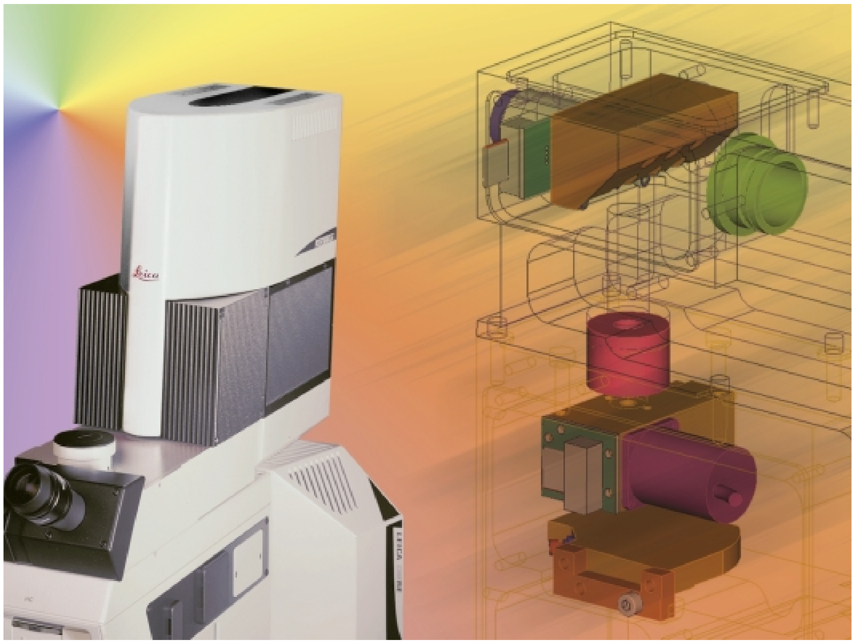

True Confocal Scanner
Leica TCS SP2

User Manual

Leica

MICROSYSTEMS

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General

How to Use this Manual

About this book
This manual is both a tutorial and a reference manual for the Leica TCS SP2. We intend this manual for the novice and the experienced user. If you are a new user, you should start with the chapter "Running the LCS Software", use the software for a while, and then read through the rest of the manual. Experienced users can search through the chapters, table of contents, and index to find special topics explained.
This book also covers the way your Leica Confocal Software and the Leica Microscope interact.
How to use this manual
We urge you to use this book, not just read it. To learn about the Leica Confocal Software, use the book as a tutorial. We show how to accomplish a variety of tasks. Work through the instructions to learn. The manual usually introduces a set of steps to accomplish a particular task with a distinctive heading.
Performing a task
Following the heading that introduces a task are either sequential steps or optional steps, each with its distinctive style:
1. Numbered paragraphs, like this one, designate step-by-step instructions. Follow them to learn how the tools work.
2. If several menu commands must be selected in sequence, an arrow separates the individual menu commands: for example, ***File* → *Save*** indicates that you click on File and select Save in the displayed pull-down menu.

Usually there is a sequence. Perform one or more of the steps to cause the designated action or actions.
We recommend reviewing this manual again after you have used the Leica TCS SP2 for a while. You will come across useful functions during the second reading that may have been overlooked during the first reading.
Online Help
Leica TCS SP2 comes with a context-sensitive online help system that explains the different functions of the system.
To retrieve the contents of the online help system:
Select ***Help* → *Contents*.** The online help system opens with an activated CONTENTS window. It shows a collapsible table of contents. Double-click an entry of the table of contents to view the corresponding information.
To retrieve information using a keyword:
Select ***Help* → *Index*.** The online help system opens with an activated INDEX window. Enter the word you want to look up. The online help shows the key word that represents the closest match to the word you entered.
To perform a full-text-search:
Select ***Help* → *Search*.** The online help system opens with an activated SEARCH window. Enter the term or definition you want to look up and click on the LIST TOPICS button. A hierarchically structured list of topics is returned. On the right side of the field where you enter the word is a menu to perform a full-text search using logical operators.

New in the description of single functions

3D projections and animations of image series (chapter on Data Processing, page 113)
Adjusting the acousto-optical beam splitter (AOBS) (chapter on Data Recording Functions, page 113)
Arithmetic and Boolean operations (chapter on Data Processing, page 183)
Converting the bit resolution of images (chapter on Data Processing, page 187)
Combining or separating image sections, images and image series (chapter on Data Processing, page 189)

Adjusting intensity, contrast and gamma correction (chapter on Data Processing, page 193)
Expanded focus through projection of image stacks onto a single image (chapter on Data Processing, page 195)
Linear filters and morphological filters (optional) (chapter on Data Processing, page 197)
Leveling and filtering of surface recordings (optional) (chapter on Data Processing, page 202)
Reducing optical crosstalk between detection channels (chapter on Data Processing, page 206)
Segmentation of image structures (optional) (chapter on Data Processing, page 208)
Viewing recordings as thumbnails in the experiment browser (chapter on Data Processing, page 145)
Controlling functions via control panel (chapter on User-Specific Adjustments, page 229)
Controlling the XY objective stage (chapter on Data Recording, page 141)

Confocal Imaging

What is confocal imaging?

Conceptualized in 1953, the Confocal Laser Scanning Microscopy has only in the past 10 years become a practical technique. Today it is the technique of choice for biological research, chemical analysis, and materials testing. The results of many years of research and development in many different areas are combined in such an instrument: microscopy, laser technology and optics for coherent light, video technology, electronics and computer technology.

Confocal microscopy detects structures by collecting light from a single focal plane of the sample, excluding light that is out of focus.

In a point scanning confocal system, the microscope lenses focus the laser light on one point in the specimen at a time (the **focal point**). The laser moves rapidly from point to point to produce the scanned image. Both fluorescent and reflected light from the specimen pass back through the objective.

The microscope and the optics of the scanner module focus the light emitted from the focal point to a second point, called the **confocal point**. The pinhole aperture, located at the confocal point, allows light from the focal point to pass through the detector. Light emitted from outside the focal point is rejected by the aperture.

The confocal principle is illustrated schematically for the epi-illumination imaging mode.

As in conventional epifluorescent microscopes, one lens is used as both condenser and objective. The advantage is eliminating the need for exact matching and co-orientation of two lenses. A collimated, polarized laser beam from an aperture is reflected by a beam splitter (dichroic mirror) into the rear of the objective lens and is focused on the specimen. The reflected light returning from the specimen passes back through the same lens. The light beam is focused into a small pinhole (i.e., the confocal aperture) to eliminate all the out-of-focus light, i.e., all light coming from regions of the specimen above or below the plane of focus. The achieved optical section thickness depends on several parameters such as the variable pinhole diameter and the wavelength. The in-focus information of each specimen point is recorded by a light-sensitive detector (e.g., a photodiode) positioned behind the confocal aperture. The analog output signal is digitized and fed into a computer. The detector is a point detector and only receives light from one point in the specimen. Thus, the microscope sees only one point of the specimen at a time as opposed to the conventional microscope where an extended field of the specimen is visible at one moment. Therefore, to obtain an image it is necessary either to move the illuminated point or to move the specimen. These two possibilities have given rise to two different types of confocal microscopes:

Microscopes with movable objective stage (stage scanning): The objective stage with the specimen is moved forward after every finished recording, while the optical system remains stationary with this type of scanning.

Microscopes with beam or mirror technology: The illuminated point is scanned over the fixed specimen using small, fast, galvanometer-driven mirrors as used by LEICA.

The LEICA TCS SP2 system allows you to image a single focal plane as well as a series of planes—horizontal or vertical. A single vertical section or xz-scan allows for a side view of the specimen.

If a sequence of optical sections of the specimen is combined to form an image stack and then digitally processed, it offers the advantage of using this multidimensional data set to create either a calculated two-dimensional image (projection) or a reduced scale 3D representation of the specimen using a suitable computer.

Optical resolution

The term resolution refers to the capability of distinguishing finest details in a structure. In a perfect microscope, the optical system would be free of any type of aberration. In such a hypothetical instrument, the resolution would be limited only through diffraction. One could express this as the smallest distance between two points of a specimen at which they are still visible as two separate points (Rayleigh criterion). Beyond this limit, the two points merge (i.e., their diffraction discs overlap completely or partially) and can no longer be recognized as two different points. This distance can be calculated using the size of the diffraction image of an infinitely small point of the specimen. It corresponds to the radius of the first minimum in this diffraction image. This, in turn, is related to the numeric apertures of the objective and the condenser. The numeric aperture is defined by the diffraction index of the lens and the size of the luminous cone that may enter.

Analogous to the argumentation above, the axial resolution can be defined as the radius of the first minimum along the microscope axis of the diffraction image of a point object. According to the theory for such 3D diffraction images, the optical resolution along the z-axis is smaller than the lateral optical resolution by a factor of 2. Thus, the optical resolution along the z-axis amounts to approximately one half of the resolution within the focal plane.

The LEICA TCS SP2 microscope is a no-compromise true point-scanning system with extremely high sensitivity and theoretical maximum x-, y- and z-resolution.

Scan resolution refers to image clarity as determined by the number of pixels and pixel size. The larger the number of pixels and the larger the scanning format, the more easily two close objects can be distinguished. The scan resolution is restricted to the maximum optical resolution power.

Detection

Confocal imaging, or to be more precise, the measurement of the optical properties of tiny sub-volumes of a specimen, is limited not only by the optical quality of the microscope. Other limitations are:

- The measurement of continuous specimen only in discrete sub-volumes (because of sampling and digitalization).
- The accuracy with which the sub-volumes are defined, determined by the scanning mechanism.
- The brightness of the light source in relation to the reflectivity of the specimen.
- The sensitivity and noise produced by the detector.

Another central component of the confocal microscope is the detector. Due to its very high signal-to-noise ratio, LEICA Microsystems Heidelberg uses photomultipliers as detectors.

Image processing

In the first confocal microscopes, the detector was connected to an oscilloscope with long-persistence phosphor which would display an image as it was being scanned. In the instruments of today, the signal is digitized and recorded in a computer. This makes it possible to manipulate the image in a multitude of ways. The following options are available:

- Contrast enhancement by thresholds, linear contrast stretching and gamma correction (curvature of the image intensity value versus source intensity graph).
- Superimposition of images in experiments.
- Digital filtering for edge enhancement, smoothing, noise suppression, etc.
- Reconstruction of three-dimensional views from stacks of images of optical sections. This allows, for instance, an image of an xz plane to be reconstructed from a stack of images of xy planes. Complete 3D models of the specimen can also be rendered and examined from any direction.
- Assembly of digital movies from time-sequences of microscope images.
- Quantification and Measurements.

Although this type of image manipulation does not improve the quality of the collected data, it serves the purpose of improving the visibility and facilitating the qualitative interpretation of the data.

Light source

Lasers are extremely well suited as light sources for confocal microscopy because they emit a very bright light and small divergence of the beam. In addition, they are easy to focus and stable in intensity. The stability is especially important for quantitative measurements.

Integration

The Leica TCS SP2 was designed as an integrated system. Optical and mechanical elements work seamlessly with computer hardware and software. The integrated Leica Confocal Software package supports the complete imaging process, from optical sectioning, through image processing and analysis (which is the main application), to hardcopy output.

Help via the Internet

First contact your local Leica subsidiary or the local dealer.
You can also find information on Leica Microsystems Heidelberg GmbH and confocal microscopy on our web site at
http://www.confocal-microscopy.com.
Or send an e-mail to llt.support@leica-microsystems.com.

Legal Notes

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SAFETY

ℹ

> *The terms used of the laser safety class refer to the standard EN 60825-1. Within the scope of the CDRH, the laser class 4 should be replaced by IV in the text, the laser class 3B by IIIb, the laser class 3A by IIIA, the laser class 2 by II and the laser class 1 by I.*

This instrument is designed and manufactured to comply with applicable performance standards for Class 3B laser products as defined by USHHS, CDRH/FDA, OSHA and EN standards and regulations known to be effective at the date of manufacture.
Every hazardous situation cannot be anticipated, therefore, the user must exercise care, common sense, and observe all appropriate safety precautions applicable to Class 3B lasers and high-voltage electrical equipment during installation, operation and maintenance.
Deviating from published operating or maintenance procedures is not recommended. Operation and maintenance procedure changes are performed entirely at the user's risk.

SOFTWARE LICENSE

The software described in this document is furnished under a License Agreement which is included with the product. This agreement specifies the permitted and prohibited uses of the product.

Safety Notes

This section introduces the standard safety precautions, warnings and safety cautions.

General Safety Directions
Your safety is extremely important. Read and observe all warnings and safety cautions listed in this manual and attached to the instrument before operating any Leica instruments. Serious injuries can occur, and equipment and data can be damaged if the safety warnings and safety cautions are not followed.

Risk of Operation
The risk for the performance of the device is assumed by the operator or the owner of the product. The owner or operator will be fully liable for all consequences which may result, if the device is opened by persons other than authorized Leica service staff, if it is not serviced or maintained properly or if it is used for purposes other than those described in the accompanying documentation and the online help facility. Leica Microsystems Heidelberg GmbH will not be liable for damages resulting from non-observance of the above information. The above information does not, in any way, implicitly or explicitly, modify the warranty and liability clauses contained in the general terms and conditions of Leica Microsystems Heidelberg GmbH.

Warnings, Safety Cautions, and Notes
The warnings, safety cautions, and notes in this manual use the following format.

⚠

*Such a **warning** alerts you of an operating procedure, practice, condition, or statement that must be strictly observed to avoid death or serious injury to the persons working on the equipment.*

⚠

*Such a **safety caution** alerts you to an operating procedure, practice, condition, or statement that must be strictly observed to prevent equipment damage or destruction, or corruption or loss of data.*

ℹ

***Notes** are statements that either provide extra information about a topic or contain special instructions for handling a particular condition or set of circumstances.*

Special Conditions of the Installation Site
Even though the system was tested and complies with the permissible limits of electromagnetic radiation (EMC), a residual risk of effecting other devices cannot be ruled out.

