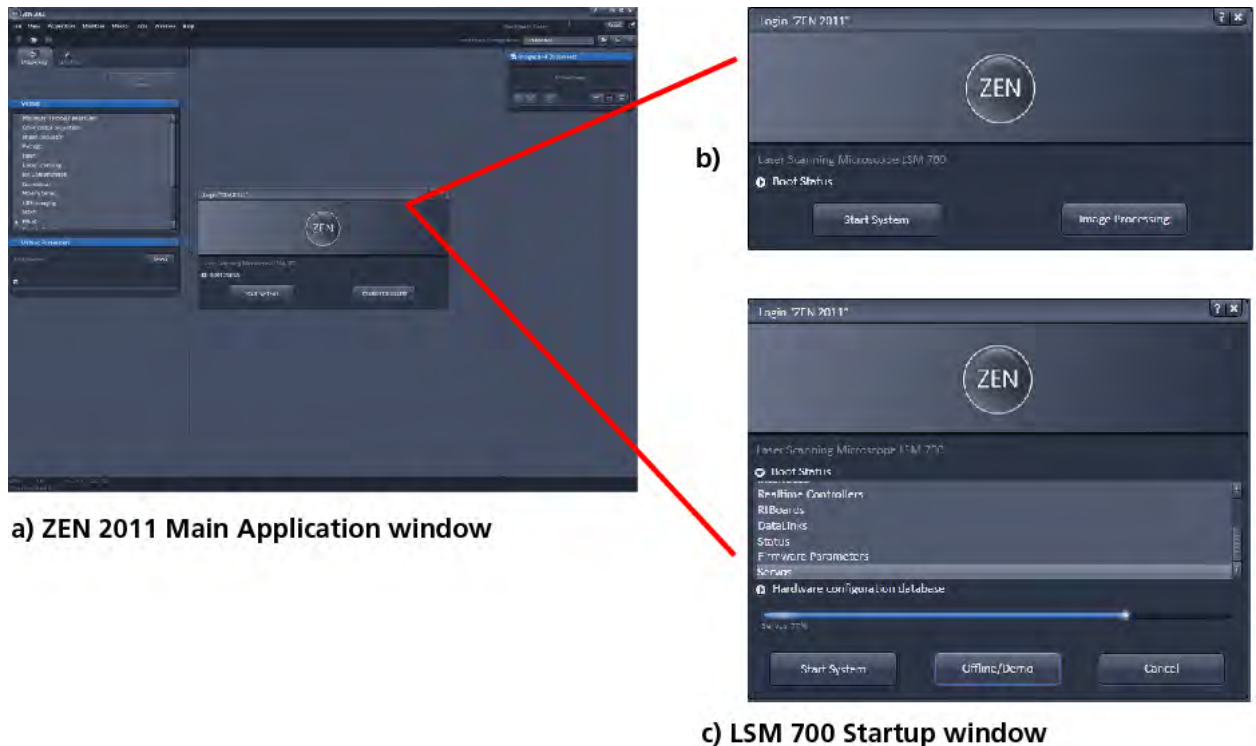
- If attached, switch on the scanning stage via the controller.

## Starting the ZEN software




- Double click the **ZEN 2011** icon on the WINDOWS desktop to start the Carl Zeiss LSM software.

The **ZEN Main Application** window and the **LSM 700 Startup** window appear on the screen (Fig. 3).

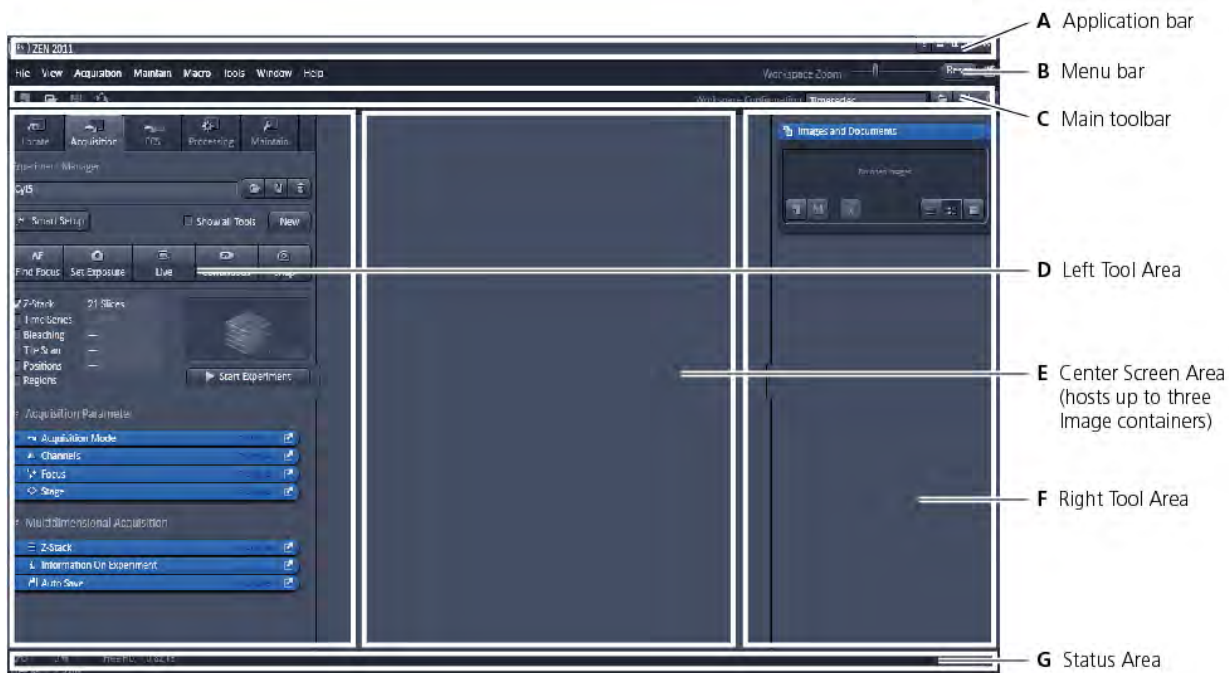


**Fig. 3** ZEN Main Application window at Startup (a) and the LSM 700 Startup window (b and c)

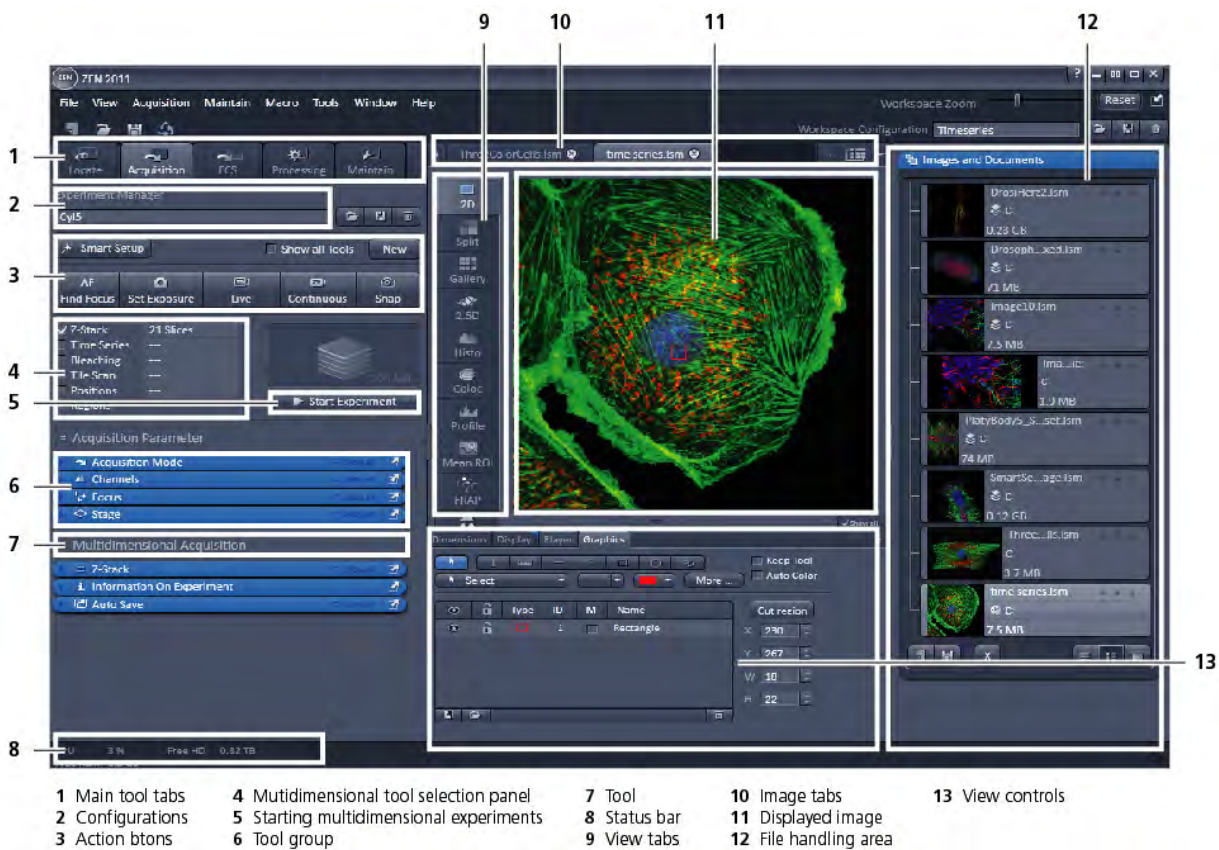
In the small startup window, choose either to start the system online (**Start System** hardware for acquiring new images) or in **Image Processing** mode to edit already existing images. Toggle the little  symbol to view the Boot Status display and get the additional **Offline/Demo** button option:

- 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 **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**



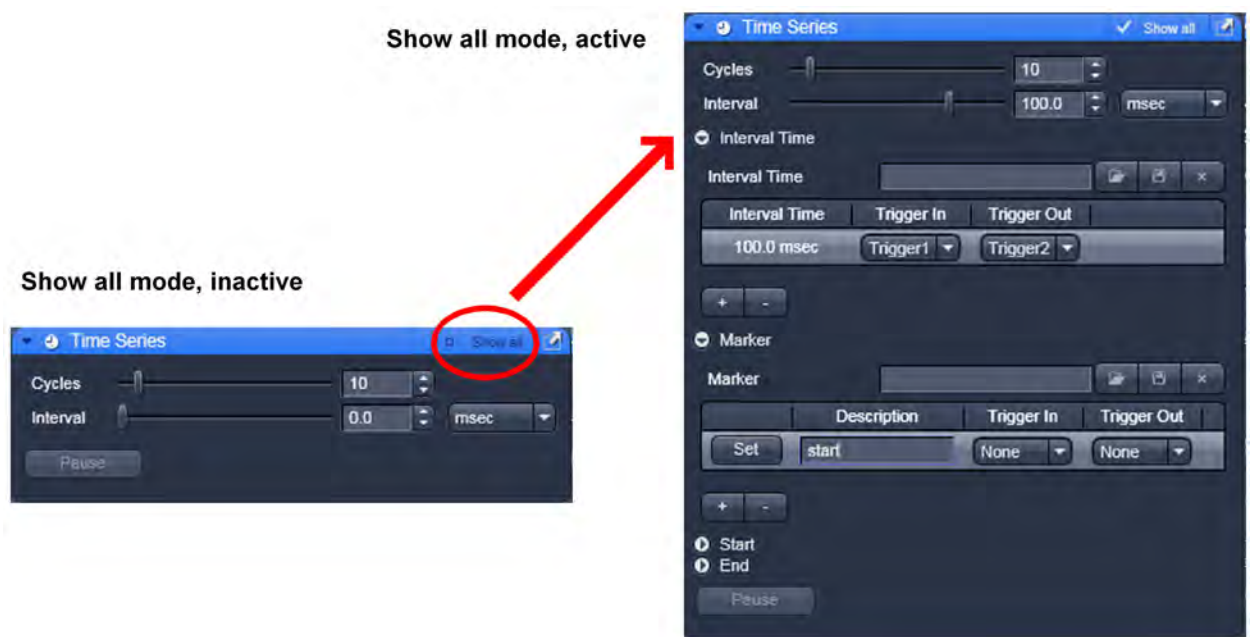
**Fig. 5 ZEN Main Application window after startup with several images loaded**

## Introduction to ZEN – Efficient Navigation

The **ZEN 2011** 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 **Locate** 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).

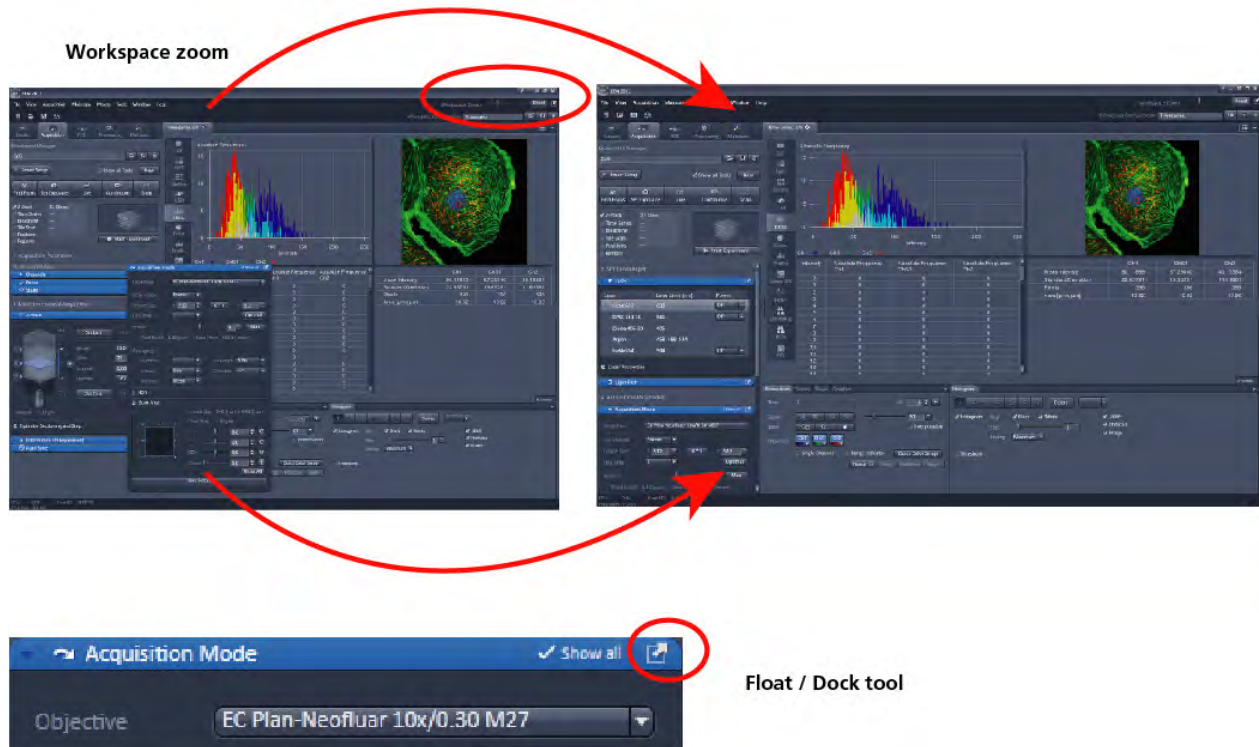
Color and brightness of the interface have been carefully adjusted to the typical light conditions of the imaging laboratory, guaranteeing optimal display contrast and minimal stray light for high-sensitivity detection experiments. The **ZEN** software is optimized for a 30" TFT monitor but can also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



**Fig. 6** Show all mode

A focus in the development of **ZEN 2011** 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 2011** 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 2011** 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 2011** 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 **Set Exposure**  button. For an empty image document press the **New**  button.