This system must not be mounted next to medical life-saving equipment.

Before moving the confocal system, it should be thoroughly cleaned. The same also applies to the removal of components. This applies in particular to systems that are located in biomedical research labs.

This is necessary to remove a possible contamination and, thereby, avoid carry-over of dangerous substances and pathogens and its accompanying risk of persons. Pay not only attention to surfaces, but especially to fans and cooling devices since dust can frequently accumulate at these locations.

The use of the instrument for unintended purposes as well as unauthorized opening of the instrument can create the risk of electrical voltage as well as laser radiation.

The terms used of the laser safety class refer to the standard EN 60825-1. Within the scope of the CDRH, the laser class 4 should be replaced by IV in the text, the laser class 3B by IIIb, the laser class 3A by IIIA, the laser class 2 by II and the laser class 1 by I.

Laser Class

This instrument is designed and manufactured to comply with applicable performance standards for Class 3B laser devices as defined by USHHS, CDRH/FDA, OSHA and EN 60825-1 standards and VBG 93 regulation for prevention of accidents known to be effective at the date of manufacture.
Every hazardous situation cannot be anticipated by the manufacturer, therefore, the user must exercise care, common sense, and observe all appropriate safety precautions applicable to Class 3B lasers and high-voltage electrical equipment during installation, operation and maintenance.
Operation and maintenance procedure changes are performed entirely at the user's risk.
The Leica TCS SP2 used in accordance with the instructions is harmless.

⚠

> *The user is responsible for compliance with current safety regulations. For safety reasons, only trained Leica service personnel is permitted in the laser area.*

Technical Safety Measures

In accordance with the laser safety directives EN 60825 (European standard) and CDRH (USA) Leica Microsystems Heidelberg GmbH employs various safety measures. Warning labels have been affixed near all apertures or removable parts where exposure to laser light is possible.
Furthermore, several **laser interlock-switches** were installed.

Observe the specification of the standard EN 60825-1: "Safety of laser products, Part 1. Equipment classification, requirements and user's guide", which requires that a laser safety officer or a laser protection advisor must be named for installations using instruments of class 3B. It is the user's responsibility to review and designate appropriate controls for the use of the equipment.

⚠

> *Never look directly into a laser beam or a reflection of the laser beam. Exposure to the laser beam can cause irreversible damage to your vision. A reflected laser beam is just as intensive as a direct beam.*
>
> *The user is responsible for the safe operation and maintenance of this instrument at all times. Class 3B laser product and electrical safety compliance is assured only when all safety devices, interlocks, and laser containment systems are in serviceable condition and operating. Disabled or damaged safety devices or systems will expose personnel to Class 3B laser radiation of sufficient power to cause severe eye injury, burns or property damage.*
>
> *Every interference with or any deactivation of one of the safety systems and/or one of the safety locks results in the loss of warranty claims.*

Power cord set

The power cord set supplied with your instrument meets the requirements of the country where you purchased the instrument. If you use the instrument in another country, you must use a power cord set that meets the requirements of that country.

The following wiring is specified by the manufacturer:

The power cord of the microscope must be plugged into the supplied connector strip. If a high-pressure mercury lamp is used, it should be connected to a separate power supply. This prevents it from continuously being switched on and off together with the control computer and the scan electronics and, therefore, increases the service life of the source of illumination.

This equipment is designed for connection to a grounded (earthed) outlet. The grounding type plug is an important safety feature. To reduce the risk of electrical shock or damage to the instrument, do not disable this feature.

To reduce the risk of fire hazard and electrical shock, do not expose the unit to rain or humidity. Do not open the cabinet. All maintenance is to be performed by an authorized Leica technician.

Do not allow any liquid to enter the instrument cabinet, or come into contact with any electrical components. The instrument must be thoroughly dry before you connect power, or turn the instrument on.

General Safety Notes

In accordance with general safety regulations as well as laser device safety regulations comply with the following stipulations:

Observe the operating instructions!
Use the product only according to the information given in this documentation and in the online help facility of the Leica Confocal Software. Carefully read the complete operating instructions before you start the system. As a preparation for your single work steps always read the relevant description of function in the online help and in particular observe the safety regulations indicated for handling the system. You can get an overview of the single functions in the contents file of the online help.

Use the product only for purposes described in this documentation and in the online help facility of the Leica Confocal Software.
The system is designed for making confocal laser scan images and quantitative measurements in the discipline of material science. Using the Leica TCS SP2 for any other purposes or applications is inexpert and inadmissible handling. The user assumes the entire risk for all experiments with this system and for the consequences resulting from such experiments. This is particularly true if the device has been opened or modified by the user.

Observe the maintenance instructions.
Please read the chapter 'Maintenance and Cleaning' in this manual.

Do not perform any adjustments on the equipment that are not described in this manual or in any subsequent addendum to the manual.
Only service technicians authorized by Leica Microsystems Heidelberg GmbH are allowed to carry out repair and service work.

Electrically connect only those electrical devices to the product that are mentioned in this accompanying documentation. Never electrically connect devices that are not mentioned in this documentation.
Before connecting any such devices to the product, consult the local Leica Service Agency or Leica Microsystems Heidelberg GmbH directly.

Do not use an S70 microscope condenser.
In the worst-case scenario, the large working distance and the low numeric aperture of the S70 condenser could result in impermissible high laser radiation loads if the safety notes are not observed (see "Special Safety Notes for Users"). Therefore, only S1 and S23 Leica condensers should be used.

Special Safety Remarks for Users
Leica Microsystems Heidelberg GmbH has done everything possible to maximize user safety and minimize health risks applying special safety measures in accordance with several safety directives. The user must operate the TCS SP2 in accordance with the instructions in order to ensure safety. Several important points are listed below:

Do not change objectives during scanning operations
How to change objectives:
Ensure that the Scan mode is switched off. Be sure that there is no laser light in the focal plane.
Turn the objective nosepiece so that the objective lens to be exchanged appears outside of the optical axis and points outwards. Do not apply any force to the objective nosepiece, as this may permanently reduce the resolution of the turret scan.
Screw in the new objective lens and turn the objective nosepiece back into the optical axis.

All unused positions on the objective nosepiece must be capped!

Do not insert objects into the beam path during scanning operations!

Insert the specimen correctly into the specimen holder!

Do not change specimen during scanning operations!
How to exchange a specimen:
Ensure that the Scan mode is switched off. Be sure that there is no laser light in the focal plane. Close the safety shutter at the tube.
Exchange the specimen.

Laser Safety

The word 'LASER' is an acronym for **L**ight **A**mplification by **S**timulated **E**mission of **R**adiation. The first laser was demonstrated in 1960 and used a ruby as the lasing medium. Lasers have been used in many applications from surgery to bar code readers at supermarket checkouts, laser pointers, laser printers to CD players.
Lasers can produce a very intense and very narrow (collimated) beam of electromagnetic radiation in the wave range of 150 nm to 1 mm. This radiation is generally in the form of intense visible light. Because laser light is not an ionizing type of radiation, interaction with matter is generally at the surface. The eye and the skin are very sensitive organs for laser radiation exposure. The resultant effects vary depending on the type of laser (wavelength of the radiation) and beam energy output. Laser energy of the proper wavelength and power may be focused by the lens of the eye onto the retina causing irreversible damage. If laser radiation energy is high enough, skin burns may also result if extremities or other body parts are placed in the laser beam. The following table summarizes the various regions of the electromagnetic spectrum produced by lasers and the organs that are at risk if exposure occurs.

Spectrum Region	Wavelength Range	Organ Effected
	UV-C 100 nm to 280 nm	All absorbed in cornea and conjunctiva
Ultraviolet	UV-B 280 nm to 320 nm	Almost all absorbed in cornea and conjunctiva
	UV-A 320 nm to 380 nm	Lens (lens cloudiness)
Visible	380 nm to 760 nm	Retina
	IR-A 760 nm to 1400 nm	Retina, lens, skin
Infrared	IR-B 1400 nm to 3000 nm	Cornea and skin
	IR-C 3000 nm to 10^6 nm	Cornea and skin

Lasers are frequently identified based on the type of laser medium. The Laser Hazard Classification is determined by the wavelength(s), maximum duration of exposure, and the average power output of the laser.

Laser hazard classification

> *Never look directly into a laser beam regardless of the type of laser and the power used.*

Class 1
Harmless laser (usually embedded).
Class 2
Low power visible laser which, due to the reflex response, does not normally present a hazard unless viewed directly.
Class 3A
Lasers that are generally harmless if inadvertently viewed directly, including intrabeam viewing; however, these lasers may represent a danger if collector optics are used.
Class 3B
Lasers that can produce an eye injury if viewed directly, including intrabeam viewing of specular reflections.
Class 4
Laser that can produce an eye injury or skin burns from direct, specular or diffuse reflections. Danger of burns may develop.

Embedded systems

Class 2, 3A, 3B or 4 lasers or laser systems contained in a protective housing and operated in a lower classification mode may be classified at a lower classification. Specific control measures may be required to maintain the lower classification.

Laser Hazards

The danger from lasers can be divided into the following major categories:

1. Eye injuries such as burns of the retina or cornea.

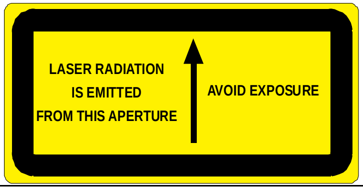
> *RETINAL INJURY IS IRREVERSIBLE*

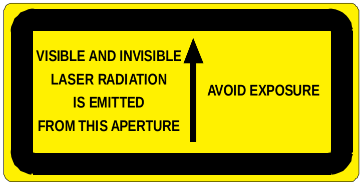
2. Skin hazards such as burns.
3 Electrical hazards from high voltage equipment.
4. Fire and explosion hazard.

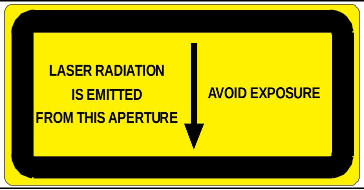
Specification of laser radiation in use

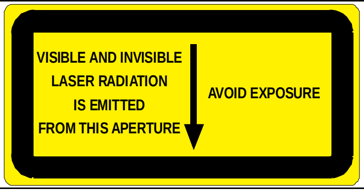
Laser type	Wavelength [nm]	Maximum luminous power at laser output [mW]	Maximum luminous power in focal plane [mW]	Pulse duration
Ar-UV	351, 364	< 50	< 2.5	continuous wave (non-pulsed)
Diode	405	< 25	< 4	continuous wave (non-pulsed)
Diode	430	< 10	< 4	continuous wave (non-pulsed)
HeCd	442	< 30	< 3	continuous wave (non-pulsed)
ArKr	488, 568, 647	< 125	< 10	continuous wave (non-pulsed)
Ar	458, 476, 488, 514	< 200	< 50	continuous wave (non-pulsed)
HeNe	543	< 1.5	< 0.5	continuous wave (non-pulsed)
Kr	568	< 25	< 5	continuous wave (non-pulsed)
HeNe	633	< 15	< 4	continuous wave (non-pulsed)

The following laser safety labels are used

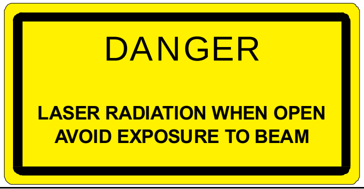
<table>
<tr><th colspan="2">1. at all openings where light can be emitted</th></tr>
<tr><td>For laser emission in the visible spectral region (Ar/ ArKr/ Kr/ HeNe/ HeCd/ diode)</td><td>For laser emission in the ultraviolet spectral region (Ar-UV)</td></tr>
<tr><td>
LASER RADIATION
IS EMITTED
FROM THIS APERTURE
AVOID EXPOSURE

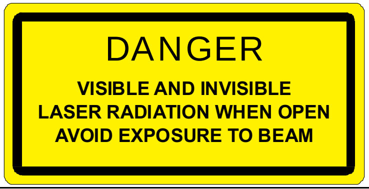
</td><td>
VISIBLE AND INVISIBLE
LASER RADIATION
IS EMITTED
FROM THIS APERTURE
AVOID EXPOSURE

</td></tr>
<tr><td>
LASER RADIATION
IS EMITTED
FROM THIS APERTURE
AVOID EXPOSURE

</td><td>
VISIBLE AND INVISIBLE
LASER RADIATION
IS EMITTED
FROM THIS APERTURE
AVOID EXPOSURE

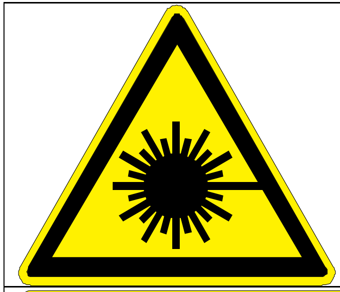
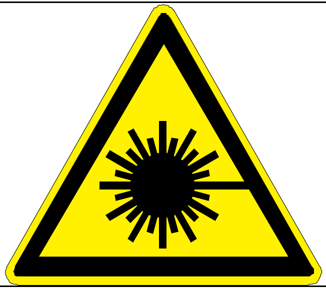
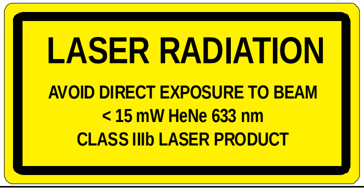
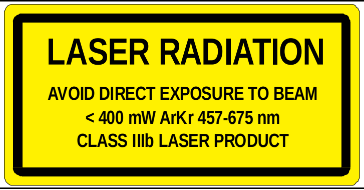
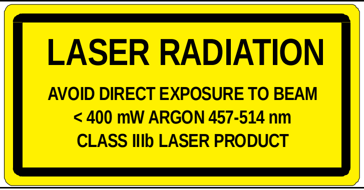
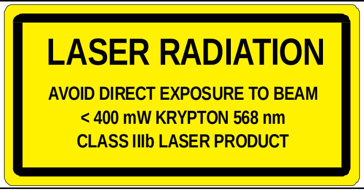
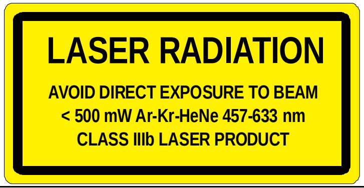
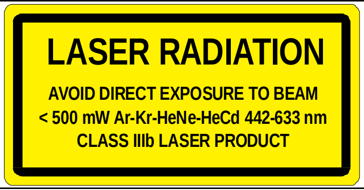
</td></tr>
</table>