The new document is immediately presented in the **Documents** panel of the Right Tool 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 in case 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.

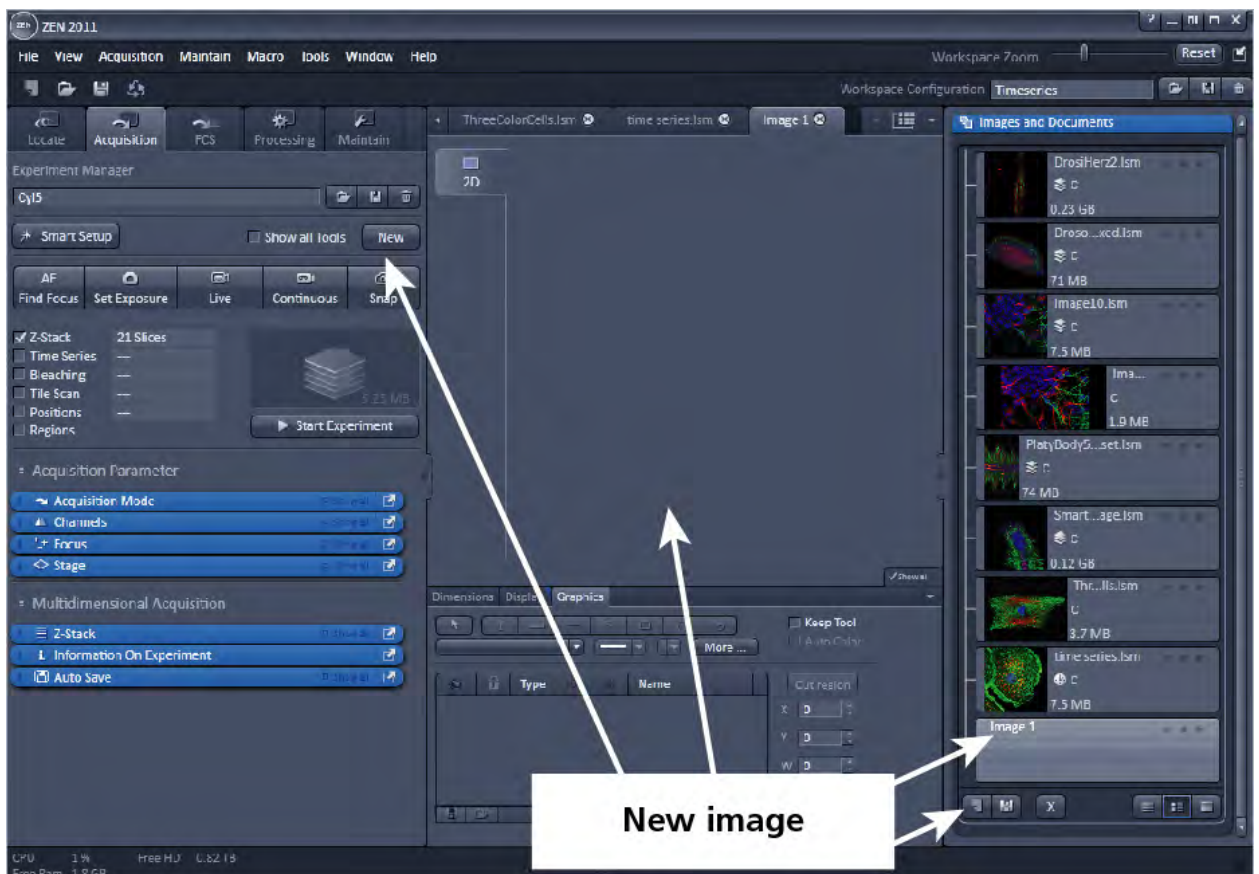
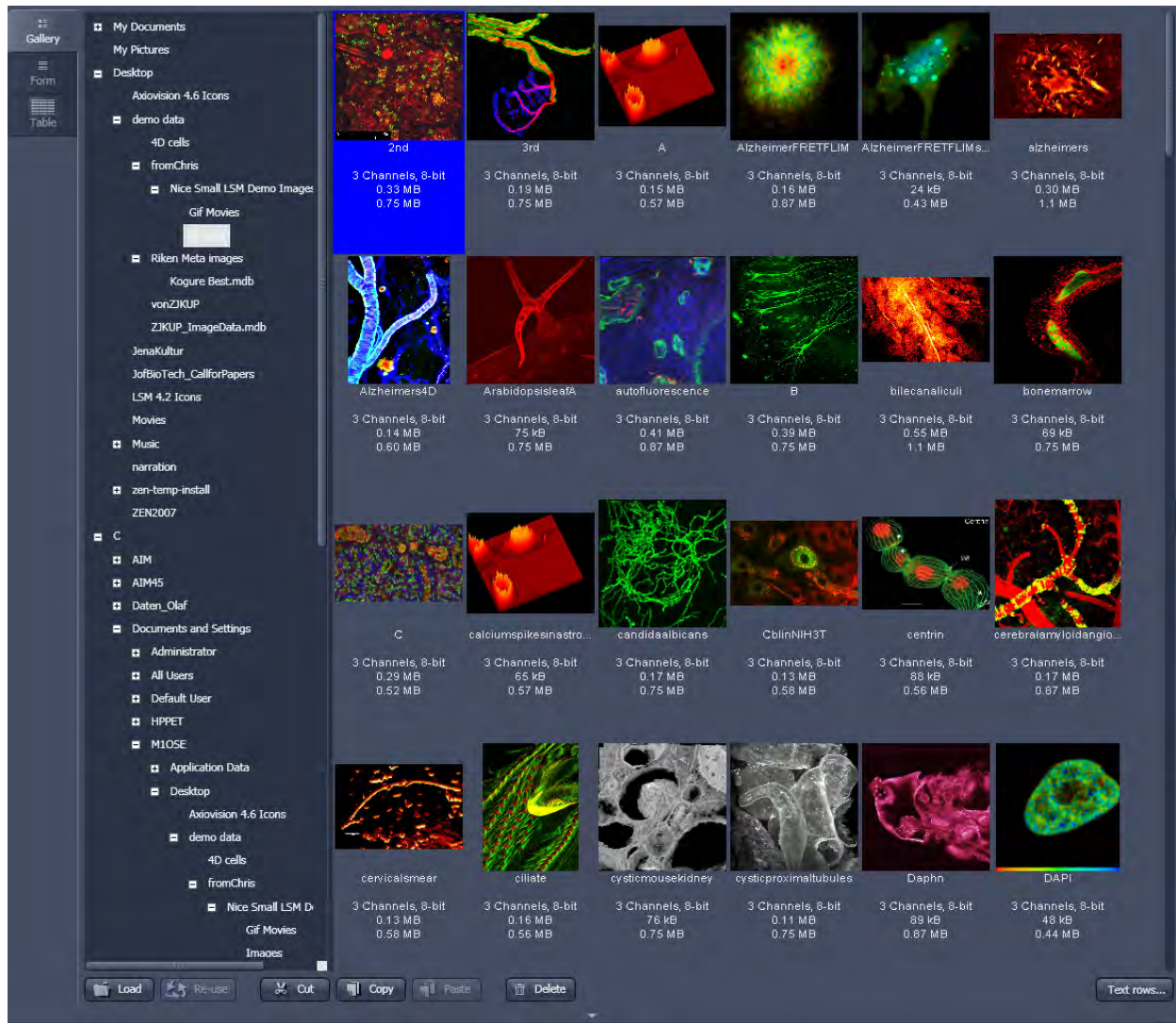


Fig. 8 New image document in the Open Images Areas

Advanced data browsing is available through the **ZEN File Browser** (Ctrl + F or from the **File** menu). The 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.



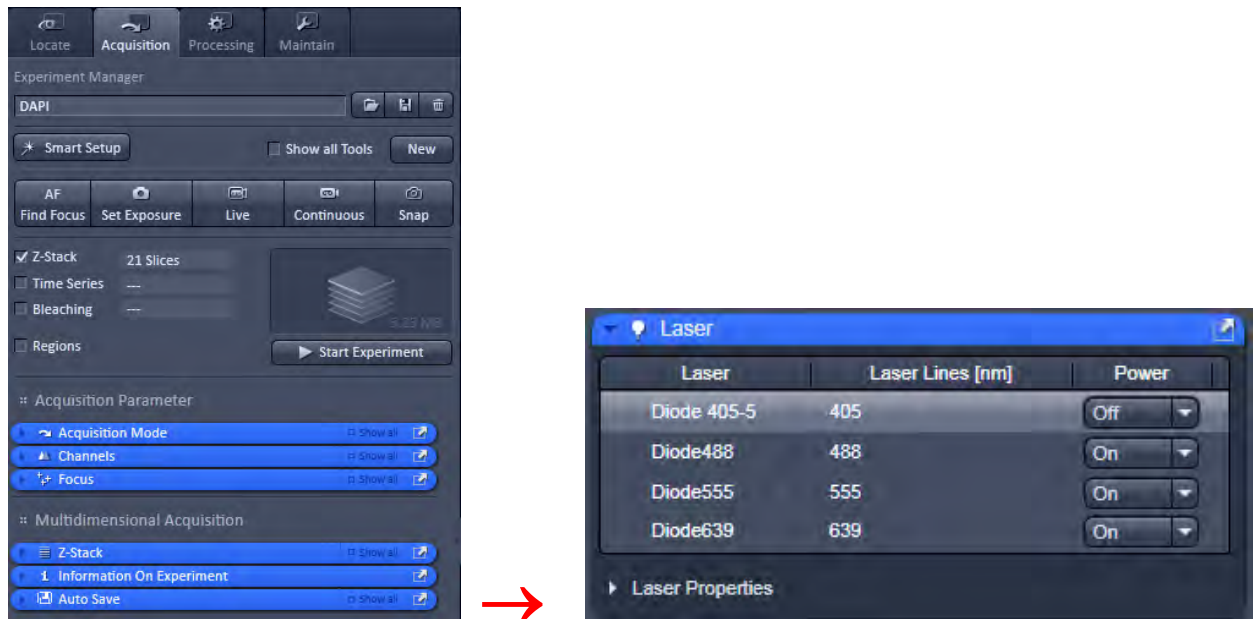
**Fig. 9** File Browser



## Turning on the lasers

ZEN 2011 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 all tools** tick box and open the **Laser** tool. All available lasers can be operated within this tool (Fig. 10).



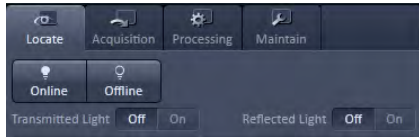
**Fig. 10** Laser Control tool

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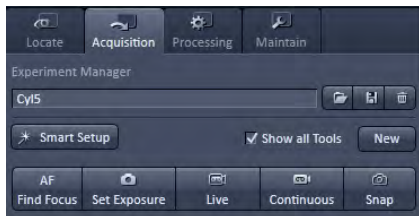
## Setting up the microscope

### Changing between direct observation and laser scanning mode

On the **Locate** tab, the basic switch between manual operation of the microscope (observation of the specimen with the eye) and laser scanning mode is located.



- Click on the **Locate** tab to access the microscope controls. 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 **Locate** tab is set in the active mode (**Online**) again. When leaving the **Locate** tab the software remembers the state of the **Online/Offline** buttons.



- Click on the **Acquisition** tab to start imaging (laser scanning mode). After pressing one of the start buttons the system automatically switches laser scanning mode.

## Setting up the microscope and storing settings

Click on the **Locate** 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

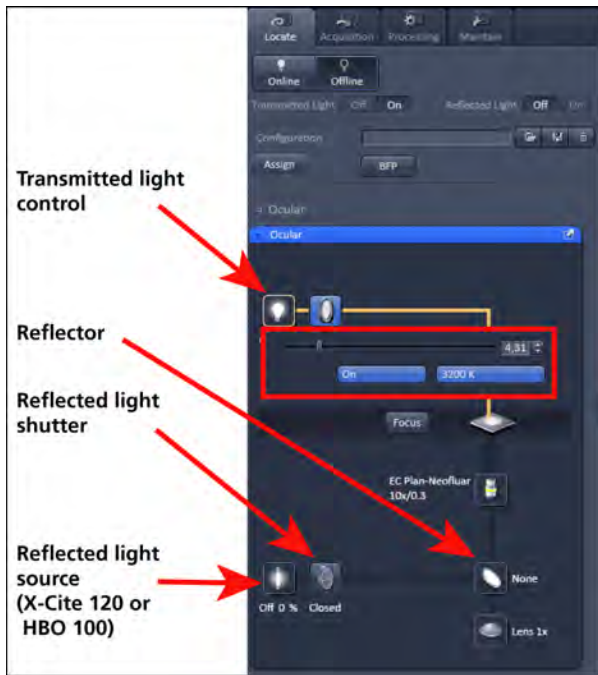
- Open the graphical pop-up menu by clicking on the **Objective** symbol and select the objective lens for your experiment (Fig. 11).
  - The chosen objective lens will automatically move into the beam path.

### 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.



**Fig. 11** Microscope Control window, e.g.: Axio Observer





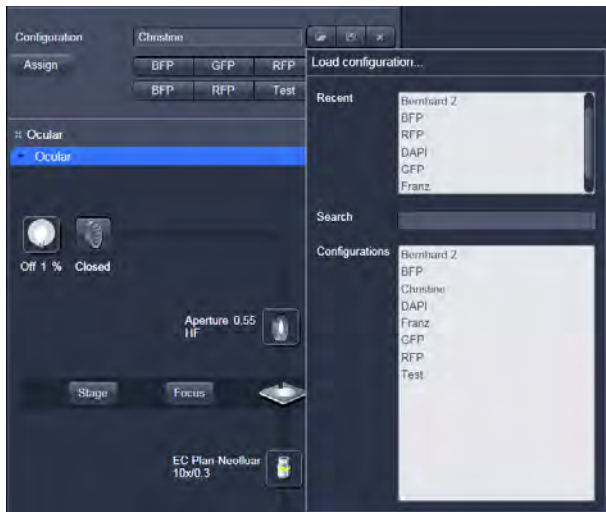
**Fig. 12** Microscope Control window with Transmitted Light pop-up menu

### Setting the microscope for reflected light

- Click on the **Reflected light source** 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 pressing the save button  and typing a configuration name in the pop-up window – confirm the name by clicking **OK**. Fast restoration of a saved configuration is achieved by selecting the configuration from the pull-down list. The configuration is then immediately loaded and possible hardware changes are performed. Configurations can be selected from a pull down list and deleted by pressing the delete  button.



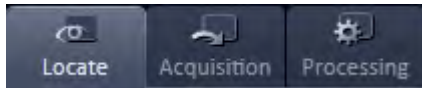
**Fig. 13** Saving and loading Microscope configurations

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

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

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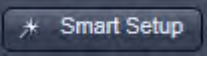
## Configuring the beam path and lasers

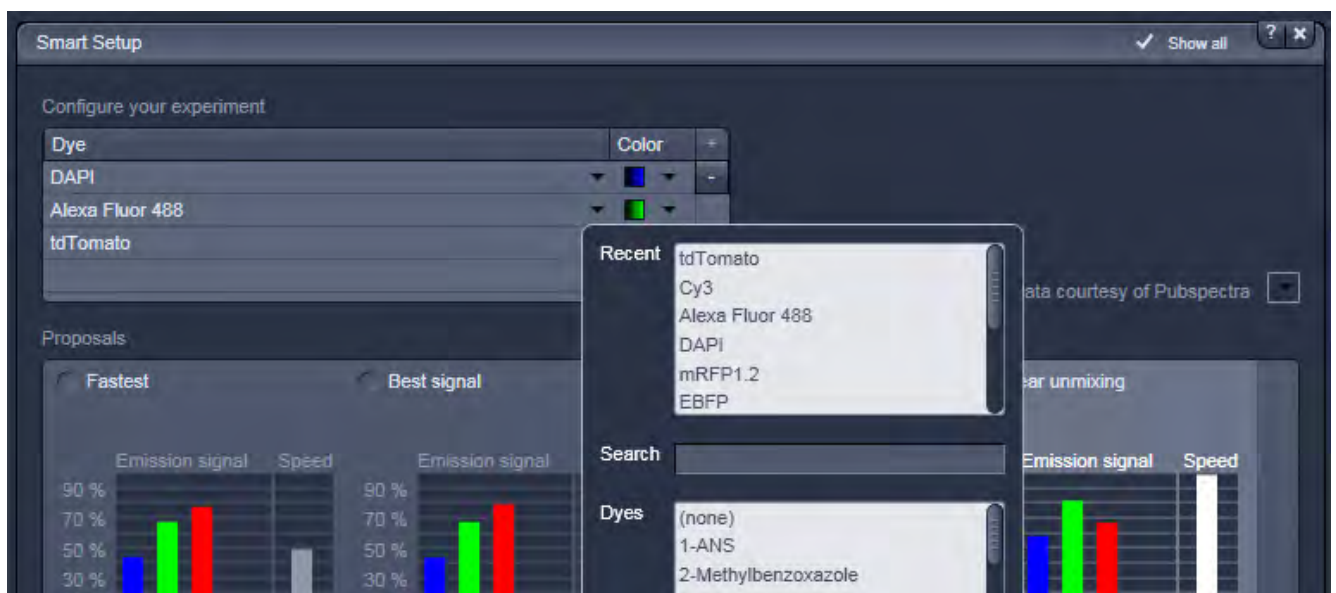


- Click the **Acquisition** button.

### 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.

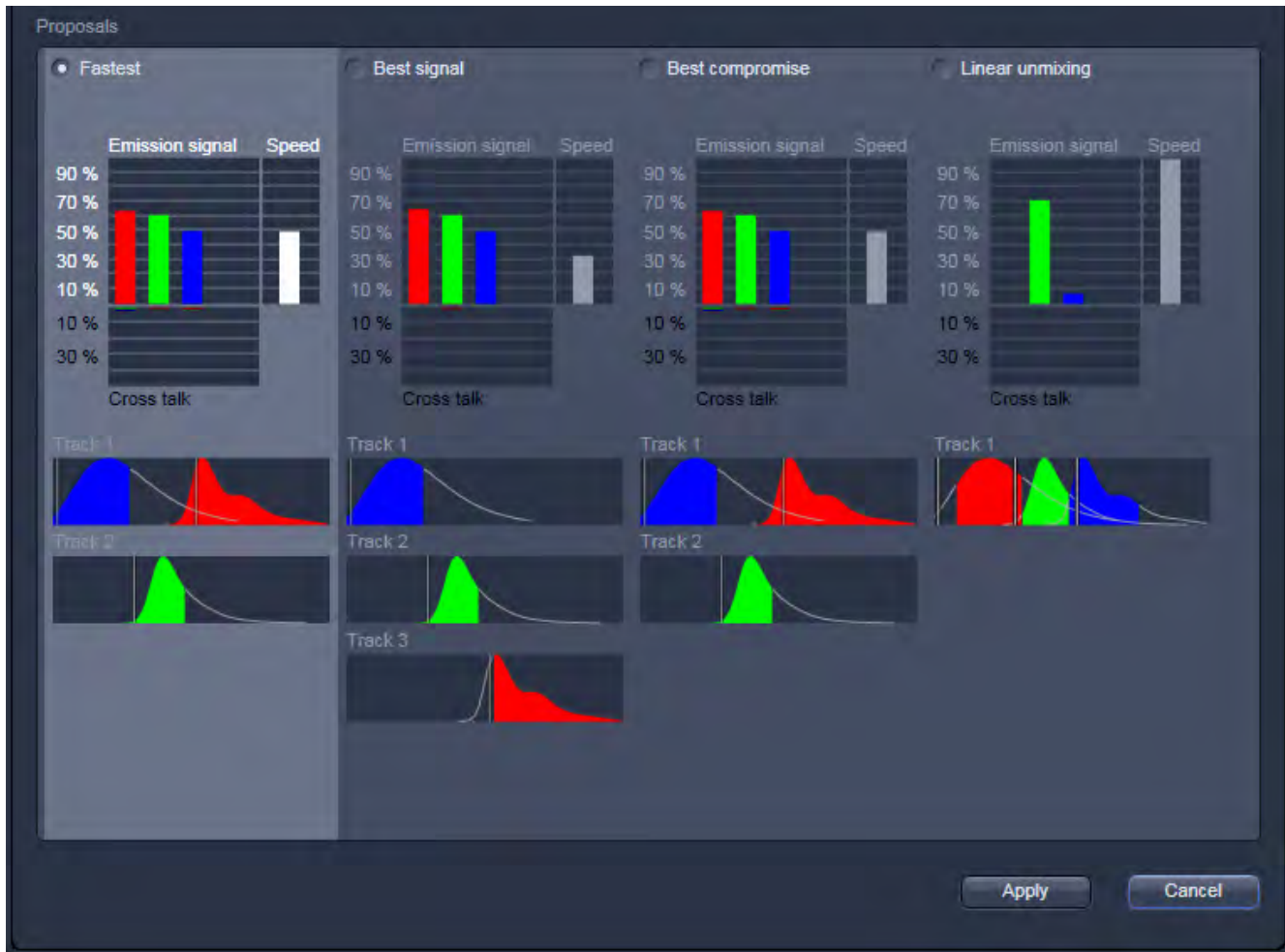
- Click on the **Smart Setup** button  to open the smart setup window. This window can be accessed any time from the software to change dye combinations.
- Click on the arrow in the dye list and simply choose the dye(s) you want to use in your experiment from the list dialogue. In this dialogue, the 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 chosen, the system is set in the lambda mode automatically.

Pressing the **Set Exposure**  button will then optimize the settings of the **Gain** for the given laser power and pinhole size. Further image optimization from this point can be done easily.

## Setting up a configuration manually

For the manual setup of the system (without using the smart setup functionality) tick the **Show all tools** tick box on top of the **Acquisition** tab. The tool group **Setup Manager** will appear containing the tools **Laser**, **Imaging Setup** and **Light Path**.

**Simultaneous** scanning of single, double, and triple labeling:

- Advantage: faster image acquisition
- Disadvantage: 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 **Light Path** tool in the **Setup Manager** tool group and the **Channels** tool in the **Acquisition Parameter** tool group to setup the imaging configuration. De-activate the **Show all** mode.

The open **Light Path** is shown in Fig. 16.

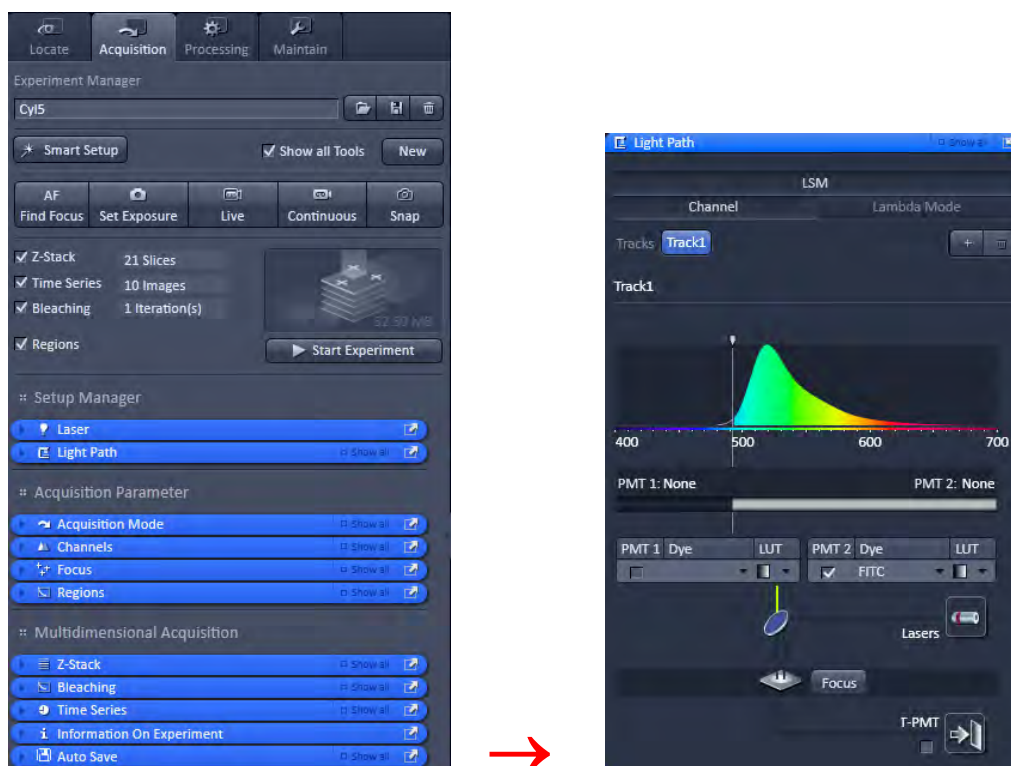


Fig. 16 Light Path tool for a single track (LSM 700)

## Settings for track configuration in Channel mode

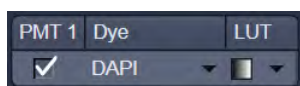
- Select **Channel** mode if necessary (Fig. 16).
- Click on the **LSM** button in the light path tool (Fig. 16).

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

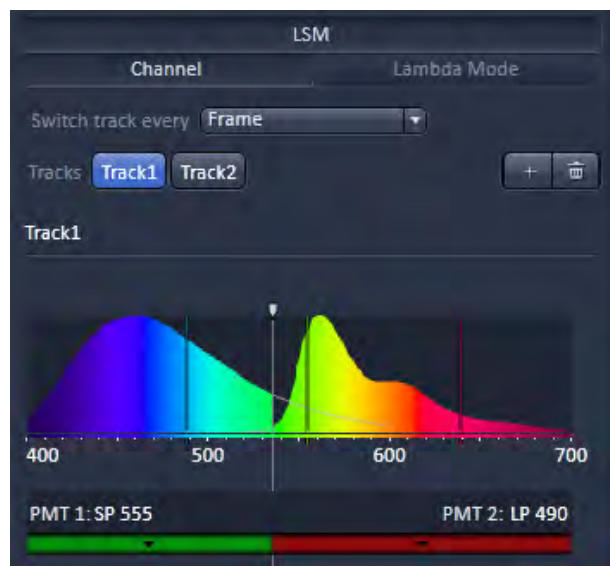
- You can change the settings of the light path tool using the following functional elements:



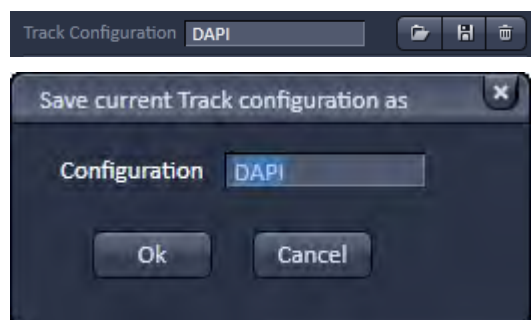
Activation / deactivation of the excitation wavelengths (check box) and setting of excitation intensities (slider). If necessary open the **Laser Control** tool (see above).



Activation / deactivation (via check box) of a channel (**PMT1, 2**), the dye to be detected with this channel (**dye**), and a color for display (**LUT**) of the acquired information.



**Fig. 17** Detection bands & Laser lines display



**Fig. 18** Track Configurations window

- The system will automatically: a) select the most appropriate position of the secondary dichroic mirror and b) choose the suitable emission filters for the dye combination entered for PMT 1 and PMT 2.
- The emission spectra and the laser lines used of the selected dyes are displayed in the light path tool.
- Click the **Laser** icon to select the laser lines and set the attenuation values (transmission in %) in the light path tool.
- The Detection Bands & Laser Lines are also displayed in a spectral panel (Fig. 17) to visualize the activated laser lines for excitation (vertical lines) and activated detection channels (colored horizontal bars).
- For storing a new track configuration open the **Channels** tool in the **Acquisition Parameter** tool group, click and enter a desired name in the box (Fig. 18). Press **OK** to confirm.
- For loading an existing configuration click on and select a configuration from the list box.
- For deleting an existing configuration click and select it from the list box. Press **Ok** to confirm the deletion.



---

## 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.

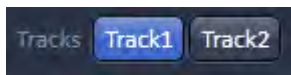
The maximum of four tracks with up to three channels can be defined simultaneously and then scanned one after the other. Each track is a separate unit and can be configured independently from the other tracks with regard to channels, Acousto-Optical Tunable Filters (AOTF), emission filters and dichroic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

The following functions are available in the **Light Path** tool of the **Setup Manager** tool group (, Fig. 17 and Fig. 18).