<table>
<tr><th colspan="2">2. on all housing parts that can be opened with tools and emit laser radiation</th></tr>
<tr><td>For laser emission in the visible spectral region (Ar/ ArKr/ Kr/ HeNe/ HeCd/ diode)</td><td>For laser emission in the ultraviolet spectral region (Ar-UV)</td></tr>
<tr><td>
DANGER
LASER RADIATION WHEN OPEN
AVOID EXPOSURE TO BEAM

</td><td>
DANGER
VISIBLE AND INVISIBLE
LASER RADIATION WHEN OPEN
AVOID EXPOSURE TO BEAM

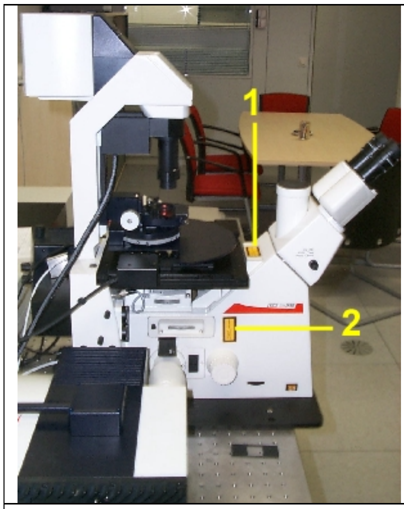
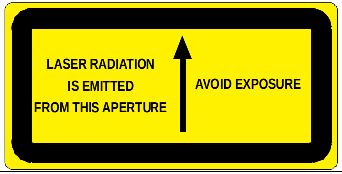
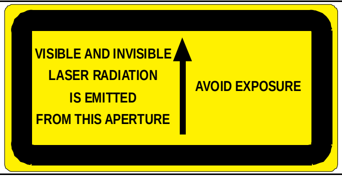
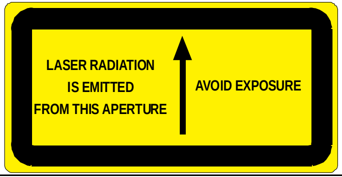
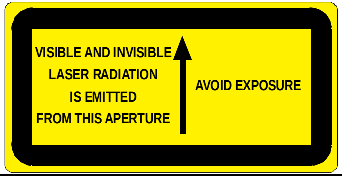
</td></tr>
</table>

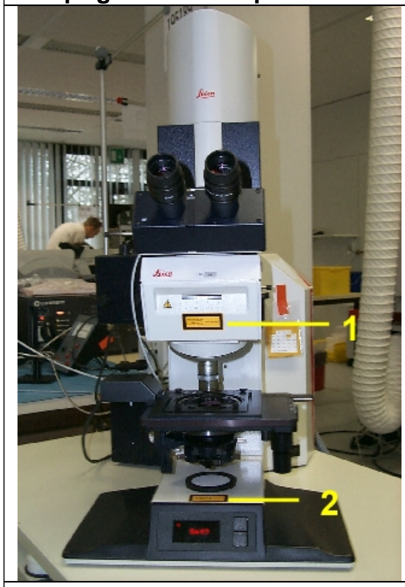
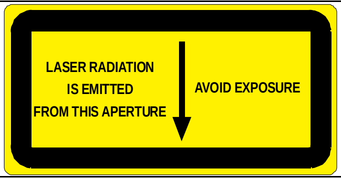
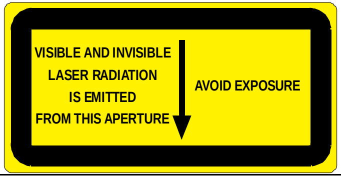
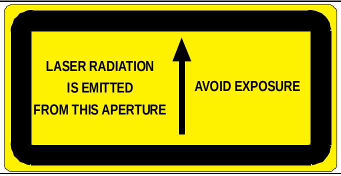
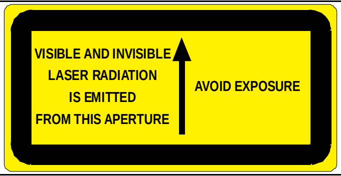
<table>
<tr><th colspan="2">3. on the cover of the supply unit</th></tr>
<tr><td>For laser emission in the visible spectral region (Ar/ ArKr/ Kr/ HeNe/ HeCd/ diode)</td><td>For laser emission in the ultraviolet spectral region (Ar-UV)</td></tr>
</table>

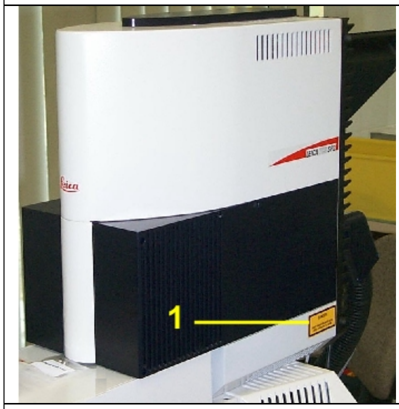
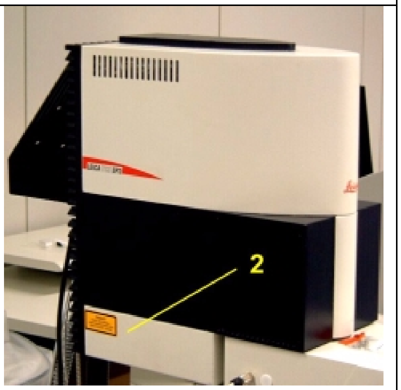
	
 LASER RADIATION AVOID DIRECT EXPOSURE TO BEAM < 15 mW HeNe 633 nm CLASS IIIb LASER PRODUCT	
 LASER RADIATION AVOID DIRECT EXPOSURE TO BEAM < 400 mW ArKr 457-675 nm CLASS IIIb LASER PRODUCT	
 LASER RADIATION AVOID DIRECT EXPOSURE TO BEAM < 400 mW ARGON 457-514 nm CLASS IIIb LASER PRODUCT	
 LASER RADIATION AVOID DIRECT EXPOSURE TO BEAM < 400 mW KRYPTON 568 nm CLASS IIIb LASER PRODUCT	
 LASER RADIATION AVOID DIRECT EXPOSURE TO BEAM < 500 mW Ar-Kr-HeNe 457-633 nm CLASS IIIb LASER PRODUCT	
 LASER RADIATION AVOID DIRECT EXPOSURE TO BEAM < 500 mW Ar-Kr-HeNe-HeCd 442-633 nm CLASS IIIb LASER PRODUCT	

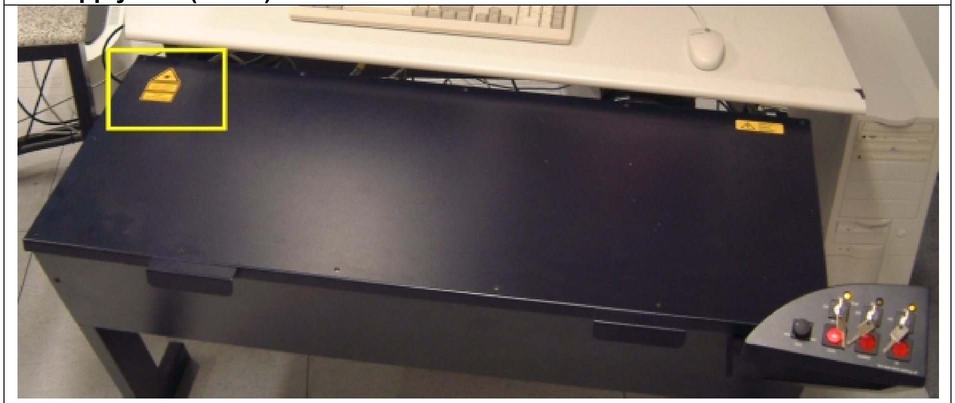
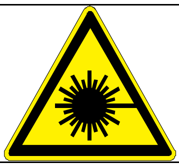
Position of laser safety labels

1. Inverted microscope	

	
Location 1	for systems WITHOUT UV laser
	 LASER RADIATION IS EMITTED FROM THIS APERTURE AVOID EXPOSURE
	for systems WITH UV laser
	 VISIBLE AND INVISIBLE LASER RADIATION IS EMITTED FROM THIS APERTURE AVOID EXPOSURE
Location 2	for systems WITHOUT UV laser
	 LASER RADIATION IS EMITTED FROM THIS APERTURE AVOID EXPOSURE
	for systems WITH UV laser
	 VISIBLE AND INVISIBLE LASER RADIATION IS EMITTED FROM THIS APERTURE AVOID EXPOSURE

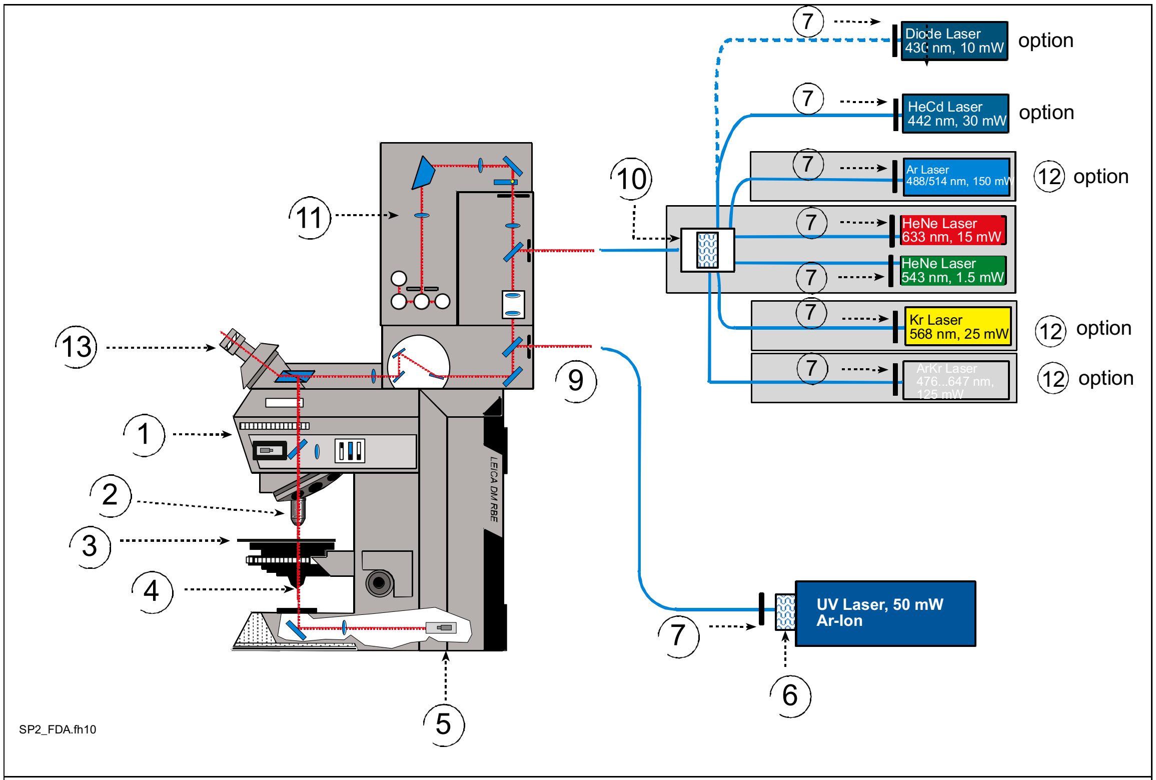
2. Upright microscope	
	
Location 1	for systems WITHOUT UV laser
	 LASER RADIATION IS EMITTED FROM THIS APERTURE AVOID EXPOSURE
	for systems WITH UV laser
	 VISIBLE AND INVISIBLE LASER RADIATION IS EMITTED FROM THIS APERTURE AVOID EXPOSURE
Location 2	for systems WITHOUT UV laser
	 LASER RADIATION IS EMITTED FROM THIS APERTURE AVOID EXPOSURE
	for systems WITH UV laser
	 VISIBLE AND INVISIBLE LASER RADIATION IS EMITTED FROM THIS APERTURE AVOID EXPOSURE

3. Scan head	
	
Position 1 and 2	for systems WITHOUT UV laser
	DANGER **LASER RADIATION WHEN OPEN** **AVOID EXPOSURE TO BEAM**
	for systems WITH UV laser
	DANGER **VISIBLE AND INVISIBLE** **LASER RADIATION WHEN OPEN** **AVOID EXPOSURE TO BEAM**

4. Supply unit (cover)	
	
Location 1	
	 DANGER **LASER RADIATION WHEN OPEN** **AVOID EXPOSURE TO BEAM**
	for systems with HeNe laser