### Switch track every selection box

- 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 is 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**.

### Tracks line



**Track** buttons: To change between the tracks settings click on the appropriate track button. The selected track is highlighted. The setting table for configuration will be shown behind.



**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.

## Scanning an image

### Setting the parameters for scanning

- Select the **Acquisition Mode** tool from the **Left Tool Area** (Fig. 19).
- 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  $\lambda$ .

The number of pixels influences the image resolution!

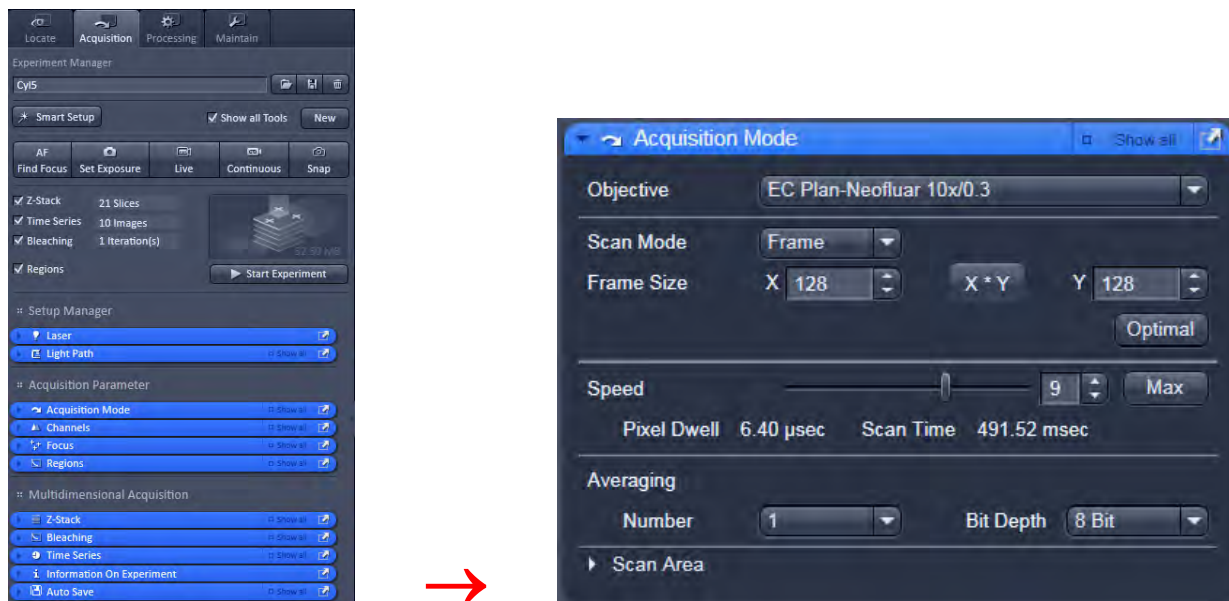


Fig. 19 Acquisition Mode tool

### Adjusting scan speed

- Use the **Scan Speed** slider in the **Acquisition Mode** tool (Fig. 19) 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 or 16 Bit (per pixel) in the **Bit Depth** pull-down in the **Acquisition Mode** tool (Fig. 19).

8 Bit will give 256 gray levels; 12 Bit will give 4096 gray levels, 16 Bit will give 65536 gray levels. Publication quality images should be acquired using 12 Bit data depth. 12 or 16 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

- 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.

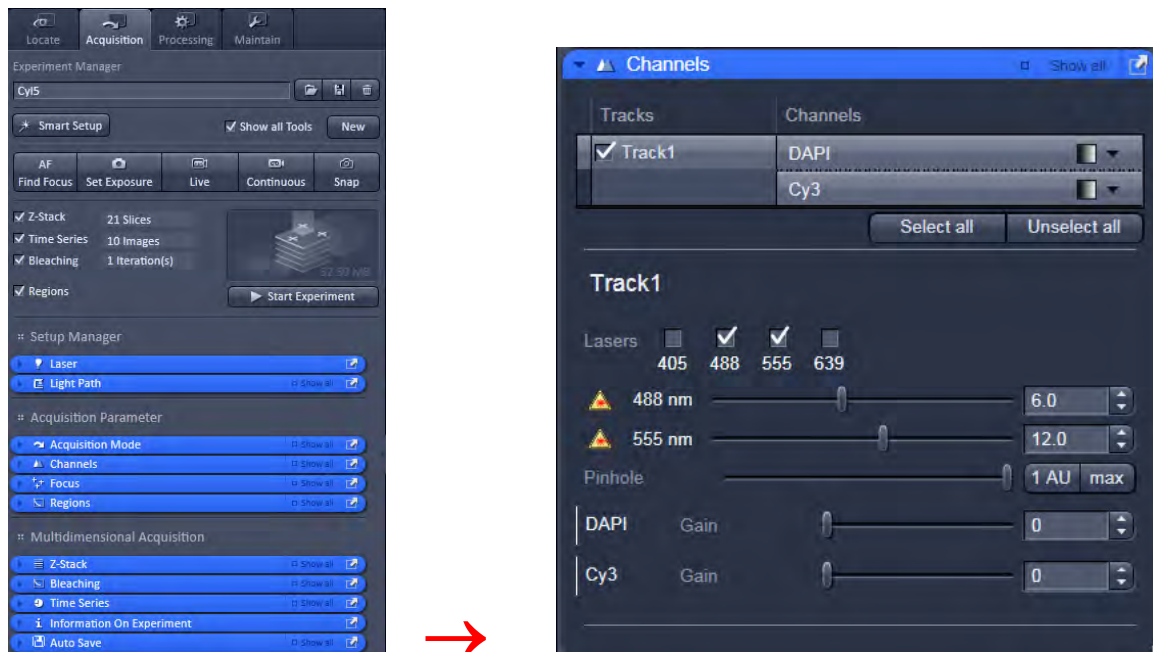


Fig. 20 Channels tool

## Image acquisition

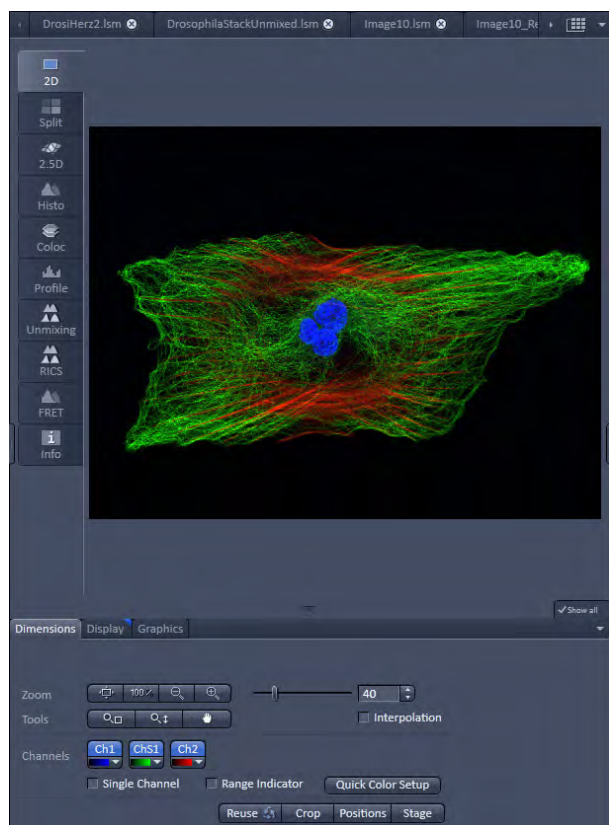
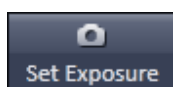


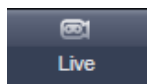
Fig. 21 Image Display

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

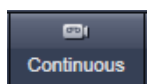
- Use one of the **AutoExposure**, **Live**, **Continuous** or **Snap** buttons to start the scanning procedure to acquire an image.
- Scanned images are shown in separate windows.
- Click on the **Stop** button to stop the current scan procedure if necessary.



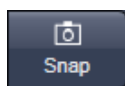
Select **Set Exposure** for automatic pre-adjustment of detector gain and offset.



Select **Live** for continuous fast scanning – useful for finding and changing the focus.



Select **Continuous** for continuous scanning with the selected scan speed.



Select **Single** for recording a single image.



Select **Stop** for stopping the current scan procedure.


## Image optimization



Fig. 22 View Dimensions Control Tab

### Choosing Range Indicator

- In the **View – Dimensions** View Option Control Tab, activate the **Range indicator** check box (Fig. 22).

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

The scanned image appears in a false-color presentation (Fig. 23).

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 AU** (Airy Unit) (Fig. 24).
- Set the **Gain** high.
- When the image is saturated, Laser Power in the **Laser** control section of the **Channels** tool (Fig. 24) using the slider to reduce the intensity of the laser light to the specimen.

### Adjusting gain

- Reduce the **Gain** (Fig. 24) until the red pixels only just disappear.

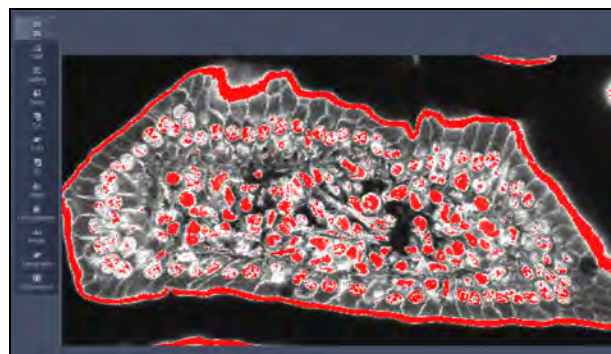
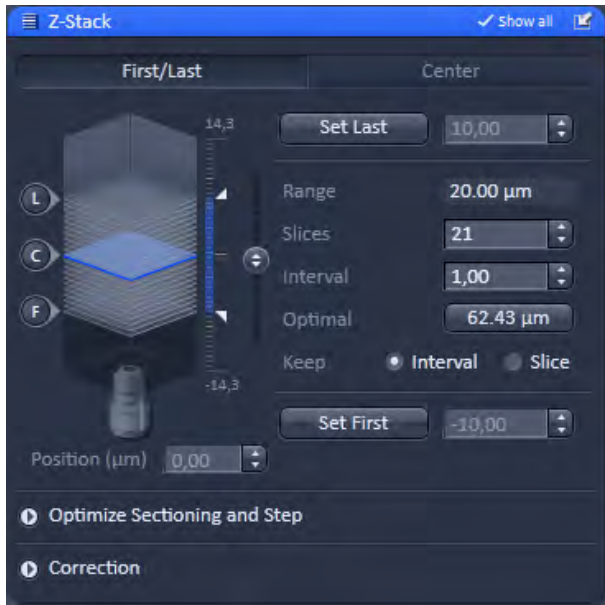


Fig. 23 Image Display




Fig. 24 Channels tool



**Fig. 25** Z Stack tool

## Scanning a Z-Stack

- Select **Z-Stack**  **Z-Stack** in the main tools area.
  - Open the **Z-Stack** tool in the **Left Tool Area**.
  - Click on the **Continuous** button in the **Action Button** area.  
A continuous XY-scan of the set focus position will be performed.
  - Use the focus drive of the microscope to focus on the upper position of the specimen area where the Z-Stack is to start.
  - Click on the **Set First** button to set the upper position of the Z-Stack.
  - Then focus on the lower specimen area where the recording of the Z-Stack is to end.
  - Click on the **Set Last** button to set this lower position.
  - Click on the **Optimal** **62.43 μm** button to set number of slices to match the optimal Z-interval for the given stack size, objective lens, and the pinhole diameter.
  - Click on the **Start Experiment** 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.

## Storing and exporting image data

- 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. 26/1), or click on the  button at the bottom of the **File Handling Area** (Fig. 26/2)

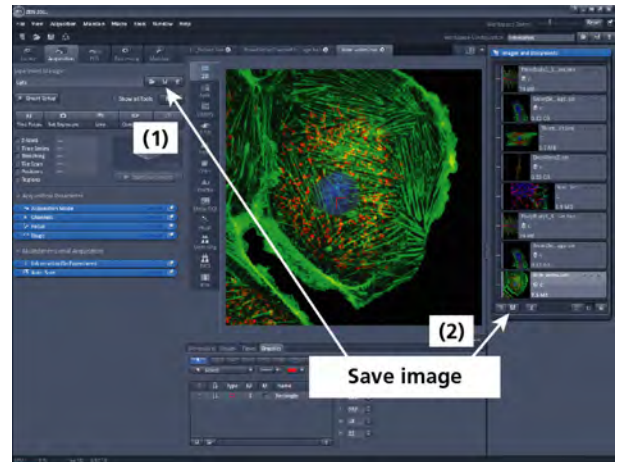


Fig. 26 Save Image buttons in ZEN

- The WINDOWS **Save As** window (Fig. 27) appears.
- Enter a file name and choose the appropriate image format.  
Note: The LSM 5 format is the native Carl Zeiss LSM image data format and contains all available extra information and hardware settings of your experiment.
- Click on the **Save** button.

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

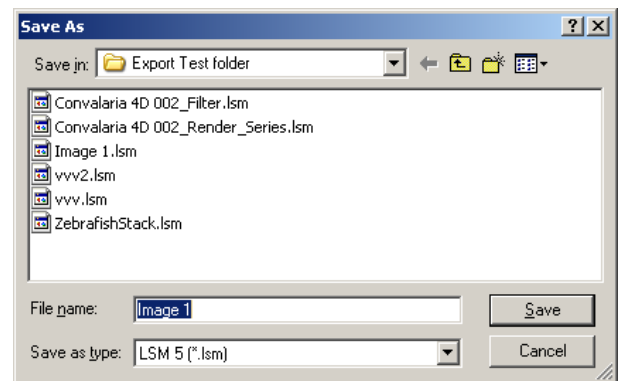


Fig. 27 Save as window

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.



Fig. 28 Export window

---

## Switching off the system

- Click on the **File** button in the menu bar and then click on the **Exit** button to leave the **ZEN 2011** software.
- Shut down the computer.
- Turn the key switch (Fig. 1/1) of the laser module to the left in the off position.
- If using the Axio Observer microscope stand, push the power button at the left hand side to switch off the microscope.
- Turn off the LSM 700 system via the switch-operated two multipoint connectors on the system.
- Switch off the HXP 120 / HBO 100 or XBO 75 lamps and, if necessary, the scanning stage and other attached components.



Carl Zeiss

# Confocal Laser Scanning Microscopy

$$\text{Resolution} = \frac{0.64 \cdot \lambda}{(n - \sqrt{n^2 - \text{NA}^2})}$$



We make it visible.

# **Confocal Laser Scanning Microscopy**

Stefan Wilhelm

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## PART 1 **Optical Image Formation**

Part 1 deals with the purely optical conditions in a confocal LSM and the influence of the pin-hole on image formation. From this, ideal values for resolution and optical slice thickness are derived.

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## PART 2 **Signal Processing**

Part 2 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.

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## PART 3 **Details**

Part 3 deals with special aspects of Part 1 and 2 from a more physical perspective.

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<b>Optical Coordinates</b>	<b>II</b>
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## Literature

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.

# Introduction

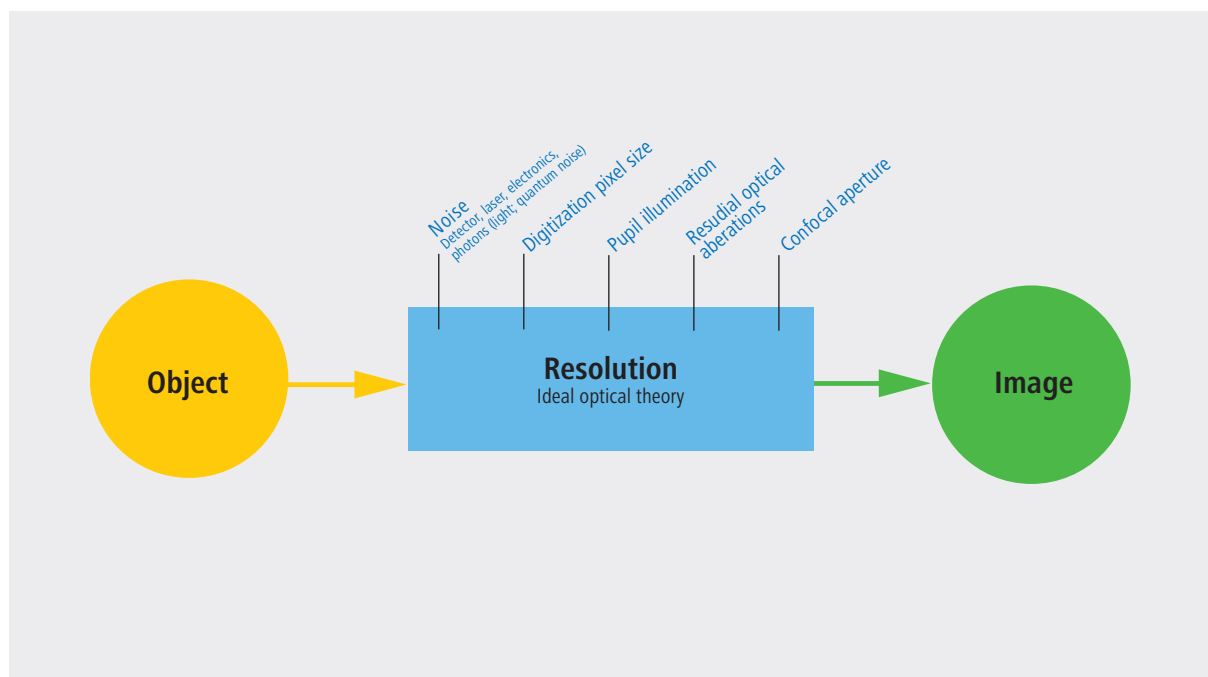
Following a description of the fundamental differences between a conventional and a confocal microscope, this monograph will set out the special features of the confocal LSM and the capabilities resulting from them. The conditions in fluorescence applications will be given priority treatment throughout.

## 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. 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.*



## 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 pointwise 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 laser scanning microscopes from Carl Zeiss. 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).

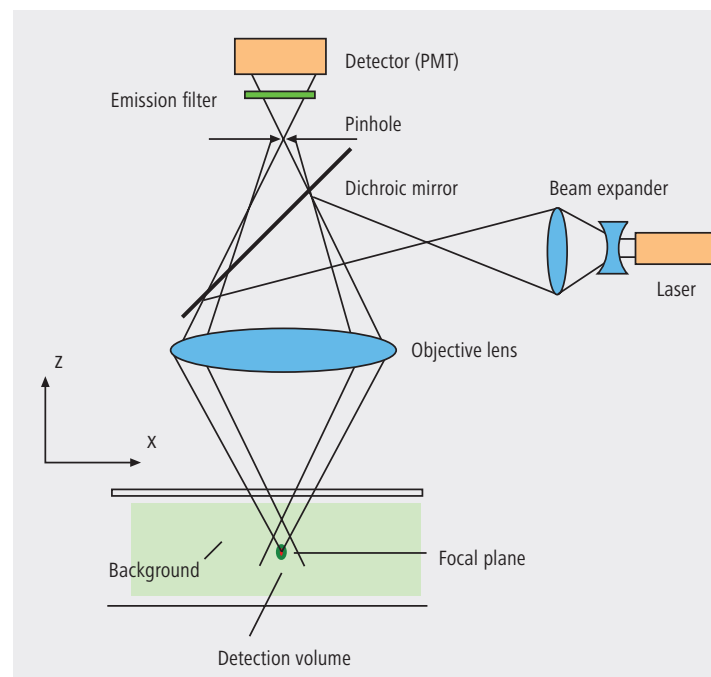
As the laser beam is focused to a diffraction-limited spot, which illuminates only a point of the object at a time, the point illuminated and the point observed (i.e. image and object points) are situated in conjugate planes, i.e. they are focused onto each other. The result is what is called a confocal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

*Fig. 2 Beam path in a confocal LSM. A microscope objective lens 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 lens. 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.*



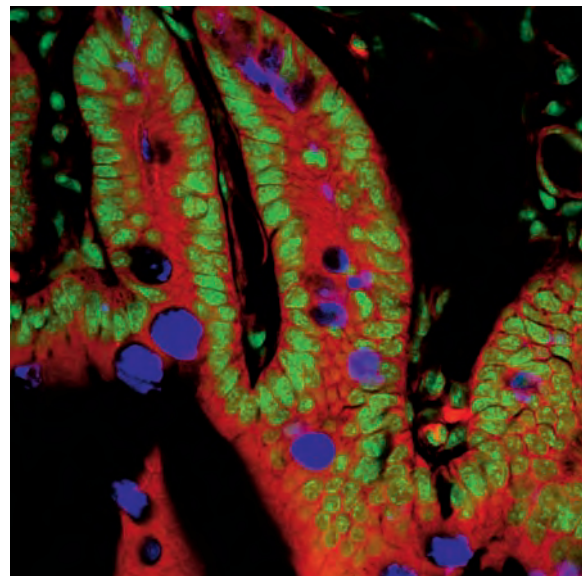
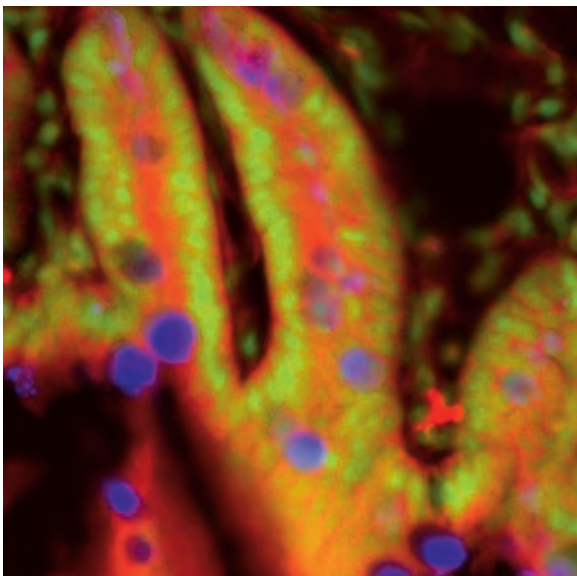
## Optical slices

With a confocal LSM and its variable pinhole it is therefore possible to exclusively image a thin optical slice out of a thick specimen (typically, up to 100  $\mu\text{m}$ ), 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 lens.

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).

Hence it follows that a confocal LSM can be used to advantage especially where thick fluorescent specimens (such as biological cells in tissue) have to be examined. 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 furthermore satisfactorily separated and can be recorded simultaneously.

Figure 3 (right) demonstrates this capability of a confocal Laser Scanning Microscope.



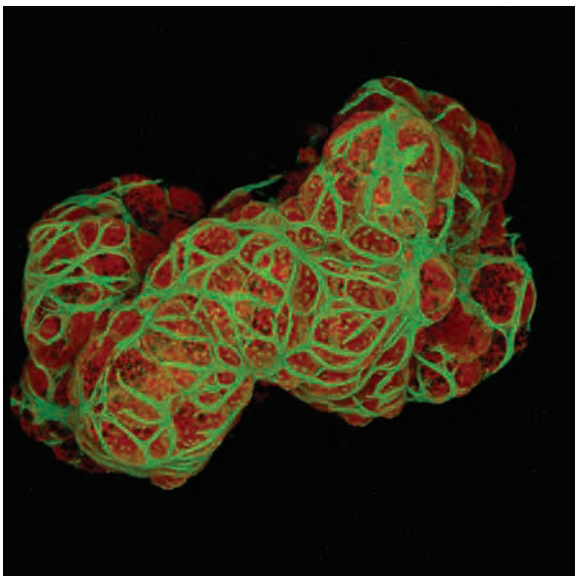
*Fig. 3 Non-confocal (left) and confocal (right) image of a triple-labeled cell aggregate (mouse intestine section). In the non-confocal image, specimen planes outside the focal plane degrade the information of interest from the focal plane, and differently stained specimen details appear in mixed color.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



### 3<sup>rd</sup> 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 Z-planes of the specimen, with the specimen being moved along the optical axis 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).



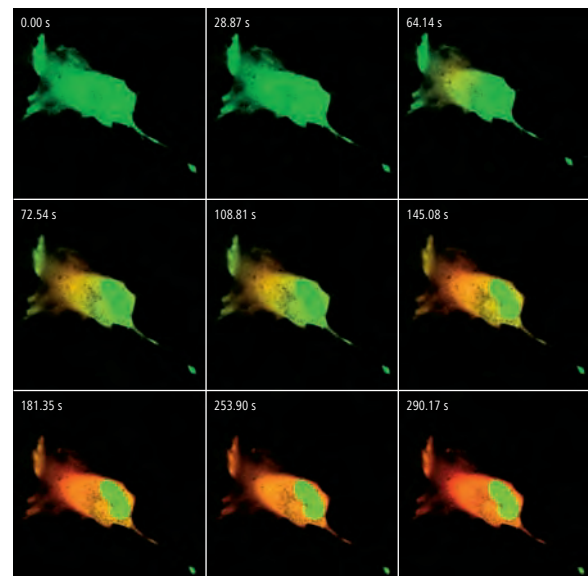
### Time series

A further field of 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 (figure 5).

### Spectral Imaging

The detection of spectral information becomes necessary when overlapping emission signals of multiple marked specimen are to be separated. By means of a special Multichannel PMT it is possible to record the spectral information of the fluorescent signal. Together with advanced computation methods one can separate signals which almost overlap completely and only differ in their spectral signature. The method behind is called "linear unmixing" and allows, for example, several fluorescent proteins to be distinguished.

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-PSF.

In the ideal, diffraction-limited case (no optical aberrations, and homogeneous illumination at all lens cross sections – see Part 3 “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 of the objective lens 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, i.e. the well-defined area in which the intensity of the 3D PSF in axial and lateral direction has dropped to half of the central maximum.

Any reference to the PSF in the following discussion exclusively refers to the half-maximum area.

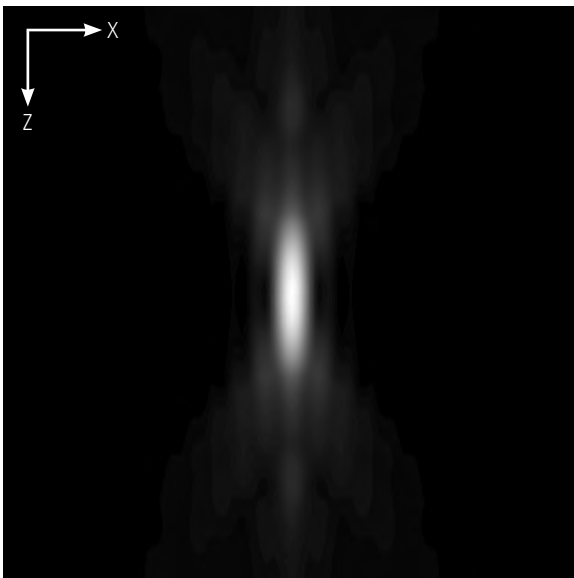
As said before the confocal LSM system as a whole generates two point images: one by projecting a point light source into the object space, the other by projecting a point detail of the object into the image space.

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). 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 lens. It is also influenced by diffraction at the objective lens pupil (as a function of pupil illumination, see details) 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., [7]).

*Fig. 6a Section through the 3D-PSF in Z direction as generated by a diffraction limited objective lens – left, and in XY-direction – right (computed; dimensionless representation); the central, elliptical maximum is distinctly visible.*



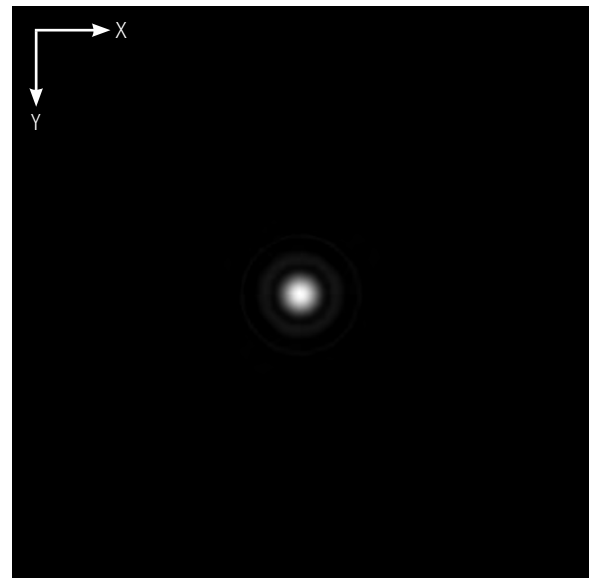
$PSF_{det}$  is also influenced by all these factors and, additionally, by the pinhole size. For reasons of beam path efficiency (see Part 2), the pinhole is never truly a point of infinitely small size and thus  $PSF_{det}$  is never smaller in dimension than  $PSF_{ill}$ . It is evident that the imaging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

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.