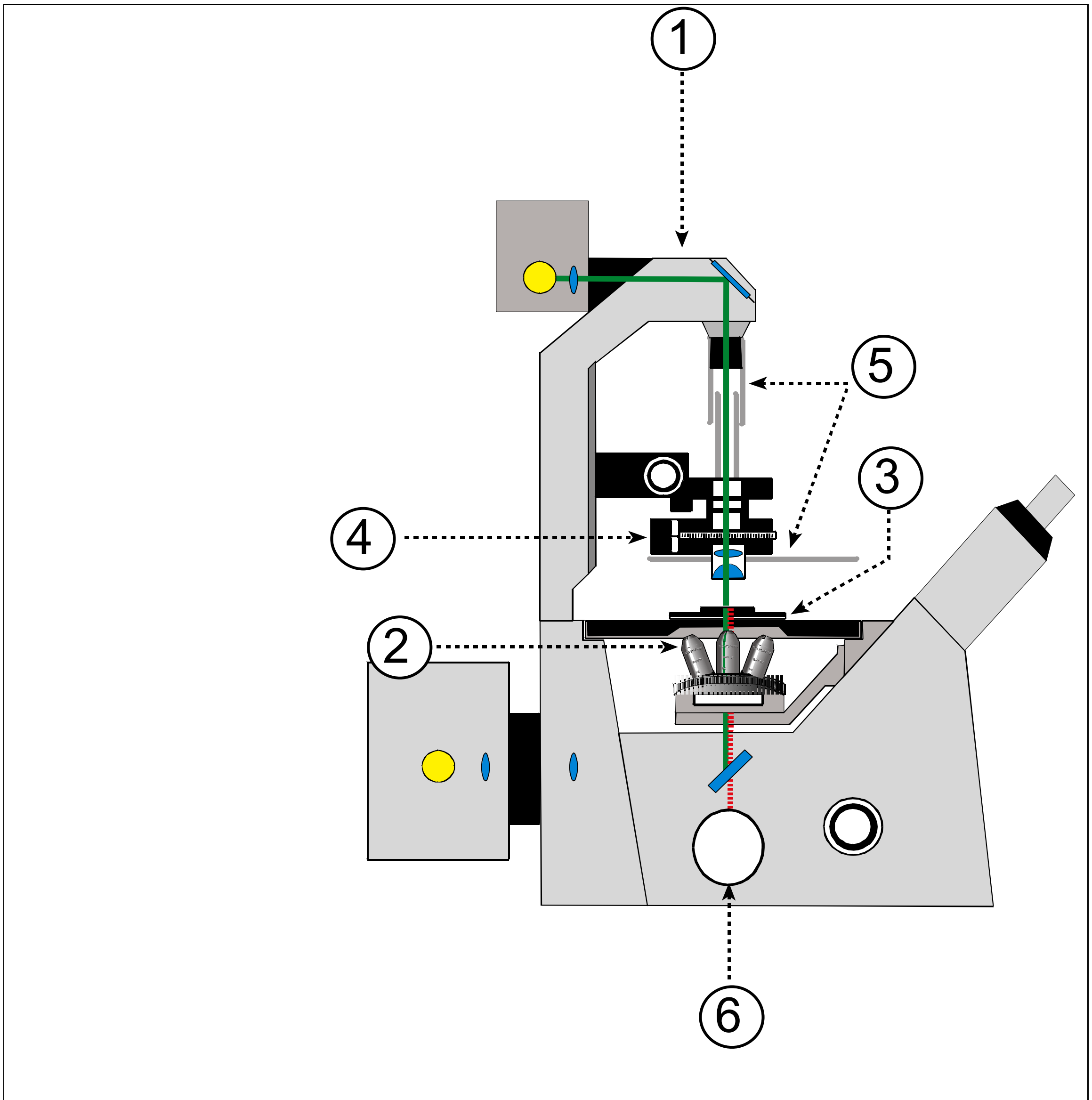
	LASER RADIATION AVOID DIRECT EXPOSURE TO BEAM < 15 mW HeNe 633 nm CLASS IIIb LASER PRODUCT
	for systems with ArKr laser
	LASER RADIATION AVOID DIRECT EXPOSURE TO BEAM < 400 mW ArKr 457-675 nm CLASS IIIb LASER PRODUCT
	for systems with Ar laser
	LASER RADIATION AVOID DIRECT EXPOSURE TO BEAM < 400 mW ARGON 457-514 nm CLASS IIIb LASER PRODUCT
	for systems with Kr laser
	LASER RADIATION AVOID DIRECT EXPOSURE TO BEAM < 400 mW KRYPTON 568 nm CLASS IIIb LASER PRODUCT
	For systems with HeNe laser, Ar laser and Kr laser
	LASER RADIATION AVOID DIRECT EXPOSURE TO BEAM < 500 mW Ar-Kr-HeNe 457-633 nm CLASS IIIb LASER PRODUCT
	For systems with HeNe laser, HeCd laser, Ar laser and Kr laser
	LASER RADIATION AVOID DIRECT EXPOSURE TO BEAM < 500 mW Ar-Kr-HeNe-HeCd 442-633 nm CLASS IIIb LASER PRODUCT

System overview
Upright microscope


7
Diode Laser
430 nm, 10 mW
option
7
HeCd Laser
442 nm, 30 mW
option
7
Ar Laser
488/514 nm, 150 mW
12
option
7
HeNe Laser
633 nm, 15 mW
HeNe Laser
543 nm, 1.5 mW
7
7
Kr Laser
568 nm, 25 mW
12
option
7
ArKr Laser
476...647 nm,
125 mW
12
option
11
10
13
9
1
LEICA DM RBE
2
3
4
UV Laser, 50 mW
Ar-Ion
7
6
5
SP2_FDA.fh10

1: Upright microscope stand, 2: Objectives, 3: Microscope specimen stage, 4: Condenser, 5: Transmitted light detector (non-descanned), 6: UV modulator (for UV systems only), 7: Laser safety shutter, 9: embedded optical fibers (all optical fibers leading to the scan head), 10: Beam collection, VIS modulator, 11: Scan head, 12: Laser box, 13: Eyepieces

System overview
Inverted microscope



1: Inverse microscope stand, 2: Objectives, 3: Microscope specimen stage, 4: Condenser, 5: Aluminum disc as beam collector, protective sleeve between condenser and transmitted light detector, 6: Connection for scan head (scan head identical to that used with upright microscope stand)

Starting the operating system

You do not have to start the operating system—it starts automatically when you turn on your PC.You will first see a splash screen.

Next you have to log on to your computer. As you can see from the instructions in the box, pressing the **Ctrl**, **Alt** and **Delete** keys at the same time will log you on.

After pressing the Ctrl, Alt, and Delete keys, the **Logon** information dialog box appears.

Typing your password identifies you as a valid user for this computer.

The default user name for the Leica TCS SP2 system is "TCS_User."

A standard password was not set. It is recommended setting up a separate user ID for each user (system administrator). This will create individual directories that can be viewed only by the respective user.Since the LCS software is based on the user administration of the operating system, separate files are created for managing user-specific profiles of the LCS software. For information about setting up users, please refer to the chapter "Setting Up Users" in this manual.

After logging on with your user ID, you may change your password by pressing the keys **Ctrl**, **Alt**, and **Delete** at the same time.

Then click on **Change Password**. The **Change Password** dialog box displays.

Type your current password in the **Old Password** field (passwords are case sensitive, so be sure you use the right case). Then press the Tab key. Pressing the Tab key moves the cursor to the next field. Type your new password, then press the Tab key again. Confirm your new password by re-entering it. This will eliminate any typing errors. This is especially important since the characters you type appear as asterisks on the screen.

> *If you miss-type the confirmation password, you will see a warning dialog. Try again!*

Then click the OK button. Your new password will be in effect the next time you log on.

> *Caution: Do not forget your password if you set one! Without the right password you cannot access your computer anymore.*

The Welcome dialog box is now displayed. Take a moment to read the "Did you know..." tip and then click the **Close** button to begin using the operating system.

Using a Mouse

You need a mouse to work most efficiently with the operating system. Here are the mouse actions you need to know:

Point means to move the mouse pointer onto the specified item by moving the mouse. The tip of the mouse pointer must touch the item.

Click on an item means to move the pointer onto the specified item and press and release the mouse button once. Unless specified otherwise (i.e. right-click), use the left mouse button. Clicking usually selects an item.

Double-click on an item means to move the pointer to the specified item and press and release the left mouse button twice quickly. Double-clicking usually activates an item.

Drag means to move the mouse pointer onto the item, hold down the mouse button, and move the mouse while holding down the button. Unless specified otherwise (i.e. right-drag), use the left mouse button.

The user interface of the operating system

The basic user interface of the operating system is referred to as "**Desktop**". It provides a background for the items it contains.

The initial icons on the Desktop allow the user to view and interact with the system in a logical way.

The background on which all the pictures and boxes rest is the desktop.

The taskbar shows the windows and programs that are open. You can switch between open windows and programs by clicking the name on the taskbar.

The Start button opens a menu system from which you can start programs. Click on the Start button, then click on your selection from each menu that appears.

Some icons appear on your desktop. You can activate one by double-clicking on it.

We now take a brief tour of the items you see on the screen.

A standard desktop item is the **My Computer** icon. **Double-clicking** this icon opens the My Computer window.

The 'My Computer' window gives you easy access to the major components like hard and floppy disk drives of your computer system or workstation. For example, by double-clicking the **Hard disk [C:]** icon you can see the contents of your PC's hard drive. This allows the user to view local resources as objects. In addition, the 'My Computer' window gives you access to the control panel (Control Panel symbol) and the printer of the control computer, if a printer is installed. If you installed one of the additional applications such as 'Dial-Up Networking' at installation time, it will also appear in the 'My Computer' window.

You can use the **Control Panel** icon in the 'My Computer' window to view and change any system component. The Control Panel contains numerous icons that allow you to control your system. The particular icons that you see on your PC may be slightly different from those illustrated, due to that fact that you may have different hardware installed, and may or may not be connected to a network or modem. Yet another reason could be that you have installed other operating system options.

Double-clicking the **Network Neighborhood** icon displays the Network Neighborhood dialog box which gives you information about who and what is connected to your workstation. It provides an easy mechanism for browsing any network systems and resources that you may be able to connect to in a way that is independent of the actual type of network vendor. Traditionally, if a system needed to be simultaneously connected to different types of network, the way in which each could be connected and viewed would be vendor-specific. The operating system is capable of displaying a common view of your entire network even though it may actually comprise resources from Windows NT, Novell NetWare, Banyan Vines, or others!

The **Inbox** icon is used if Microsoft Exchange is active on your system. The operating system has built-in electronic mail services based on Microsoft Mail (MS Mail) and Microsoft Exchange. If there is already an MS Mail post office on the same network that the system is connected to, the operating system mail client can connect directly to it. The Inbox lets you access your messages.

The **Recycle Bin** icon represents the holding place for deleted items. As long as files are in the Recycle Bin they can easily be recovered if they have been accidentally deleted. The operating system will preserve files until the system runs out of free disk space. When this happens, the operating system will prune the contents of the Recycle Bin on a first-in first-out basis.

Files that are overwritten due to an application using a duplicate filename will not be saved to the Recycle Bin.

Double-clicking the Bin displays its contents. The empty window confirms that there are no items in the Recycle Bin.

The Start menu

A single click of the left mouse button on the Start button opens the start menu and presents the seven major option categories for starting work on the system.

A single click of the right mouse button opens a small but powerful control menu containing the options Open, Explore and Find.

Their functionality is described below.

Starting a Program

The Start menu contains the various categories where your applications and work are stored. You can move further into the various subcategories by positioning the mouse over the category that you are interested in to automatically open the next subcategory. You do not have to click the mouse!

The **Programs** command displays the Programs menu. This menu lists all of the applications installed and available to you. Some options are marked by an arrow. This arrow indicates that a submenu follows. Drag the mouse cursor over the **Accessories** command to see its submenu. The Accessories submenu lists the set of program built into the operating system.

TIP: If you drag an object either from the Desktop or Windows Explorer and drop it directly onto the Start button, a link to that object will automatically appear in the Start menu.

There are many ways to start a program. The following method is the simplest one:

1. Click on the Start button.
2. Click on Programs.
3. Click on the group that contains the program you want to start (for instance, LCS).
4. Click on the program you want to start (for instance, Leica LCS).

Another way to start a program is to open a document that you created in that program. The program automatically opens when the document opens. Double-click on a document file in My Computer or Windows Explorer to open it. Or click on the Start button and select a recently used document from the Documents menu.

You can also start a program by double-clicking on its shortcut icon on the desktop. Shortcut icons are links to other files. When you use a shortcut, Windows simply follows the link back to the original file. Whenever you use a document or program frequently, you might consider creating a shortcut for it on the desktop. To do so, just use the right mouse button to drag an object out of Windows Explorer or My Computer. After releasing the mouse button, a menu appears. Select the option Create Shortcut(s) Here. Some programs automatically create a shortcut during their installation procedure.

The operating system does not actively track a link between an original and a shortcut. For instance, if you create a shortcut of a program, and subsequently move (rather than copy) the original to a different folder, then the shortcut may no longer function.

The Startup folder has a special function: All programs located in this folder are automatically started whenever the operating system is started.

The Documents menu shows the names of the 15 files you created most recently. You can open any of these files and its related application at the same time by clicking the file's name in this menu.

Document files that are opened within an application (typically by selecting the File/Open command within the application) will not be displayed here. Only documents opened directly from the Desktop will be displayed here.

The Settings menu offers three commands for changing your system's settings. You can directly access the Control Panel and Printing folders. Also accessible is the Task Properties window.

Being able to access the core system configuration utilities in this way is particularly useful when an application is already in the foreground and you want to make a quick change.

The Find command features an easy way to locate all system resources. Within the Find category you can perform searches for three distinct types of search as described below:

The Taskbar

The taskbar—positioned at the bottom of the screen—provides a constant view of which applications are running on the system and an easy way of switching between them. As you add to the number of concurrently running applications, the taskbar automatically re-sizes its iconized view of the applications to ensure that they can always be seen. To switch from one running program to another, simply click on the second program as displayed in the taskbar.

The taskbar also provides constant additional information such as the system time and volume control if a sound card is installed. All of these functions can be adjusted by the user.

Setting the Time and Date

The current Date, Time and Time Zone information can be set from the Date/Time icon within the

Control Panel. This setting is important since the operating system stamps the date and time on all of your files as you create and modify them. The two options can be selected by clicking on the appropriate tab.

To change the Date and Time

Click on the appropriate date or use the controls to change the month or year. The time can also be changed by first selecting the digital display and then using the up and down arrows.

To change the Time Zone

Select the appropriate Time Zone from the drop down list at the top of the screen. Notice that the option to automatically adjust the clock for daylight savings time is selected. On some systems you can also drag the highlighted area on the world map and drop it on the correct location.