*Fig. 6b The central maximum in this illustration is called Airy disk and is contained in the 3D-PSF as the greatest core diameter in lateral direction.*



# Resolution and Confocality

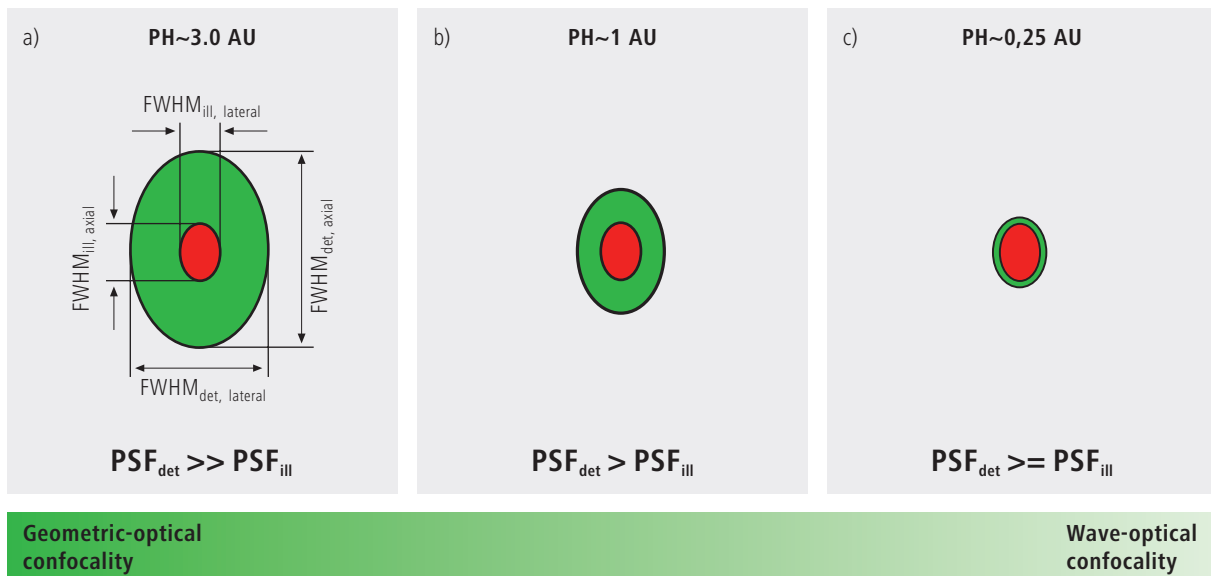
Wherever quantitative data on the resolving power and depth discrimination of a confocal LSM are specified, it is necessary to distinguish clearly whether the objects they refer to are point-like or extended, and whether they are reflective or fluorescent. These differences involve distinctly varying 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 the pinhole diameter, the data and computation methods for resolution and depth discrimination will 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,  $PSF_{det}$  (green) shrinks until it approaches the order of magnitude of  $PSF_{ill}$  (red).



## Resolution

Resolution, in case of large pinhole diameters ( $PH > 1AU$ ), 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 objective  
 $\lambda_{exc}$  = wavelength of the excitation light

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

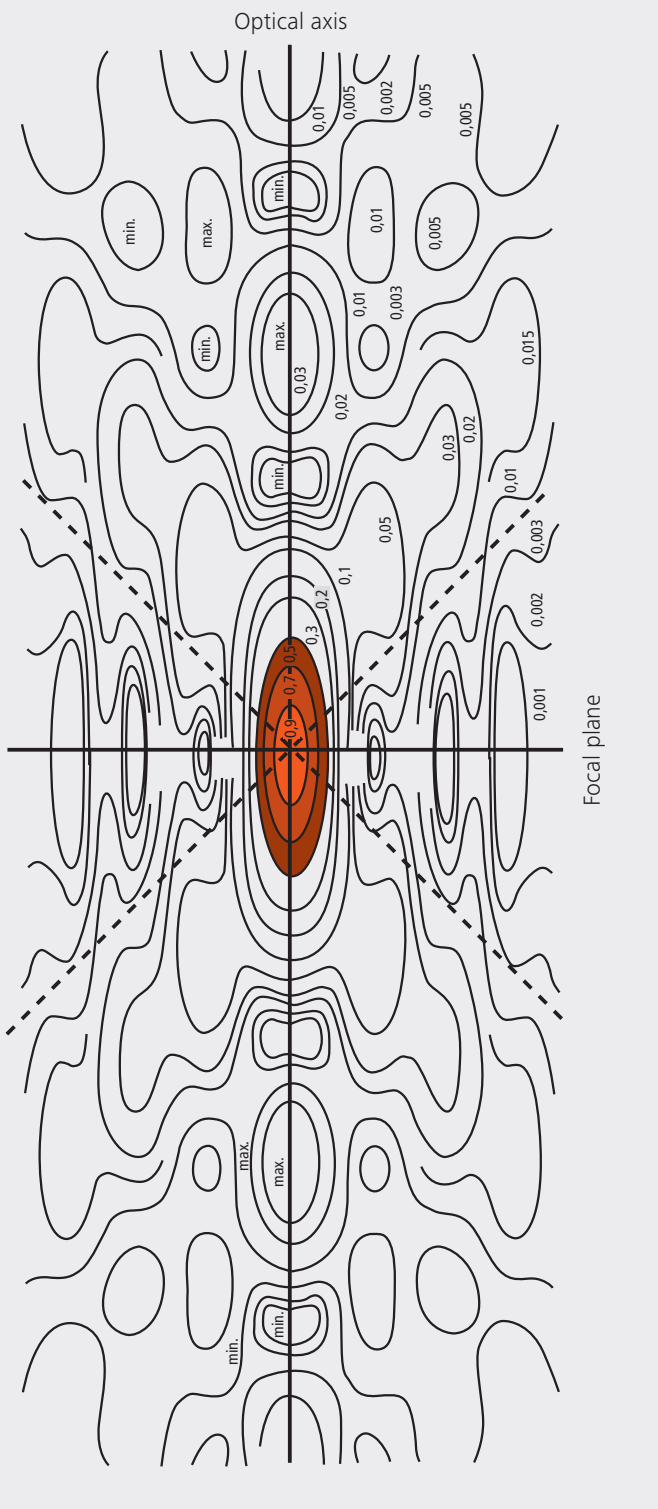
$$\approx \frac{1.77 \cdot n \cdot \lambda_{exc}}{NA^2} \quad (2a)$$

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., [1]). 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 the PSF so far be further illustrated by the figure on the left. It shows a section through the resulting diffraction pattern surrounding the focus on the illumination side ( $PSF_{ill}$ ).

The lines include areas of equal brightness (isophote presentation). The center has a normalized intensity of 1. The real relationships result by rotation of the section about the vertical (Z) axis. Symmetry exists relative to the focal plane as well as to the optical axis. Local intensity maxima and minima are conspicuous. The dashed lines mark the range covered by the aperture angle of the microscope objective used.

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

*Fig. 8 Isophote diagram of the intensity distribution around the illumination-side focus ( $PSF_{ill}$ ). The intensity at the focus is normalized as 1. (Born & Wolf, 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:

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

$\lambda_{em}$  = emission wavelength  
 $PH$  = object-side pinhole diameter [ $\mu m$ ]  
 $n$  = 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 lens 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 waveoptical 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).

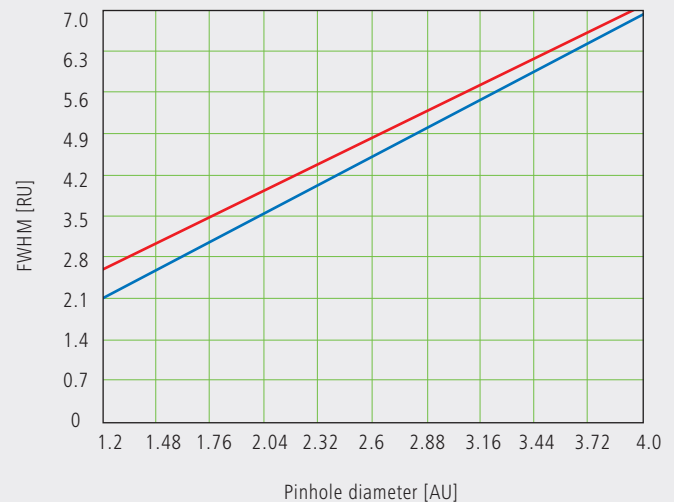


Fig.9 Optical slice thickness as a function of the pinhole diameter (red line). Parameters:  $NA = 0.6$ ;  $n = 1$ ;  $\lambda = 520$  nm. The X axis is dimensioned in Airy units, the Y axis (slice thickness) in Rayleigh units (see also: Details "Optical Coordinates"). In addition, the geometric-optical 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  $\lambda_{exc}$  and the emission wavelength  $\lambda_{em}$ . This is done by specifying a mean wavelength<sup>1</sup>:

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

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 (2) 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 parameters (NA,  $\bar{\lambda}$ , n) influencing the optical slice thickness, the type of specimen also affects the measurement result.**

<sup>1</sup> For rough estimates, the expression  $\bar{\lambda} \approx \sqrt{\lambda_{em} \cdot \lambda_{exc}}$  suffices.

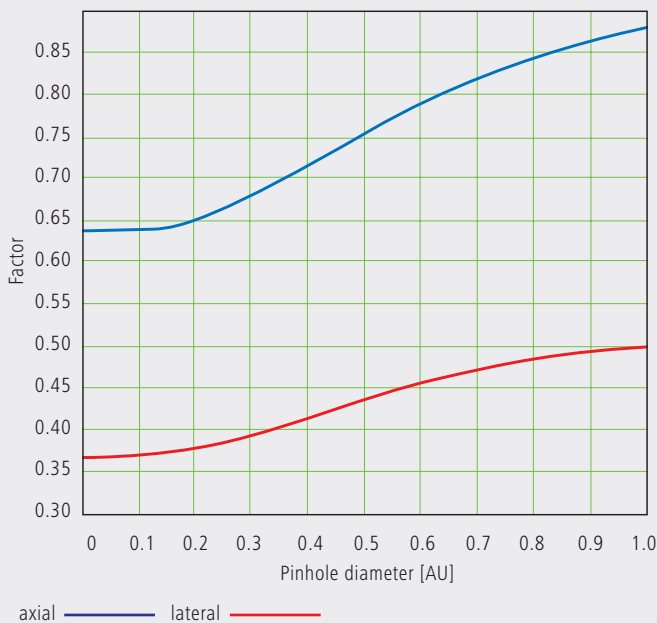


From equations (7) and (7a) it is evident that depth resolution varies linearly with the refractive index  $n$  of the immersion liquid and with the square of the inverse value of the numerical aperture of the objective lens  $\{NA = n \cdot \sin(\alpha)\}$ . To achieve high depth discrimination, it is important, above all, to use objective lenses 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 case 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.

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



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.

To conclude the observations about resolution and depth discrimination (or depth resolution), the table on page 17 provides an overview of the formulary relationships developed in Part 1. In addition, figure 11a (page 16) 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 11 (b–d), equation (7) is plotted for different objects and varied parameters ( $NA$ ,  $\bar{\lambda}$ ,  $n$ ).

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

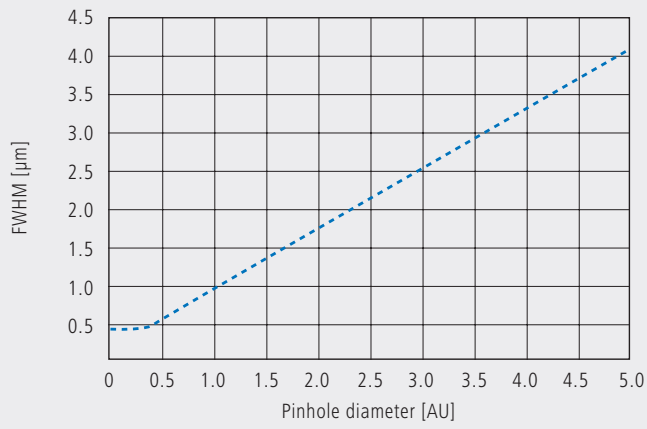
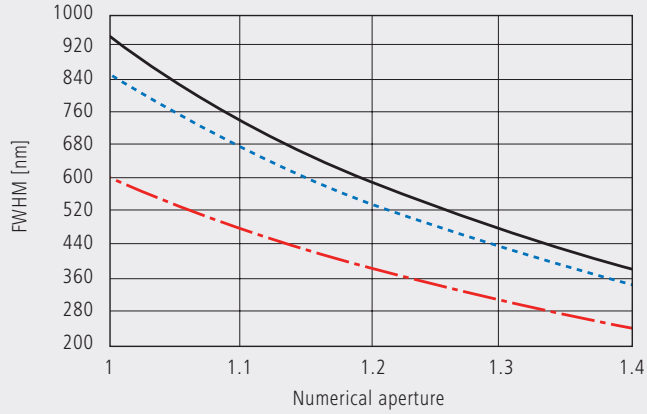


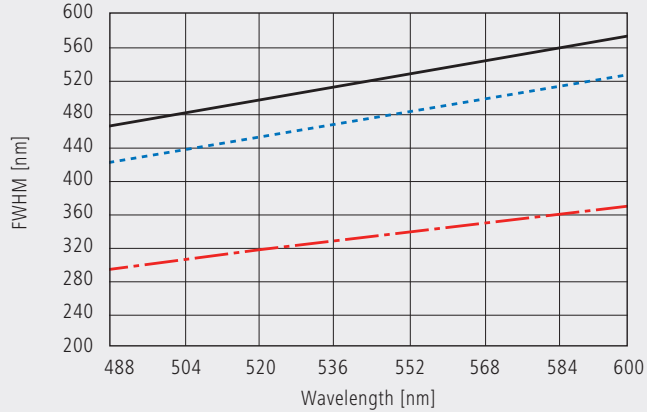
Fig. 11  
a) Variation of  
pinhole diameter

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



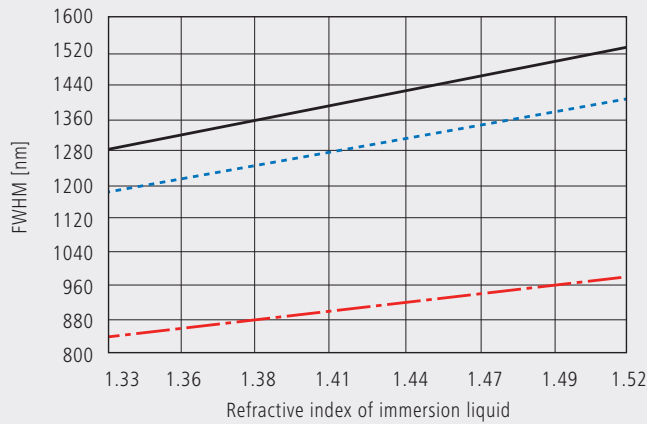
b) Variation of  
numerical aperture

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



c) Variation of  
wavelength ( $\lambda$ )

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



d) Variation of  
refractive index

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

# Overview

Conventional microscopy	Confocal microscopy $1 \text{ AU} < \text{PH} < \infty$	Confocal microscopy $\text{PH} < 0.25 \text{ AU}$
<p>1. Optical slice thickness <b>not definable</b>            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 thickness<sup>1)</sup></p> $\sqrt{\left(\frac{0.88 \cdot \lambda_{em}}{n \cdot \sqrt{n^2 - NA^2}}\right)^2 + \left(\frac{\sqrt{2} \cdot n \cdot \text{PH}}{NA}\right)^2}$ <p>Corresponds to the FWHM of the intensity distribution behind the pinhole (<math>\text{PSF}_{\text{det}}</math>). 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 <math>\mu\text{m}</math>.</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. <math>\bar{\lambda}</math> stands for a mean wavelength – see text 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 of field.</p>	<p>2. Axial resolution</p> $\frac{0.88 \cdot \lambda_{exc}}{(n \cdot \sqrt{n^2 - NA^2})}$ <p>FWHM of <math>\text{PSF}_{\text{ill}}</math> (intensity distribution at the focus of the microscope objective) in Z direction.</p> <p>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</p> <p>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 <math>NA &lt; 0.5</math></p> $\frac{1.77 \cdot n \cdot \lambda_{exc}}{NA^2}$	<p>3. Approximation to 2. for <math>NA &lt; 0.5</math></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 XY direction.</p>	<p>4. Lateral resolution</p> $\frac{0.51 \cdot \lambda_{exc}}{NA}$ <p>FWHM of <math>\text{PSF}_{\text{ill}}</math> (intensity distribution at the focus of the microscope objective) in XY 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 XY 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)  $\text{PH} < \infty$  is meant to express a pinhole diameter of  $\approx 4\text{--}5 \text{ AU}$ .