Changing the date and time information within operating system will update the battery backup CMOS clock in your system.

> *Depending on the shell configuration, systems connected to a network may get a time and date update from a network server every time they log on. If the server's time is incorrect, your workstation's time will be wrong, too. Please inform your network administrator.*

Getting Help

The operating system includes a powerful help system. In addition to Help menus in every window, there is a standalone Help feature available from the Start menu. To access it, click your mouse on the Start button, and click on Help.

There are three tabs in this box: Contents, Index and Find. The Contents tab appears on top. To move to a tab, click on it.

Contents

The Contents tab displays individual Help topics. The topics are organized into categories and are represented by small book icons. Double-click on any book to open it. Sub-books and documents appear. Double-click on sub-books and documents to open them.

Index

The Index tab displays an index of all available topics. Type the word you want to look up. The Index list scrolls to that part of the alphabetical listing. When you see the topic on the list that you want to read, double-click on it.

Find

Rather than searching for information by category, the Find tab offers a full text search. Enter the word(s) or phrase to be searched for under Help in the text entry box. The text entry box is linked to a list of words in your Help files, and any words or phrases that match will be displayed, and any words or phrases that match will be displayed. You can specify more than one word by separating words with a space. If you wish to change a search option, select Options. The first time you click on this tab, Windows tells you it needs to create a list. Click Next and then Finish to allow this. The main Find tab appears next. Type the word(s) you want to find in the top text box. Then click a word in the middle box to narrow the search. Finally, review the list of help topics at the bottom, and double-click the one you want to read.

When you are done reading about a document, click Help Topics to return to the main Help screen, or click Back to return to the previous Help topic. Click the window's Close button to exit Help.

Exiting the operating system (Shutdown)

Always use the **Shut Down** command before you turn off your PC. The Shut Down option allows the user to close the operating system and ensure all running processes can halt cleanly and are given the chance to flush any data out to the disk that may be in cache memory. Several options are available when shutting the system down.

Powering down your computer without prior shutting it down may result in severe data loss.

Setting Up Users

1. Logging on as Administrator

To log on as administrator use the ID: "Administrator" and the password: "Admin"

2. Opening the User Manager

Select ***Start→Programs→Administrative Tools→User Manager*.**

3. Defining a new user

Enter the following information in the dialog window being displayed:

1. Username
2. Password (must be re-entered in the next line for confirmation purposes)

From the check boxes displayed, select the following two:

a.) "User must change password at next logon" (this allows the new user to define his or her own password at logon)

b.) "Password never expires" (this allows a defined password to be valid either until it is changed in the User Manager or the user is deleted)

Select the "Profiles" option in the bottom section of the dialog. In the "Local path" field enter the following path for storing user-specific files:

d:\users\username ("username" is an open parameter which must be replaced by the currently defined user name).

Factory-installed hard disks are set up with two partitions (C:\ and D:\). The user directory should be set up on partition D:\.

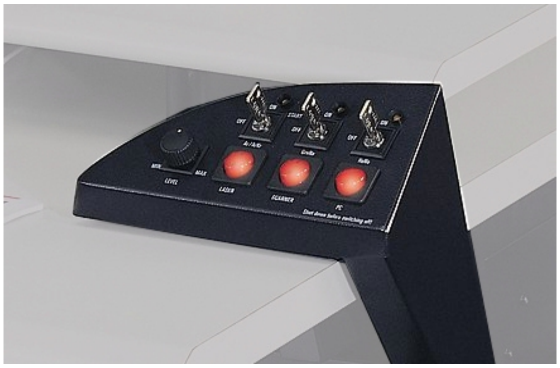
Starting the Confocal System

1. Check whether the hardware dongle is plugged into the parallel port of the computer.
The LCS software can NOT be started without the provided dongle.

2. Switch on the microscope(s) and the light sources.
All manual Leica microscope stands (DM-LM, DM-IRB, DM-R, DM-LFSE) can be switched on and off using the switch located at the side of the stand.
All automatic Leica microscope stands (DM- LFSA, DM-RXA, DM-RXA2, DM-RE, DM-IRBE) can be switched on and off using the switch located at the side of the stand.
The Leica microscope stands DM-LFSA, DM-IRE2 and DM-RXA2 can be switched on and off using the separate electronics unit (LEICA CTR MIC Electronics box).

> *All fluorescent lamps are equipped with individual power supplies. For this reason, they can only be switched on and off at the power supply.*

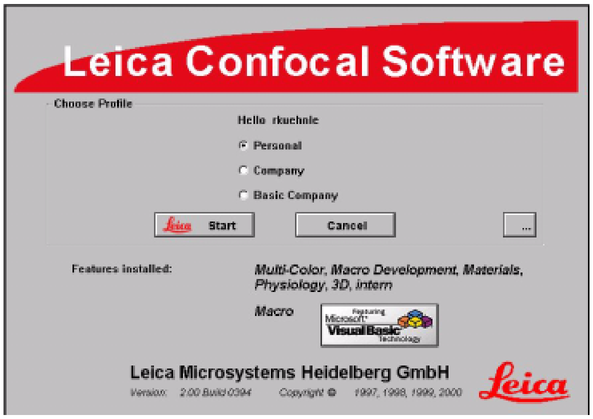
3. Activate the switches on the control panel for the workstation (PC), confocal scanner (Scanner) and lasers (Lasers).



4. Log on to the computer.
Use your personal user ID if one has been set up. This ensures that the user-specific settings are saved and maintained for this user only. If the system administrator has not yet assigned a personal user ID, log on as "TCS_User". A password is not required.

5. Start the LCS program.
To do so, click on the program icon on the desktop of the computer.

6. Next, select a software profile.
A separate profile can be created for each user containing the structure of the graphic user interface as well as the definition of user-specific settings of the confocal system.



Leica Confocal Software

Choose Profile

Hello rkuehnle

● Personal

○ Company

○ Basic Company

Features installed: Multi-Color, Macro Development, Materials, Physiology, 3D, intern

Macro

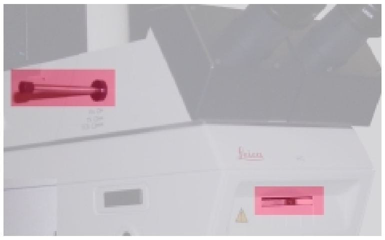
Leica Microsystems Heidelberg GmbH

Version: 2.00 Build 0394 Copyright © 1997, 1998, 1999, 2000

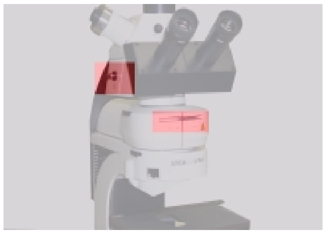
> *Do not perform the next step until the initialization of the hardware components is complete.*

7. Affix a specimen and adjust the specimen using the conventional microscopy mode.
First, focus on a position of your specimen to be observed. Next, set the Köhler illumination (see the chapter on "Köhler Illumination" page 37).

a.) Adjusting to conventional observation for upright microscopes of series DM-Rxxx, DM-LFxx



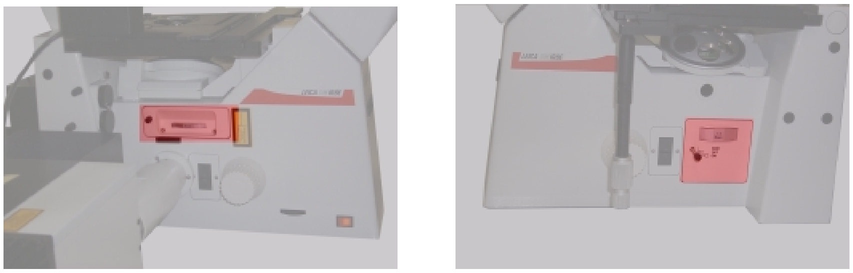
DM_Rxx microscope series:



DM-LFSx microscope series:

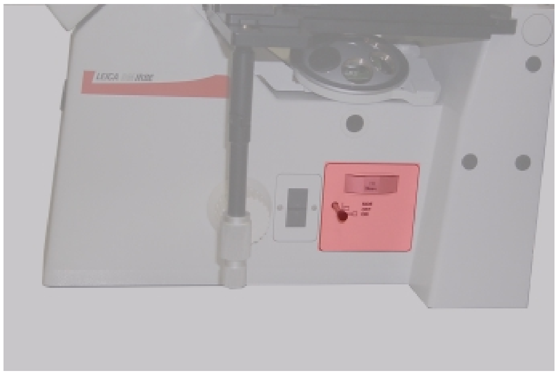
Set the switch rod to "VIS" and turn the fluorescence filter wheel to the corresponding filter cube Pos.: 1 DAPI (optional), Pos2: TRITC, Pos3: FITC, Pos4: open position (scan)

b.) Adjusting to conventional observation for inverted microscopes of series DM-IRxx



DM_IRxx microscopes:
Pull out the switch rod on the left side completely (removed) and turn the fluorescence filter wheel to the corresponding filter cube
Pos.: 1 DAPI (optional), Pos2: TRITC, Pos3: FITC, Pos4: open position (scan)
Push in the switch rod on the right side completely

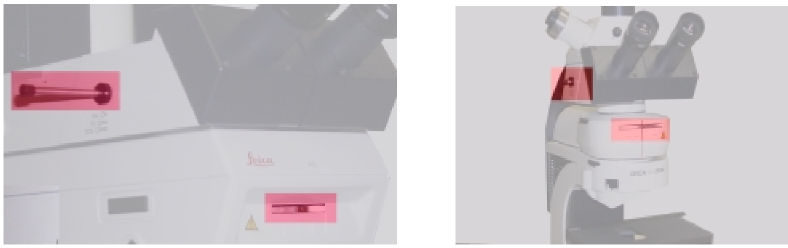
In the case of a UV system: Turn the objective tube lens to the "Scan" position.



DM_IRxx microscopes:
Turn tube lens wheel on the right side to "Scan."

8. Changing to confocal operation.

a.) Switching to confocal observation for upright microscopes of the DM-Rxxx, DM-LFxx series



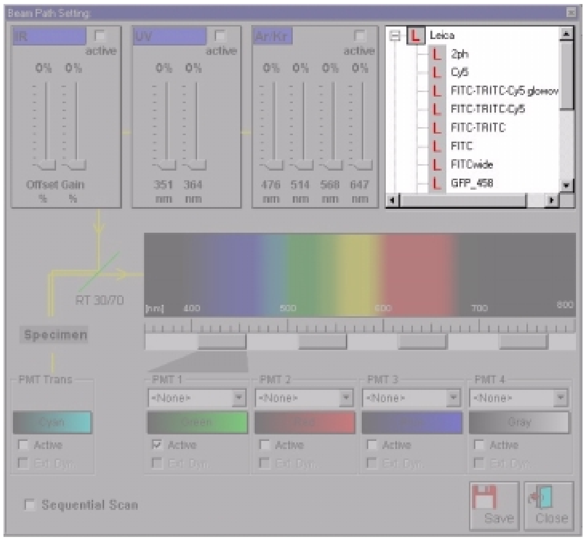
DM_Rxx microscope series: *DM-LFSx microscope series:*
Set the witch rod and fluorescence filter wheel to "Scan"

9. Selecting a specific set of acquisition parameters (application).

Press the "Beam" button in the "Acquisition" section

Beam

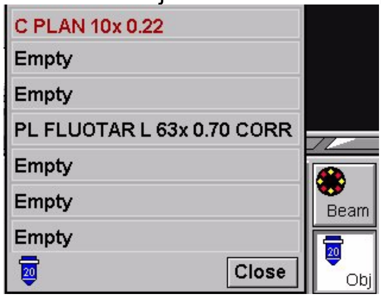
Select a method in the open "Beam Path Setting" dialog window (top right window)


Beam Path Setting
IR
UV
Ar Kr
active
Offset Gain
351 364 nm
476 514 568 647 nm
Leica
2ph
Cy5
FITC-TRITC-Cy5 glomov
FITC-TRITC-Cy5
FITC-TRITC
FITC
FITCwide
GFP_458
RT 30/70
Specimen
PMT Trans
PMT 1
PMT 2
PMT 3
PMT 4
<None>
Cyan
Green
Gray
Active
Sequential Scan
Save
Close

A method is a set of hardware settings (IPS: Instrument Parameter Setting) specifically identifying a certain acquisition technique and a special type of sample preparation. For example, the FITC/TRITC designation refers to the settings for a two-channel recording (simultaneous) for the two fluorescent dyes FITC and TRITC). It is always possible to define and store your own methods in addition to the factory-set methods. For details, refer to the chapter on "Software User Configurations".

10. Selecting the microscope objective.

Press the "Obj." button in the "Acquisition" section


C PLAN 10x 0.22
Empty
Empty
PL FLUOTAR L 63x 0.70 CORR
Empty
Empty
Empty
Close
Beam
Obj

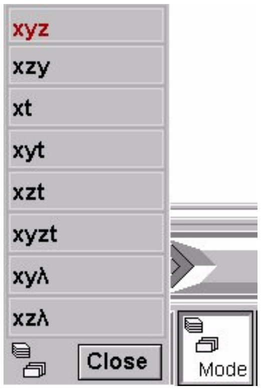
Select the objective lens to be used in the opened dialog window.

With automatic microscope stands, the selected microscope objective is automatically moved into the beam path.

Other stands require a manual operation.

11. Selecting the scan mode.

Press the "Mode" button to select the scan mode.


xyz
xzy
xt
xyt
xzt
xyzt
xyλ
xzλ
Close
Mode

The scan mode is used to select the type of data stack to be recorded. The following options are available:

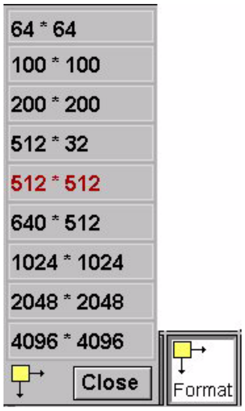
a.) spatial scan mode (xyz, xzy)

b.) time scan mode (xt, xyt, xzt, xyzt)

c.) spectral scan mode (xyλ, xzλ)

12. Selecting the scan format.

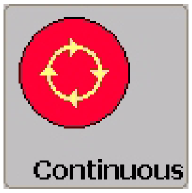
Press the "Format" button to select the scan format.