# 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 factor of influence 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 13, page 20).

The object information gained by the scanning process is, in most cases, detected by a photomultiplier tube (PMT), which 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 cathode of the PMT converts optical information (photon) into electrical information (photoelectron), which is intensified in the following dynode cascade. The resulting continuous electric signal is periodically sampled by a subsequent analog-to-digital (A/D) converter and thus transformed into a discrete, equidistant succession of measured data (pixels) (figure 12).

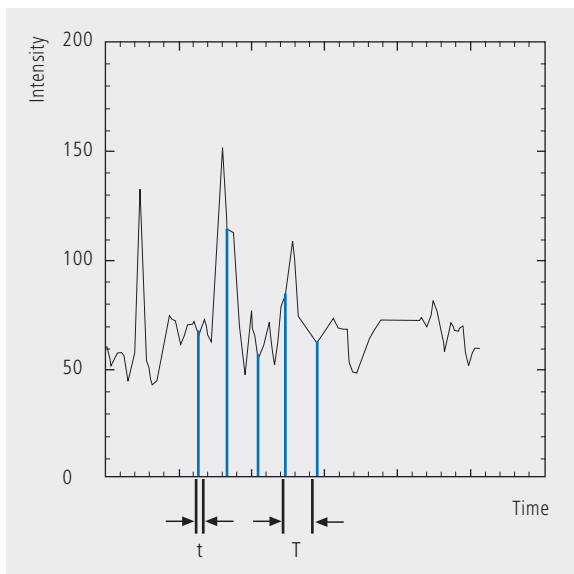
### Types of A/D conversion

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. 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<sub>lat</sub>) 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  $d_{\text{pix}} = 0.5 \times \text{FWHM}_{\text{lat}}$ . With a two-point object (see explanation on page 18), 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 22).

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).

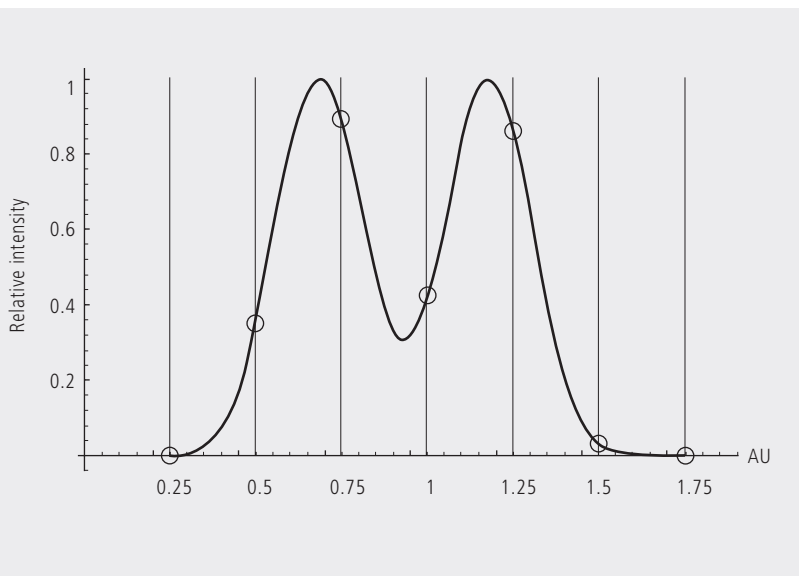
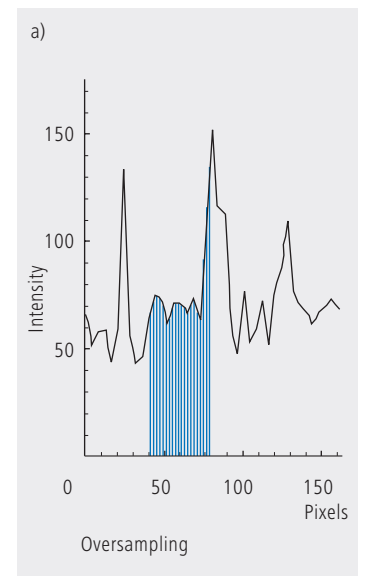


Fig. 14 Oversampling, correct sampling and undersampling of a continuous signal.



## 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 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 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<sub>obj</sub>**    objective magnification

### System constant

LSM 510, LSM 5 PASCAL	8.94 mm
LSM 700	6.36 mm
LSM 710 with Axio Observer	10.08 mm (rearport) 9.31 mm (sideport)
LSM 710 with Axio Imager	10.08 mm (rearport) 9.31 mm (tube)
LSM 710 with Axio Examiner	6.85 mm

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

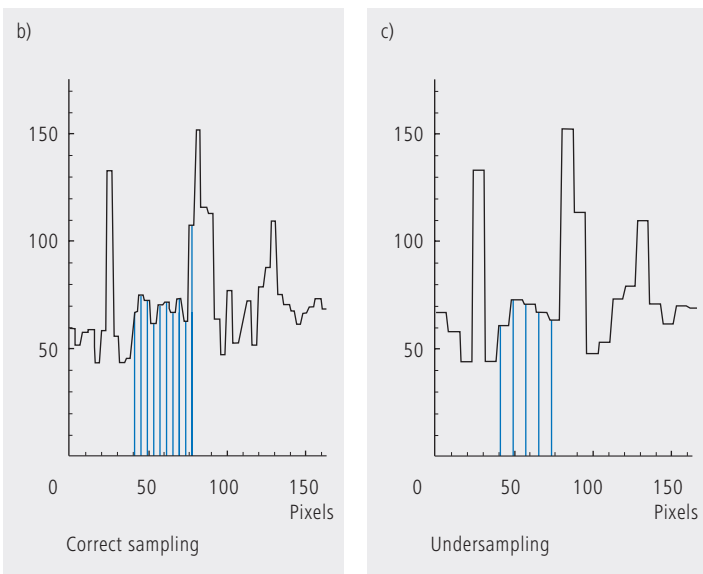
$$Z \geq \frac{3.92 \cdot NA \cdot \text{system constant}}{\text{number of pixels} \cdot \text{magnification}_{obj} \cdot \lambda_{exc}}$$

$NA$  = numerical aperture of objective  
 $\lambda_{exc}$  = excitation wavelength

As an example, with the LSM 710 / Axio Observer (sideport), a 40x objective lens ( $NA=1.2$ ), a wavelength of 488nm and 512 pixels per scanline, the full resolving power (correct sampling) is achieved with a scanning zoom of 4.38; the corresponding pixelsize then is 103.8nm. With lower factors of the scanning zoom the pixel size itself will be the limiting factor for resolution (pixel resolution). Higher factors will cause oversampling. Hence, the zoom factor influences not only the total magnification but also the resolution properties of the system.

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

(With the LSM 710 and LSM 780 the number of pixels (X/Y) per image can be freely selected between 4 x 1 and 6144 x 6144).



# 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 said before 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 in figure 15 show 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 lateral resolution, is minimum in this case.

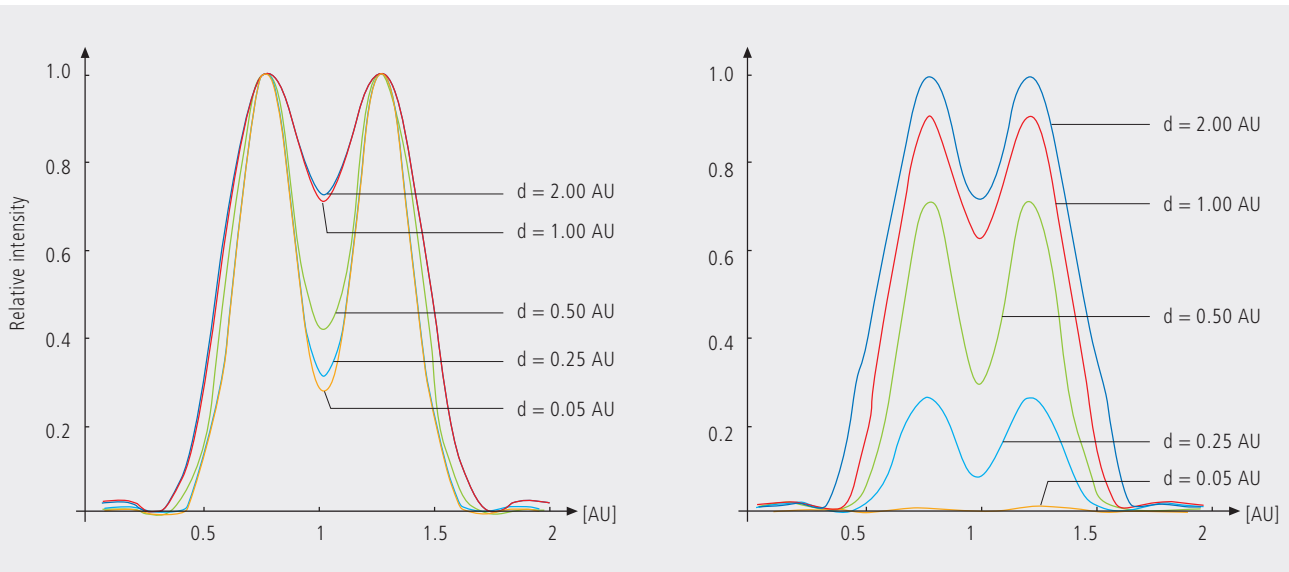
With a pinhole diameter  $<1$  AU, resolution improves (better point separation thanks to a deeper dip), which is penalized by a drastic loss in energy.

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 to PH = 0.05.*





### 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.

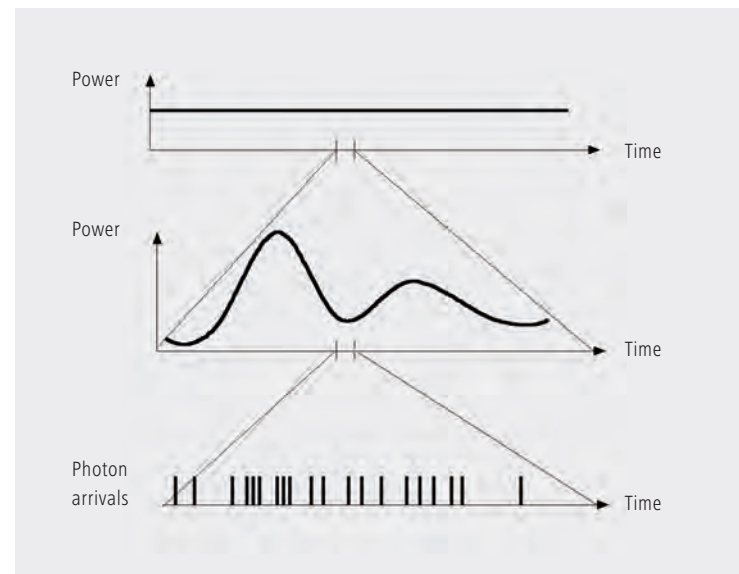
As a possible consequence of the shot noise of the detected light, it may happen, for example, that noise patterns that change because of photon statistics, degrade normally resolvable object details in such a way that they are not resolved every time in repeated measurements. On the other hand, objects 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.

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.

Figure 17 (page 24) 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.



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.

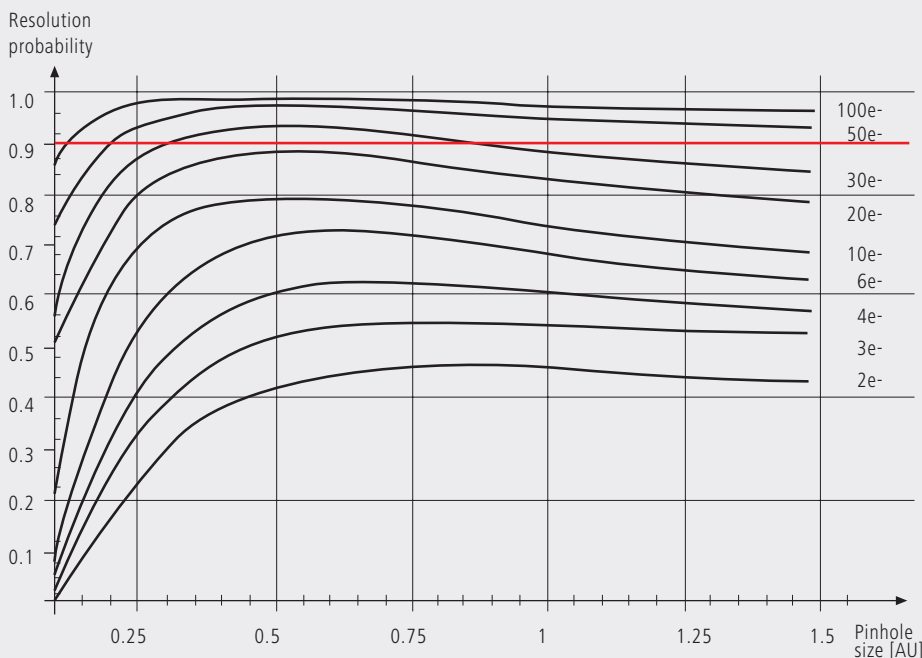


Fig. 17 The graph shows the computed resolution probability of two self-luminous points (fluorescence objects) spaced at 1/2 AU, as a function of pinhole size and for various photoelectron counts per point object (e-). The image raster conforms to the Nyquist theorem (critical raster spacing = 0.25 AU); 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  $\mu$ 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 about  $\sqrt{2}$  (3 dB). The advantage of the averaging method is the lower laser 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 ( $\text{SNR} = \sqrt{n \cdot N}$ ;  $N = \text{constant}$ ,  $n = \text{number of scans averaged}$ ). By comparison, a longer pixel time directly improves the photon statistics by a greater number  $N$  of photons detected per pixel ( $\text{SNR} = \sqrt{N}$ ,  $N = \text{variable}$ ), but there is a greater probability of photobleaching or saturation effects of the fluorophores (see also details "Fluorescence").

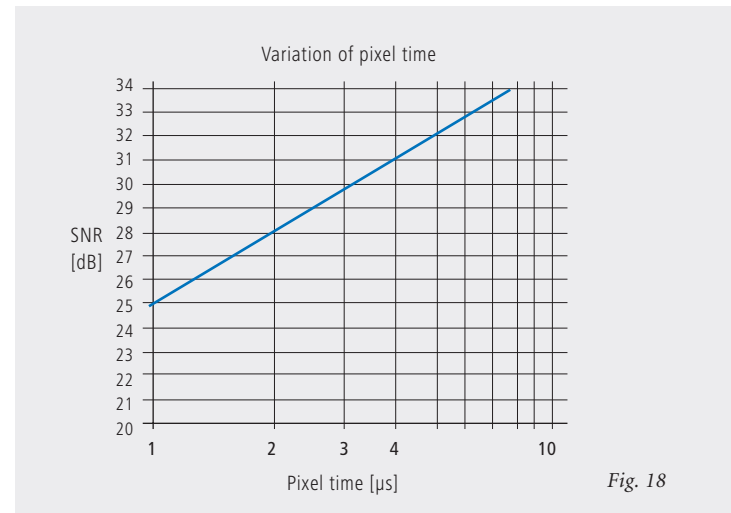


Fig. 18

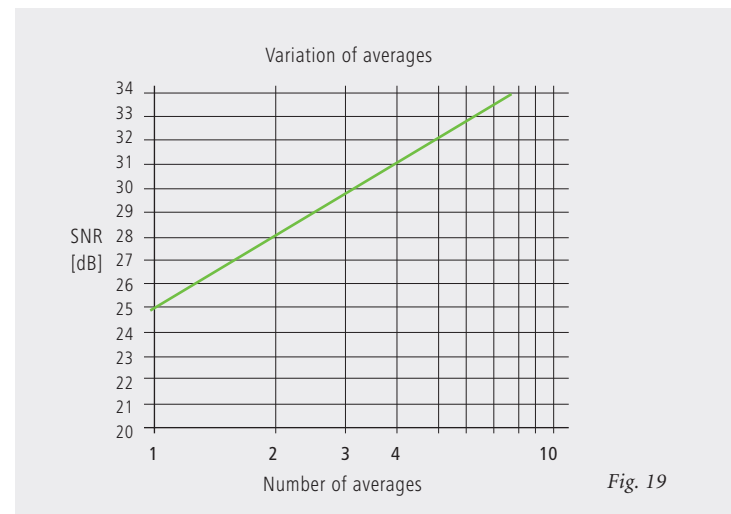
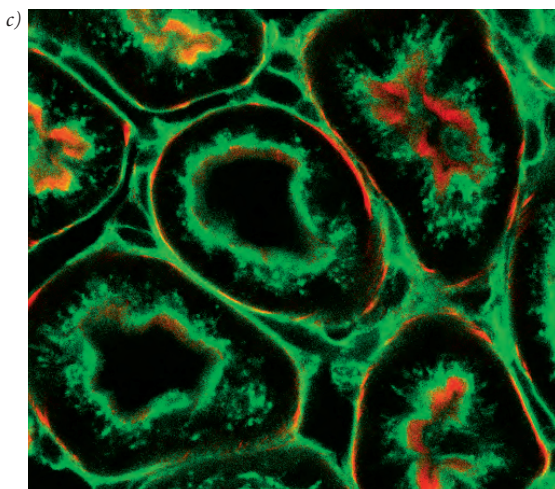
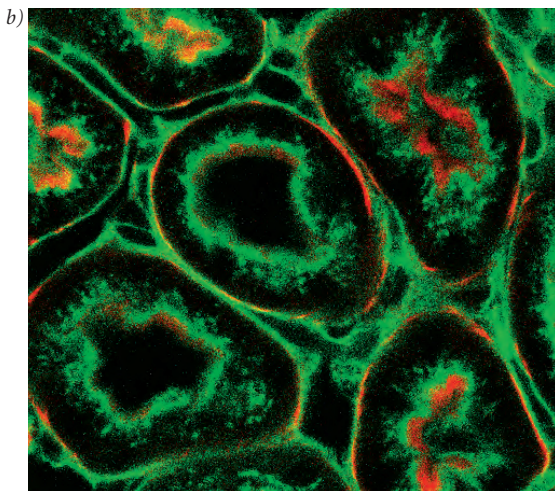
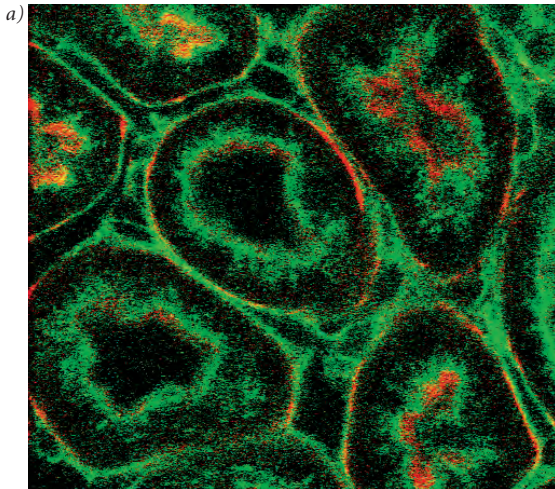


Fig. 19

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 2 (6dB) if the element separating the excitation and emission beam paths is not a neutral 80/20 beamsplitter<sup>1</sup> but a dichroic beamsplitter optimized for the particular fluorescence.

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.

*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  $\mu$ s in a), 6.4  $\mu$ s (no averaging) in b), and 6.4  $\mu$ s plus 4 times line-wise averaging in c).*

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

# Glossary

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

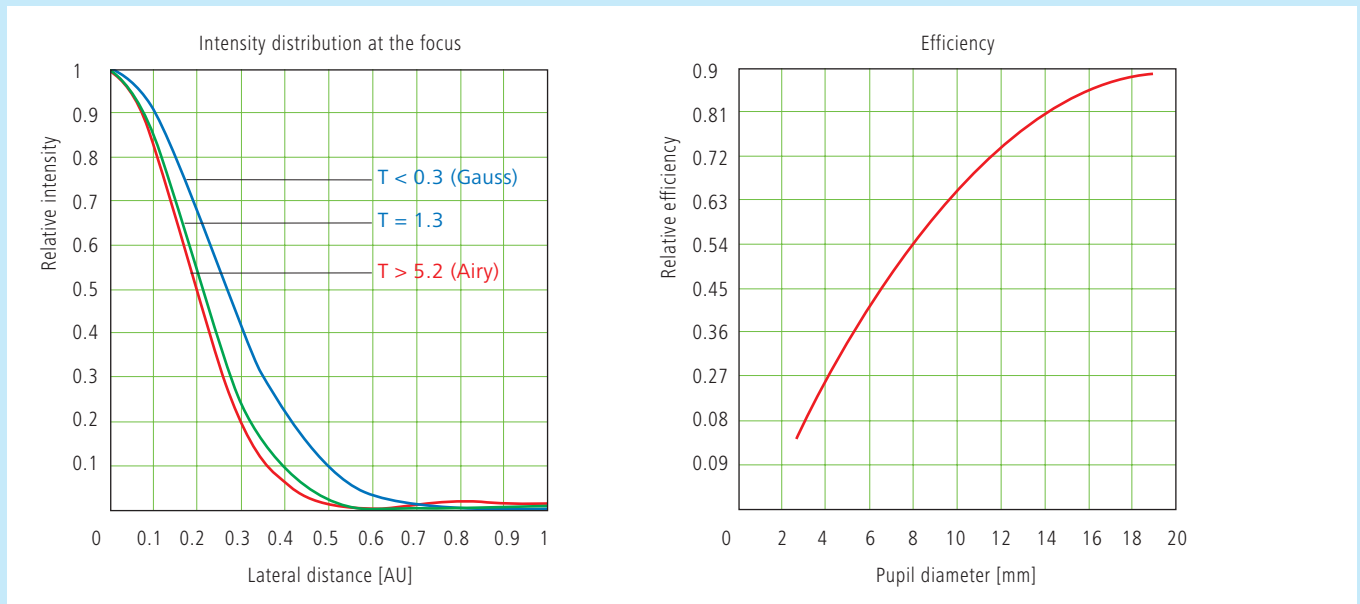
# Pupil Illumination

All descriptions in this monograph suggest a confocal LSM with a ray geometry providing homogeneous illumination at all lens cross sections. It is known that the intensity distribution generated in the focus of the objective lens, is the Fourier transform of the intensity distribution in the objective's pupil plane (backfocal plane). Hence it follows, that a homogeneous distribution in the pupil plane results in a focal distribution following the Airy function (also known as Airy disk) [In Carl Zeiss microscope objectives, the pupil diameter is implemented by a physical aperture close to the mounting surface of the lens].

The Airy distribution is characterized by a smaller width at half maximum and thus by a higher resolving power than a Gaussian distribution. Fig. A left shows the normalized intensity distribution at the focal plane as a function of different truncation factor's T (T is the ratio of the laser beam diameter ( $1/e^2$ ) and the pupil diameter of the objective lens). 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. Furthermore any truncation of the illuminating beam cross-section at the pupil plane causes a certain energy loss and thus a decrease in efficiency. Figure A (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 lens 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 with a reasonable loss in efficiency.

Fig. A



The truncation factor T is defined as the ratio of laser beam diameter ( $1/e^2$ ) and pupil diameter of the objective lens used:

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

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

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

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



## Optical Coordinates

In order to enable a representation of lateral and axial quantities independent of the objective lens 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  
 $\bar{\lambda}$  = mean wavelength  
 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):

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

$n$  = 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  $\mu$ 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/cm<sup>2</sup>. This corresponds to an excitation photon flux of  $6.34 \cdot 10^{23}$  photons/cm<sup>2</sup> sec. In conventional fluorescence microscopy, with the same objective, comparable lighting power (xenon lamp with 2 mW at 488 nm) and a visual field diameter of 20 mm, the excitation photon flux is only  $2.48 \cdot 10^{18}$  photons/cm<sup>2</sup> 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 ( $\sigma$ ), the fluorescence quantum yield (Qe) and the excitation photon flux (I) as follows:

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

In principle, the number of photons emitted increases with the intensity of excitation. However, the limiting parameter is the maximum emission rate of the fluorochrome molecule, i.e. the number of photons emittable per unit of time. The maximum emission rate is determined by the lifetime (= radiation time) of the excited state. For fluorescein this is about 4.4 nsec (subject to variation according to the ambient conditions). On average, the maximum emission rate of fluorescein is  $2.27 \cdot 10^8$  photons/sec. This corresponds to an excitation photon flux of  $1.26 \cdot 10^{24}$  photons/cm<sup>2</sup> sec. At rates greater than  $1.26 \cdot 10^{24}$  photons/cm<sup>2</sup> 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  $\mu$ W to roughly 1mW. Figure B (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 B (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/ $\mu$ 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  $F \sim 23$  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, 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.

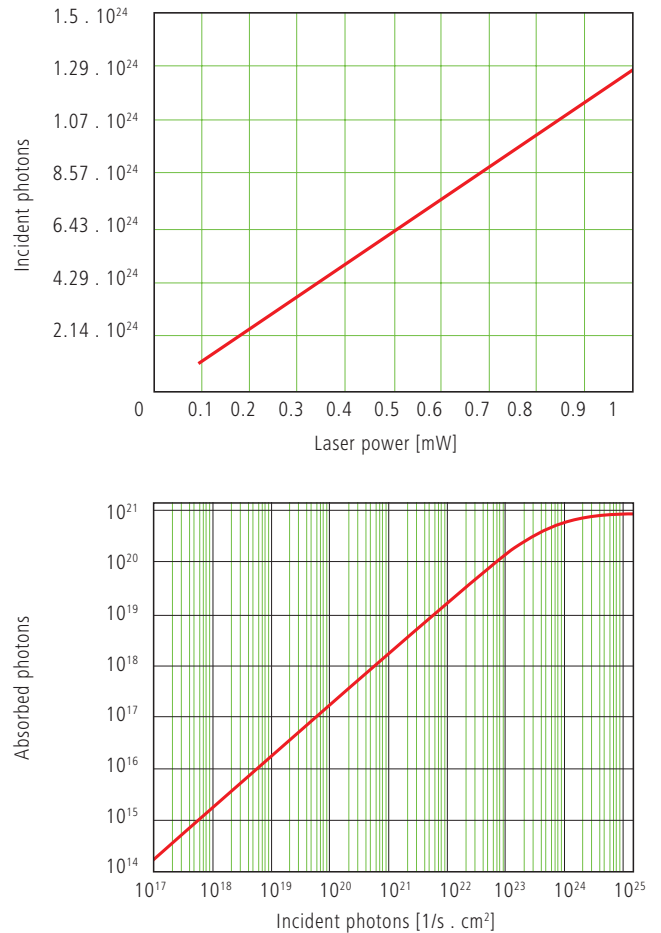


Fig. B Excitation photon flux at different laser powers (top) and excited-state saturation behavior (absorbed photons) of fluorescein molecules (bottom).

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  $1 \text{ nMol/l}$ , 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)

Shot noise 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,

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

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  $\lambda$ ; 1 photon =  $h \cdot c / \lambda$ ; c = light velocity; h = Planck's constant

## Secondary emission noise

Secondary emission noise is 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 of the detector 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  $\Delta N$  from the average (N+N<sub>d</sub>) of the photoelectrons and dark electrons registered, is

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

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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We gratefully acknowledge the assistance of Bernhard Gröbler,  
Hartmut Heinz and all other staff members who contributed  
to this brochure.

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Information subject to change.  
Printed on environmentally friendly paper  
bleached without chlorine.  
60-1-0030/e — printed 11. 10  
ISBN: 978-3-940885-02-9





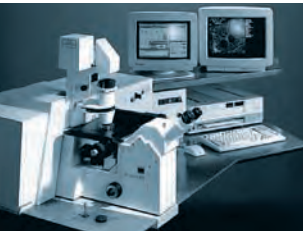


## Highlights of Laser Scanning Microscopy



**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.



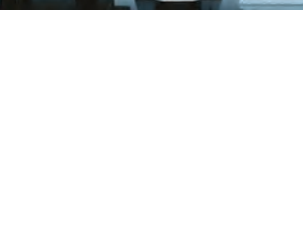
**1992**

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.

**2004**

The LSM 5 LIVE for ultrafast acquisition.

**2008**

The LSM 710 and LSM 700 – the new generation of sensitivity and flexibility.

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.



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