64 * 64
100 * 100
200 * 200
512 * 32
512 * 512
640 * 512
1024 * 1024
2048 * 2048
4096 * 4096
Close
Format

The scan format determines the number of grid points used by the scanner to scan the scan area.

13. Optimizing the acquisition parameters in continuous scan.

Press the "Continuous" button to start the continuous scan.

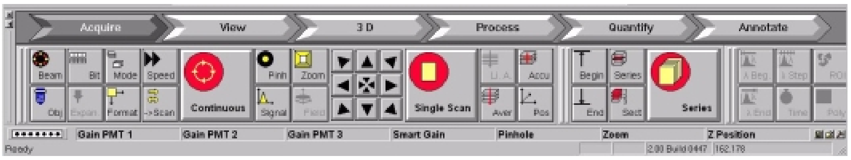

Continuous

As soon as the continuous scan runs, you can use the control panel and its defined acquisition parameters to optimize image quality.
The optimization parameters include:
a.) the exact z-position within the specimen
b.) the amplification factor of the selected detector
c.) the diameter of the detection grid mask
d.) the zoom factor

14. Acquisition of a three-dimensional spatial data set (3D series).

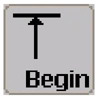
14.1. Define the upper limit of the data set to be acquired.
Use the corresponding knob for adjusting the z-position of the control panel.

The default setting of the z-position is position 7 of the control panel.


Acquire
View
3 D
Process
Quantify
Annotate
Beam
Bit
Mode
Speed
Obj
Expan
Format
->Scan
Continuous
Pinh
Zoom
Signal
Field
Single Scan
LI. A
Accu
Aver
Pos
Begin
Series
End
Sect
Series
λ Beg
λ Step
ROI
λ End
Time
Poly
Gain PMT 1
Gain PMT 2
Gain PMT 3
Smart Gain
Pinhole
Zoom
Z Position
Ready

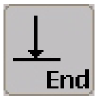
Standard layout of the control panel (bottom part of the image)

14.2. Press the "Begin" button to define the starting position of the 3D series.



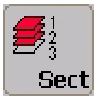
14.3. Define the lower limit of the data set to be acquired.
Use the corresponding knob for adjusting the z-position of the control panel.

14.4. Press the "End" button to define the end position of the 3D series.



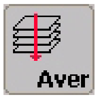
14.5. Stop the continuous scan by pressing the "Cont." button again.

14.6. Press the "Sect." button to define the number of optical sections.



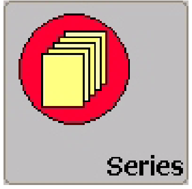
> *Additional details concerning this function can be found in the online help.*

14.7. Press the "Aver." button to define the number of sampling times (frame average).



> *This method averages the recordings of individual optical sections.*

14.8. Next, press the "Series" button to record the 3D series.



15. Saving image data.

Select ***File->Save as*** from the menu to save the data record.

> *Additional information about saving data records and saving formats can be found in the chapter "Opening and saving data sets*
>
> *", page 42.*

Setting the Köhler Illumination

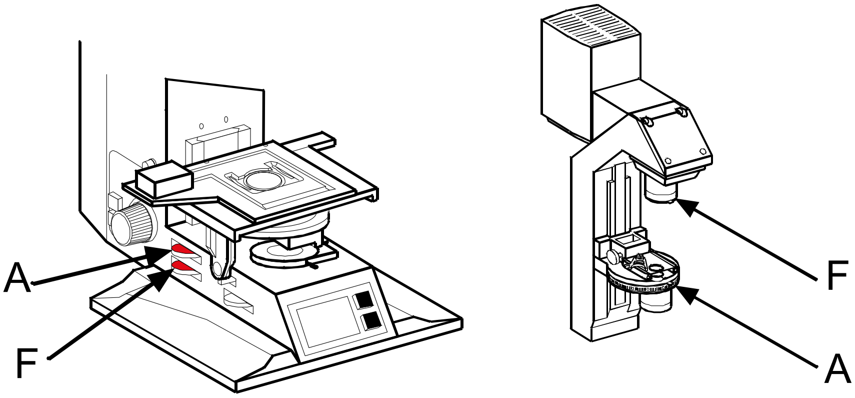
What is the Köhler illumination?

In a microscopic image, only a certain area of a specimen can be displayed (image field). Köhler illumination allows for illuminating only this particular area. The technical background for the illumination of the image field is described as follows:
If the illuminated area is smaller than the image field, the luminous cone detected by the objective lens as well as the numeric aperture becomes smaller. Since the optic resolution is directly dependent upon the numeric aperture, a lower illumination also reduces the optic resolution—which is not desired in most cases.
If the illuminated area is larger than the image field, it leads to increased scattered light. This, in turn, leads to a reduction of the image contrast, possibly resulting in the situation where optically dissolved structures of the microscopic image can no longer be observed.
Köhler illumination represents a compromise between maximum contrast and maximum resolution. The most efficient microscope objectives frequently reach their optimum optic performance only with exactly adjusted Köhler illumination.

Setting the Köhler Illumination

1. Focussing
Focus an area of the object. Neglect the quality of the illumination for the time being.

2. Opening the aperture diaphragm
Fully open the aperture diaphragm. It will be closed at a later point in time until the desired contrast is adjusted.



DM-Rxx *DM-IRxx*
A: aperture diaphragm, F: field diaphragm

3. Closing the field diaphragm
The image field darkens in most areas. You will see an unfocused light spot. If the spot disappears upon closing the field diaphragm, the field diaphragm must be centered. In this case, open the field diaphragm until you can just see the light spot at the border of the image field.

If no light spot is visible, the condenser could be set to the wrong height. You should, therefore, adjust the height of the condenser until the field diaphragm is visible.

4. Focussing

Focus the border of the light spot by adjusting the height of the condenser.

5. Centering

Turn the centering screws of the condenser until the light spot is centered in the middle of the image field. The centering is easier if you slightly open the field diaphragm to enlarge the light spot.

6. Opening the field diaphragm

Open the field diaphragm until the light spot just disappears at the border of the image field.

7. Closing the aperture diaphragm

Close the aperture diaphragm until you have set the desired image contrast (open to approximately 70% of the maximum diameter).

8. If you change the objective

It may become necessary to readjust the Köhler illumination after you have changed the objective.

Maintenance and Cleaning

Please refer to the corresponding manuals for information on how to maintain the Leica research microscope.

The instructions and additional information relating to the components of the TCS SP2 confocal system are summarized below.

Selecting an installation site

Do not expose the system to draft. Ensure that the TCS SP2 is not installed next to elevators, air conditioners or other inlets and outlets. For this reason, the installation site should be carefully planned.

Protecting the microscope from dust and grease

When not in use, the system should be covered with a plastic foil (part of delivery) or a piece of cotton cloth. The system should be operated in a room which is kept as dust and grease-free as possible. Dust caps should always be placed over the objective nosepiece positions when no objective is in place.

Exercising care in the use of aggressive chemicals

You must be particularly careful if your work involves the usage of acids, lyes or other aggressive chemicals. Make sure to keep such substances away from optical or mechanical components.

Cleaning the optical system of the microscope

The optical system of the microscope must be kept clean. Under no circumstances should users touch the optical components with their fingers or anything which may bear dust or grease.

Remove dust by using an air puffer (not solvent-based) or a fine, dry hair pencil. If this method fails, use a piece of cloth, moistened with distilled water. Persistent dirt can be removed from glass surfaces by means of pure alcohol, chloroform or naphtha.

If an objective lens is accidentally contaminated by unsuitable immersion oil or by the specimen, please contact your local Leica representative for advice on the use of certain solvents for cleaning purposes. Take this seriously, because some solvents may dissolve the glue which holds the lens in place.

Do not open objectives for cleaning.

Oil should be removed from oil immersion lenses after use. First, remove the immersion oil using a clean cloth. Once most of the oil has been removed with a clean tissue, a piece of lens tissue should be placed over the immersion end of the lens. A drop of recommended solvent should be applied, and the tissue gently drawn across the lens surface. This should be repeated as often as necessary to attain total cleanliness. Use a clean piece of lens tissue each time.

Cleaning the microscope surface

Use a linen or leather cloth (moistened with naphtha or alcohol) to clean the surfaces of the microscope housing or the scanner (varnished parts).

Never use acetone, xylene or nitro thinners as they attack the varnish.

All LEICA components and systems are carefully manufactured using the latest production methods. If you encounter problems in spite of our efforts, do not try to fix the devices or the accessories yourself, but contact your Leica representative.

Before moving the confocal system, it should be thoroughly cleaned. This applies in particular to systems that are located in biomedical research labs.

This is necessary to remove any existing contamination and to prevent any carry-over and endangering of others. Pay not only attention to surfaces, but especially to fans and cooling devices since dust can frequently accumulate at these locations.

The Leica Confocal Software: Overview

Starting the Software
Requirements for starting the software
The LCS software is copy-protected to prevent it from being used on two computers at the same time. This protection system allows all additional application packages to be used. The protection system consists of a dongle that must be inserted into the parallel port of the workstation. The functionality of the parallel port (e.g., for printing, etc.) is not affected. To use the software on a second computer, the dongle must be attached to its parallel port.

> *If you remove the dongle from the workstation of the confocal system, the software cannot be started, thereby preventing operation of the confocal system.*

The LCS software can be started in two operating modes: hardware mode and simulation mode. In hardware mode, all hardware components are accessed and initialized by the software. For this reason, you should switch on the hardware first and then wait about 20 seconds before starting the software.
In simulation mode, the software runs independently of the hardware. This mode is intended for secondary installations on another computer, for example for training or offline analysis of existing data.

Starting the software
Select **Start** → **Programs** → **Leica Confocal Software**. The initial screen of the Leica Confocal Software appears. This window allows you to select from three profiles.

Company
This option starts the Leica Confocal Software using the default factory settings. This means that the configuration and the position of the Toolbars is preset. These settings cannot be changed.

Personal
This option lets you use a user-defined configuration profile. The user name is dependent upon the account used to log on to the operating system. If the custom profile does not yet exist at the first initialization, the default factory settings are applied to the personal profile.

Last Exit
This option loads the configuration profile that was last active.

For advanced users:
If you have more than one configuration profile, you can load them at startup by clicking the button with the three black dots, which is located at the right lower edge of the profile options. You may also use this option to reset your custom configuration profile to the standard factory profile.
Clicking the Start button starts the Leica Confocal Software using the corresponding configuration profile.

> *After a delay, the software starts automatically and loads the currently selected configuration profile.*

The Experiment Software Concept
The Leica Confocal Software allows you to join image data or edited images into groups. Each group is called an "experiment" and is stored in a special data format (*.lei). This makes it possible to store both the original experimental image data and the image viewing data together. Additional information can be found in the chapter "Organizing Data by Grouping Experiments".

The Basic Structure of the User Interface

The appearance of the **G**raphical **U**ser **I**nterface—henceforth referred to as GUI—depends strongly on the configuration profile being used. A number of elements of the GUI, however, are standard. The GUI provides the following standard elements:

The Menu

The menu contains the submenus File, View, Macro, Tools, Window and Help. The submenus contain commands and information for general display, settings and user customization. They do not provide any of the functions for directly controlling the scan functions. These functions are located in the TCS menu (***View* → *Menu* → *TCS Menu***). The menu line cannot be customized.

The Viewer Window (TCS_Viewer)

The Viewer window displays the image data, experimental conditions and user data. The image window can be configured (see the chapter on "Modifying the User Interface and Defining User-Specific Settings"). The image window shows not only the confocal image data records, but also the experiment data and system settings. You can open a new experiment image window by selecting ***File* → *New***.

The TCS Menu (TCS_Menu)

The TCS menu contains buttons for the individual device functions. It is subdivided into individual work steps. The number of work steps available depends on the installed software. The standard set of work steps consists of data recording (Acquire), image viewing (View), surface reconstruction (3 D), measurement functions (Quantify), image processing and analysis functions (Process), and documentation functions (Annotate). If the TCS menu is not displayed using the current configuration profile, you can activate it by selecting ***View* → *Menu* → *TCS Menu***.

The Toolbars

This area allows you to insert and customize individual (function) buttons. The advantage of the container is that it can be switched on or off with its entire contents of buttons. For this purpose, proceed as follows: ***View* → *Menu* → *Container***.

The Document Viewer Window (Experiment Overview)

The Document Overview window displays the recorded experiments and their contents in a directory tree. Open this viewer window by selecting ***View* → *Experiment Overview***.

The Status bar

The Status bar is found at the bottom edge of the Leica Confocal Software user interface. It displays:
- the progress while loading image data (progress bar)
- the version number of the software
- the name of the system configuration (system type)

For details about these functions, refer to the chapter on "The LCS Software Functions."

Opening and saving data sets

Readable File Formats

The following file formats can be opened and viewed in the Leica Confocal Software:

Experiment (*.lei):

The format of this type of file is Leica-specific and binary. This format is provided for the data of entire experiments.

TIFF files (*.tif):

These are Leica image files in single and multiple TIF format. Both image files in the previously used TCS formats and external files in RGB TIF format can be read.

Annotation (*.ano):

The format of this type of file is also Leica-specific and binary. It is used for saving annotation sheets. The elements on the annotation sheet, such as images, texts and graphic images, are each available as individual objects.

When these files are loaded, both the image data and the experiment settings are loaded.

Automatically Applying the Study Parameters of an Experiment

With the Leica Confocal Software, hardware settings that have been saved for previous experiments or single images can be applied to new experiments. This allows you to carry out different experiments using constant settings. To carry over settings from a previous experiment, open the viewer window for the data record that contains the settings you want to apply. There, click the "Apply" button, which, in the factory default configuration profile, is located in the toolbar.

If you do not find the "Apply" button in one of the open windows, you can load it into any of the windows by selecting ***Tools → Customize****. In the dialog window that appears, select the "Commands" tab under the file category. With the left mouse button, click the "Apply" button and drag it while holding the mouse button pressed to the desired window. Release the left mouse button to insert the button at the current location.*

Saving Images.

As described above under "Readable File Formats", individual images and experiments can be saved using the same data formats.

Select ***File → Save*** to save your images and experiments. The first time an experiment is saved, the "Save as" dialog opens automatically, allowing you to specify a file name. In addition to defining a suitable file name, you can also select a file format here. Experiments can only be saved in the Leica-specific *.lei format. When you are saving experiments, you may be able to save existing individual images in *.tif or *.raw format.

If you are saving an experiment or image that has already been saved before, the old data will be overwritten. If you do not want to do this, select **File →Save as** *to save the new or changed data to a new file with a new name.*

Data Organization by Grouping Experiments

The concept of the Leica Confocal Software allows you to group individual images, image series and the results of edited images into single groups, or experiments. The Experiment Overview window provides a general overview of the open experiment. To open the Experiment Overview window, select ***View → Experiment Overview***. Create a new experiment by selecting ***File → New*** or ***File → New (Template)***. Files that were previously saved are handled as separate experiments when opened.

Compiling Experiments

After you have defined a new experiment by selecting ***File → New*** or ***File → New (Template)***, you can start filling it with data.

Images that have been recorded in continuous scan mode are overwritten automatically when the next scan is started. If you want to keep a single recording as a permanent part of an experiment, you should use the single scan function.

An experiment contains data acquired with the "single scan" or "series scan" function. If you carry out image processing functions on a data record, you can save the results as additional components of the experiment. To do this, double-click the desired image or series in the experiment overview window. Carry out the desired image processing functions (such as maximum projection or topological image or others). Mark the region within the viewer window that you want to keep as part of the experiment. Click the right mouse button (to open the context menu) and select ***Send to → Experiment***. The ***Selection (raw)*** option stores a copy of the raw data of the selected object as a new, separate component of the experiment. The ***Selection (snapshot)*** option stores an RGB image (no 3D data, just a pure snapshot) of the selected object as a new, separate component of the experiment.

Menu Functions

The following functions can be carried out from the menu bar:

File Menu

New:
This option opens a new experiment in a new window. The display properties of the new window are determined by the active defaults (see the chapter on "Default Settings and Templates").
New (Template):
This option opens a new experiment in a new window. The way in which the viewer window is displayed can be selected from a set of templates.
Open:
This option opens a previously saved experiment or a single data block (image data or annotation). The display properties of the new window are determined by the active defaults (see the chapter on "Default Settings and Templates").
Open (Template):
This option opens a previously saved experiment, a single data block or documentation files. The way in which the viewer window is displayed can be selected from a set of templates.
Close:
This option closes the current experiment. It does not save the experiment automatically before closing it.

> *You are not asked whether you want to save the experiment before it is closed.*

Close all:
This option closes all open experiments. It does not save the experiment automatically before closing it.

> *You are not asked whether you want to save the experiment before it is closed.*

Save:
Saves the current experiment.
Save as:
Saves the current experiment under a different name.
Recent files:
Opens one of the recently opened files.
Print:
Opens a dialog for printing the content of the currently active window.
Exit:
Terminates the program.

The View Menu

Menus:
Use this option to switch TCS menu and the toolbar on and off.
Status bar:
This option switches the display of the status bar on and off at the bottom edge of the user interface of

the LCS software.

Experiment Overview:

This option opens a window displaying the individual experiments and their components (data records, graphs, etc.) in the form of a tree. Double-click an experiment component within the Experiment Overview window to bring the corresponding viewer window to the foreground.

Viewer Options:

Use this item to configure various options for displaying data records in the Viewer window, such as the display angle and calculation methods for displaying image data, etc.

The Macro Menu

Macros:

Depending on the software installed, either use this option to edit macros directly or just list and run them.

> *You can edit and modify macros only if the complete, integrated VBA development environment (IDE) is installed.*

Record a New Macro:

This starts the automatic macro recorder.

For more information, refer to the documentation provided with the optional macro development environment.

Pause Recording:

This pauses the macro recorder.

Stop Recording:

This stops the macro recorder.

Visual Basic Editor (optional):

This option launches the software for developing macros and programs based on Visual Basic for Applications (VBA). Here you can edit and modify macros and even develop entire programs.

The Tools Menu

Legend Info:

This option opens the dialog window for entering user-specific data. This data (e.g., name of institute, name of specimen, etc.) is saved with each data record and can be displayed next to the acquired images in the legend.

Objective:

This option opens the dialog window for defining applied objectives. The objectives you use can be made known to the software by copying them using "drag and drop" from the list provided in this dialog window.

> *The correct information of the objective is important since some calculated values depend on it (e.g. scan field dimensions), automatic adaptation of the detection pinhole.*

Microscope:

Use this option to select the Leica microscope you are using. This setting influences the way in which the microscope is controlled (for example, the motorized objective nosepiece).

Settings:

Use this option to select the hardware settings that you want to be applied when opening a previously saved data record.

License:
This option allows you to install subsequently purchased licenses. A subsequent license usually is installed if a software package offered as an option is purchased.

The Window Menu

New Window:
This option opens a new Viewer window within the same experiment. For example, you can use this function to view the same data record using different views (such as topographical and overlay views).

The Help Menu

Contents:
Opens the table of contents of the online help.
Search:
Starts the search function for the online help.
Index:
Opens the index of the online help.

LCS file formats

Formats of user-specific and device-dependent data
The LCS software provides the following file formats for user-specific data:

	Directory: Leica	Directory: User
Instrument data (**I**nstrument **P**arameter **S**ettings)	*.IPS	*.IPS
Macros	Company.mac	User.mac
Templates for operator console	*.pbo	*.pbo
User profiles	CompanyProfile.pro	PersonalProfile.pro LastExitProfile.pro LastRecent.lst
Templates	*.prt	*.prt
Calibration data	Machine.lhw	
Color Look-Up Tables	*.lut	
User-specific profile as it was defined the last time in use		*.lst

In detail, the different file formats contain:

***.IPS**
Instrument parameters are saved in this format. This includes hardware-based settings such as intensity of the individual laser lines, number of channels used simultaneously, type of main beam splitter used, position and bandwidth of individual spectrophotometer diaphragms and sensitivity of detectors. In addition to the default unchangeable instrument parameter sets that allow a quick adjustment of the hardware, any user can save custom instrument parameter sets in his personal user directory. This allows you to save the instrument parameters typical for your planned use so that they can be reproduced and made available for other experiments.
***.mac**
Macros are saved in this format. Macros contain VBA (**V**isual **B**asic for **A**pplication) program code. Leica provides the individual program objects for controlling the confocal system in an object model. Additional information about the development of macros for controlling the confocal system can be found in the chapter on "The LCS Macro Language".
The software includes factory-predefined macros that can be used without modifications or modified according to your particular needs. The self-designed or modified macros are stored in the respective user-defined directory.
***.pbo**

Templates for the assignment of the control panel are saved in this format. These templates define which scan parameter is controlled by which rotary knob. This format also includes factory presets that have proven useful for most standard applications. Self-defined templates are saved in the user-specific directory.

***.pro**

Specific profiles of the user interface are saved in this format. This includes which button is located in which position on which toolbar. In addition, new toolbars can be defined or existing toolbars can be displayed or hidden. The position of the toolbars—whether embedded or freely movable—is also stored in a profile. This allows every user to save an individual user interface. For example, system administrators can use this tool to create a separate user interface for each knowledge level of the user (beginner, advanced, expert).

***.lst**

Besides the individual user-specific profiles, the user directory also contains the profile of the last session as well as a list of the last four data records applied by the user.

***.prt**

Templates for the "Viewer" window are saved in this format. This allows for specifically saving the appearance and type of displayed information. An explanation of the individual configuration options can be found in the chapter on "Viewer window".

***.lhw**

Calibration data and hardware settings of the confocal system are saved in this format. This file may be edited only by service personnel certified by Leica Microsystems Heidelberg.

> *Changes to this file may result in serious, possibly irreparable damages to the confocal system. Damages demonstrably resulting from changes to this file by the user will result in a loss of warranty for the complete system.*

***.lut**

Color look-up tables are saved in this format. They are used to assign the intensity values measured by the detectors to each color. This allows to bring out not only the true color display, but also certain intensity areas. More details can be found in the chapter on "Selecting Color Look-Up Tables."

Fixed Leica-specific file formats

The following file formats can be opened and viewed in the Leica Confocal Software:

Raw data (*.raw)

The RAW format saves data as a linear two-dimensional array in the INTEL format. The column index of the array corresponds to the fastest scan dimension (in general the x-axis), while the line index corresponds to the slower scan dimension (in general the y-axis). In an 8-bit image, each measured variable is saved as a single byte. A 12-bit recording uses 2 bytes, whereby the first byte contains the more significant bits according to the INTEL format (little endian). Each optical section is saved for each channel in a separate file with the file extension .raw using the following naming convention: *name of experiment_name of data record_channel number_z-dimension_number of optical section*.raw

Experiments (*.lei)

The format of this type of file is Leica-specific and binary. This format is provided for the data of entire experiments.

A file description is saved in this file format, referencing a series of image files in TIF format or RAW format.

Tiff files (*.tif)

These are Leica image files in single and multiple TIF format. Both image files in the previously used TCS formats and external files in RGB TIF format can be read.

Annotation (*.ano)
The format of this type of file is also Leica-specific and binary. It is used for saving annotation sheets. The elements on the annotation sheet, such as images, texts and graphic images, are each available as individual objects.

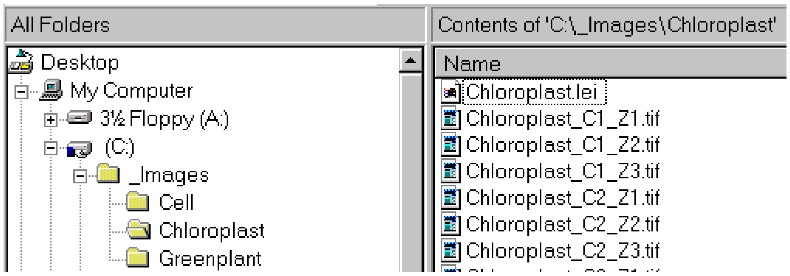
Information on whether the stored measured variable is an intensity value or a height value is saved in the corresponding LEI file.

2^7	2^6	2^5	2^4	2^3	2^2	2^1	2^0	2^{15}	2^{14}	2^{13}	2^{12}	2^{11}	2^{10}	2^9	2^8
Least significant byte according to INTEL format								Most significant byte according to INTEL format							

Displaying a 12-bit number in the INTEL format

Specification of the "Lei" file format (version beta 2.000)

When a file is saved in the LCS software, a subdirectory is first created under the current directory. The following files are saved in this subdirectory:
1. A description file whose structure is described below. This file is used for interpreting the data of an experiment through the LCS software.
2. The individual image files; naming image files is based on the following naming convention: NAME_channel number_dimensionIndex. Thus, Chloroplast_C1_Z1 means that "Chloroplast" is the name of the data record assigned by the user, that the image corresponds to the first optical section of a three-dimensional data stack (Z1), and that the data was acquired in the first detection channel (C1).



Chloroplast.lei
Chloroplast_C1_Z1.tif
Chloroplast_C1_Z2.tif
Chloroplast_C1_Z3.tif
Chloroplast_C2_Z1.tif
Chloroplast_C2_Z2.tif
Chloroplast_C2_Z3.tif

Example for saving an experiment in "Lei" format

Structure of description file

The description file can be read only by the LCS software.
It consists of a collection of various tables and contains the following structure:

Header table

Byte	Meaning	Possible values
0 ... 3	Byte arrangement	0x49494949 Intel LSB 0x4D4D4D4D Motorola MSB
4 ... 7	Identification for Leica "Lei" format	0x016033F0
8 ... 11	Version number	current: =x20000000
12 ...	Address of directory table **A**	any possible address within the complete length of the

15		file

Directory table A

Address	**Meaning**	**Entry data type**
Address of **A** = A	Number of entries in this table	DWORD
A+4	Index of the first entry (represents a logical memory block, e.g., acquisition parameter ("hardware settings") or image parameter ("dimensions"))	DWORD
A+8	Address **A1** of the 1st entry	DWORD
A+12	Index of the first entry (represents a logical memory block, e.g., acquisition parameter ("hardware settings") or image parameter ("dimensions"))	DWORD
A+14	Address **A2** of the 2nd entry	DWORD
…	...	...

The length of the directory table depends upon the number of images and image data to be saved in an experiment (acquisition parameters, number of channels, number of optical sections, etc.).
Each entry in the directory table corresponds to a logical memory block.

Block table Xn

Address	**Meaning**	**Data type**
A1 or A2 or A3.....A_i	Check digit	DWORD
A_i+ 4 Bytes	Description of the contents of the block table (currently not used)	DWORD
A_i+ 8 bytes	Version of entry	DWORD
A_i+ 12 bytes	Size of entry	DWORD
A_i+ 16 bytes	Begin of entry	The data type depends upon the entry type

The following logical memory blocks are available:

const DWORD ID_SERIES = 10;
const DWORD ID_IMAGES = 15;
const DWORD ID_DIMDESCR = 20;
const DWORD ID_FILTERSET = 30;
const DWORD ID_TIMEINFO = 40;
const DWORD ID_SCANNERSET = 50;
const DWORD ID_EXPERIMENT = 60;
const DWORD ID_LUTDESC = 70;

A memory space of 32 bits is defined for the DWORD data type.

ID_SERIES memory block

This data block contains information about the size of the complete data series combined in an experiment. It always occurs only once per experiment The data block is structured as follows:

Size [byte]	**Data type**	**Symbol**	**Description**
4	int		Internal version number
4	int	nSE	Number of image series
4	int	nIm	Length of a file name in wchar
4	int	nExt	Length of the file extension of the image file in bytes
nExt	wchar		File extension of image file

ID_IMAGES memory block

This block contains all file names of all image files that are part of a series. The data block is structured as follows:

	4	int	nFiles	Number of single images of the series
	4	int		Width of a single image
	4	int		Length of a single image
	4	int		Bits / data points (image resolution)
	4	int		Data points / pixels (display resolution)
For the next *n* image files				
	nIm * 2	wchar		Name of the next image

ID_DIMDESCR memory block

This block provides a description of an image in an n-dimensional space.

	Size [byte]	**Data type**	**Symbol**	**Description**
	4	int		Internal version number of the VOXEL description
	4	int		Voxel type, e.g., RGB or GRAY (see the following VOXEL TYPES table)
	4	DWORD		Byte size of a pixel Typical: (1 or 2) or (3 or 6 for RGB)
	4	DWORD		Resolution of scan data (8, 12 or 16 bit)
	4	int	nTC	Length of the following string in wchar (wchar = **w**ide **char**acter; character format that uses a 2-byte coding of characters unlike the ASCII format (1 byte)
	nTC * 2	wchar		Maximum value of measured variable (intensity value or length value of a distance) for a voxel
	4	int	nTC	Length of the following string in wchar
	nTC * 2	wchar		Minimum value of measured variable (intensity value or length value of a distance) for a voxel
	4	int	nTC	Length of the following string in wchar
	nTC * 2	wchar		Designation of measured variable ("I" for intensity, "z" for length value)
	4	int		Internal version number
	4	int	nDims	Dimension of the image, e.g., x_y_ch_z = 4
For the next *n* dimensions				
	4	DWORD		Identification number (ID) of the dimension (see the following list "Identification numbers (ID) for image dimensions")
	4	DWORD		Size of dimension (e.g., 512 pixel)
	4	DWORD		Distance between sub-dimensions (e.g., byte distance between the image series of two consecutive channel numbers)
	4	int	nTC	Length of the following string in wchar
	nTC * 2	wchar		Physical length with unit of length, e.g., 10 µm
	4	int	nTC	Length of the following string in wchar
	nTC * 2	wchar		Physical start position with unit of length, e.g., 10 µm
	4	int	nTC	Length of the following string in bytes
	nTC	wchar		Name of the image series
	4	int	nTC	Length of the following string in bytes
	nTC	wchar		Description of the image series

Identification numbers (ID) for image dimensions
The following identification numbers are possible:

ID (decimal)	Meaning
0	undefined
120	x
121	y
122	z
116	t for time dimension
6815843	channel number
6357100	wavelength Range
7602290	rotation
7798904	x-wide for motorized xy-stage
7798905	y-wide for motorized xy-stage
7798906	z-wide for z-stage
4259957	user1 (not specified)
4325493	user2 (not specified)
4391029	user3 (not specified)
6357095	gray-scale, e.g., of histogram
6422631	gray-scale1, e.g., of histogram
6488167	gray-scale2, e.g., of histogram
6553703	gray-scale3, e.g., of histogram
7864398	logical x (not physical, but logical position value)
7929934	logical y (not physical, but logical position value)
7995470	logical z (not physical, but logical position value)
7602254	logical t (not physical, but logical position value)
7077966	logical wavelength value (not physical, but logical value)
7471182	logical rotation value (not physical, but logical value)
5767246	logical x-wide value (not physical, but logical value)
5832782	logical y-wide value (not physical, but logical value)
5898318	logical z-wide value (not physical, but logical value)

ID_FILTERSET memory block
This memory block describes the hardware settings such as pinhole diameter or selected filters. The memory block starts with a SAFEARRAY header that describes the number and size of the entries. A complete description of the SAFEARRAY structure can be found in Appendix 1.
Presently, only three entries of the SAFEARRAY structure are used:

1. sa.cDims:
should be = 1. Presently, only one-dimensional data fields (arrays) are used.
2. sa.cbElements:
size of elements in byte
3. sa. rgsabound[0].cElements:
indicates the number of elements contained in the structure

	Size [byte]	Data type	Symbol	Description
	24	SAFEARRAY	sa	Microsoft type, see Appendix 1
for all elements that contain the structure = sa.`rgsabound[0].cElements`				
	128	wchar		Identifier for the contents of the entry

	64	wchar		Name of a short description, e.g., a TIFF description tag
	64	wchar		String (contains the contents of the string)
	16	VARIANT		Contains the data type and data value (except for the "string" data value which is described in the field above)
	4	DWORD		Separate memory space for data values
	4	DWORD		Not in use
	4	DWORD		Identifier (ID) of the memory block (for internal use only)
	4	DWORD		Test value

ID_TIMEINFO memory block

This memory block contains the time taggers for image series that were acquired with a time-dependent scan mode.

	Size [byte]	Data type	Symbol	Description	
	4	int	nDims	Indicates the number of dimensions that the description of the time tagger includes	
	4	int		Indicates which dimension was provided with a time tagger	
For the next *n* dimensions					
	4	DWORD		Identifier (ID) of the dimension (see the description of the D_DIMDESCR memory block)	
	4	DWORD		Size of the dimension (number of elements, e.g., 512 pixel)	
	4	DWORD		Distance between the individual dimension entries	
	4	int	nTS	Number of time taggers	
For the next *n*time taggers = nTS					
	64	wchar		Time tagger as string ("string" data type)	
	4	int	nTM	Number of time tags per time tagger	
For the next *m* time tags = nTM					
		4	int	nC	Number of dimensions of the time tagger description
	loop nC				
		4	int		Coordinate in this dimension
		64	wchar		Time tag as string (wchar data type)

Example:

Image with the following dimensions and dimension sizes:

x	y	ch	z
512	512	3	10

For example, the description of a time tag is 512_512_3_10. This description matches the description of the image. The time tagger description 1024_1024_3_10 for the above sample image means that the original image featured an xy-dimension of 1024*1024 and was reduced by the software (downsampling). In such cases, the time tag must be recalculated accordingly.

A time tag can be defined at any time during an n-dimensional data acquisition process (scan). The coordinate of the time tag 0_0_2_7 indicates that the time tag was set at the start of a data record (0_0_c_#) on the second channel (0_0_2_#) for the 7th optical section (0_0_2_7).

ID_SCANNERSET memory block

This memory block contains device parameters that are set at the time of the image acquisition. Further details about the SAFEARRAY structure can be found in the description of the ID_FILTERSET memory block.

	Size [byte]	Data type	Symbol	Description
	24	SAFEARRAY	sa	Microsoft type, see Appendix
for all elements that contain the structure = sa`.rgsabound[0].cElements`				
	128	wchar		Identifier for the contents of this entry
	64	wchar		Name of a shorthand notation, e.g., a TIFF description tag
	64	wchar		String (contains the contents of the string)
	20	VARIANT		Contains the data type and data value (except for the "string" data value which is described in the field above)
	4	DWORD		Separate memory space for data values
	4	DWORD		Not in use
	4	DWORD		Identifier (ID) of the memory block (for internal use only)
	4	DWORD		Test value

ID_EXPERIMENT memory block

Description of the used save format (e.g., “*.lei file with PC-Tiff images”)

	Size [byte]	Data type	Symbol	Description
	4	int		Internal version number
	4	int		Number of images in the data collection of the experiment
	4	int	nTC	Length of the following string in wchar
	nTC * 2	wchar		Short description of the format
	4	int	nTC	Length of the following string in wchar
	nTC * 2	wchar		File extension of the main file
	4	int	nTC	Length of the following string in wchar
	nTC * 2	wchar		Identifier of the single image format (e.g., PC-TIFF)
	4	int	nTC	Length of the following string in wchar
	nTC * 2	wchar		File extension for the single image format (e.g., tif / raw)

ID_LUTDESC memory block

Description of the color look-up tables (LUTs)

	Size [byte]	Data type	Symbol	Description
	4	int	nLU	Number of channels
	4	DWORD		Identifier (ID) of the dimension whose display is being colored.
For the next channels until = nLU				
	4	int		Internal version number
	1	bool		bool IsInverted
	4	int	nTC	Length of the following string in bytes
	nTC	wchar		Description of the color look-up table (LUT)
	4	int	nTC	Length of the following string in bytes
	nTC	wchar		File name of the color look-up table (if available)
	4	int	nTC	Length of the following string in bytes
	nTC	wchar		Name of the color look-up table
	4	int		Field for internal use
	4	int		Dimension of the color look-up table

SAFEARRAY Data Type

```
typedef struct FARSTRUCT tagSAFEARRAY {
    unsigned short cDims;        // Count of dimensions in this array
    unsigned short fFeatures;    // DON’T CARE
    unsigned long cbElements;    // Size of an element of the array.
                                 // Does not include size of
                                 // pointed-to data.
    unsigned long cLocks;        // DON’T CARE
    void HUGEP* pvData;          // Pointer to the data.
    SAFEARRAYBOUND rgsabound[1]; // One bound for each dimension.
} SAFEARRAY;
```

The Leica-specific file format "LEI" uses only one-dimensional data fields (arrays).

SAFEARRAYBOUND Structure

Represents the bounds of one dimension of the array. The lower bound of the dimension is represented by `lLbound`, and `cElements` represents the number of elements in the dimension. The structure is defined as follows:

```
typedef struct tagSAFEARRAYBOUND {
    unsigned long cElements;         // num of elements
    long lLbound;                    // DON’T CARE
} SAFEARRAYBOUND;
```

VARIANT and VARIANTARG

```
typedef struct FARSTRUCT tagVARIANT VARIANT;

typedef struct tagVARIANT  {
    VARTYPE vt;
    unsigned short wReserved1;
    unsigned short wReserved2;
    unsigned short wReserved3;
    union {
        unsigned char      bVal;             // VT_UI1.
        short              iVal;             // VT_I2    .
        long               lVal;             // VT_I4    .
        float              fltVal;           // VT_R4    .
        double             dblVal;           // VT_R8    .
        VARIANT_BOOL       boolVal;          // VT_BOOL.
        SCODE              scode             // VT_ERROR.
        CY                 cyVal;            // VT_CY    .
        DATE               date;             // VT_DATE.
        BSTR               bstrVal;          // VT_BSTR.
        IUnknown           FAR* punkVal;     // VT_UNKNOWN.
        IDispatch          FAR* pdispVal;    // VT_DISPATCH.
        SAFEARRAY          FAR* parray;      // VT_ARRAY|*.
        unsigned char      FAR* pbVal;       // VT_BYREF|VT_UI1.
        short              FAR* piVal;       // VT_BYREF|VT_I2.
        long               FAR* plVal;       // VT_BYREF|VT_I4.
        float              FAR* pfltVal;     // VT_BYREF|VT_R4.
        double             FAR* pdblVal;     // VT_BYREF|VT_R8.
        VARIANT_BOOL       FAR* pboolVal;    // VT_BYREF|VT_BOOL.
        SCODE              FAR* pscode;      // VT_BYREF|VT_ERROR.
        CY                 FAR* pcyVal;      // VT_BYREF|VT_CY.
        DATE               FAR* pdate;       // VT_BYREF|VT_DATE.
        BSTR               FAR* pbstrVal;    // VT_BYREF|VT_BSTR.
        IUnknown FAR*      FAR* ppunkVal;    // VT_BYREF|VT_UNKNOWN.
        IDispatch FAR*     FAR* ppdispVal;   // VT_BYREF|VT_DISPATCH.
        SAFEARRAY FAR*     FAR* pparray;     // VT_ARRAY|*.
        VARIANT            FAR* pvarVal;     // VT_BYREF|VT_VARIANT.
        void               FAR* byref;       // Generic ByRef.
     };
};
```

Files with the "LEI" format never use pointers as parameters.

VARTYPE

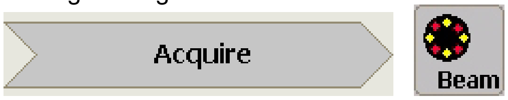
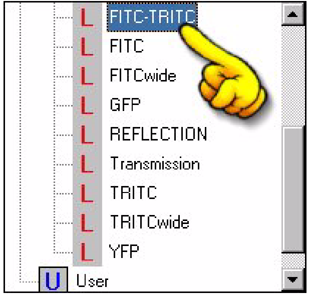
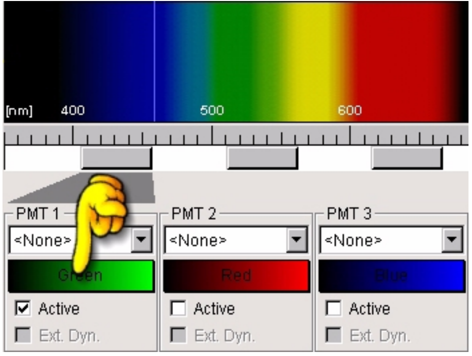
```
typedef unsigned short VARTYPE;
enum VARENUM{
    VT_EMPTY      = 0,            // Not specified.
    VT_NULL       = 1,            // Null.
    VT_I2         = 2,            // 2-byte signed int.
    VT_I4         = 3,            // 4-byte signed int.
    VT_R4         = 4,            // 4-byte real.
    VT_R8         = 5,            // 8-byte real.
    VT_CY         = 6,            // Currency.
    VT_DATE       = 7,            // Date.
    VT_BSTR       = 8,            // Binary string.
    VT_DISPATCH   = 9,            // IDispatch
    VT_ERROR      = 10,           // Scodes.
    VT_BOOL       = 11,           // Boolean; True=-1, False=0.
    VT_VARIANT    = 12,           // VARIANT FAR*.
    VT_UNKNOWN    = 13,           // IUnknown FAR*.
    VT_UI1        = 17,           // Unsigned char.

    // Other constants that are not valid in VARIANTs omitted here.

};
    VT_RESERVED    = (int) 0x8000
    // By reference, a pointer to the data is passed.
    VT_BYREF      = (int) 0x4000
    VT_ARRAY     = (int) 0x2000    // A safe array of the data is passed.
```

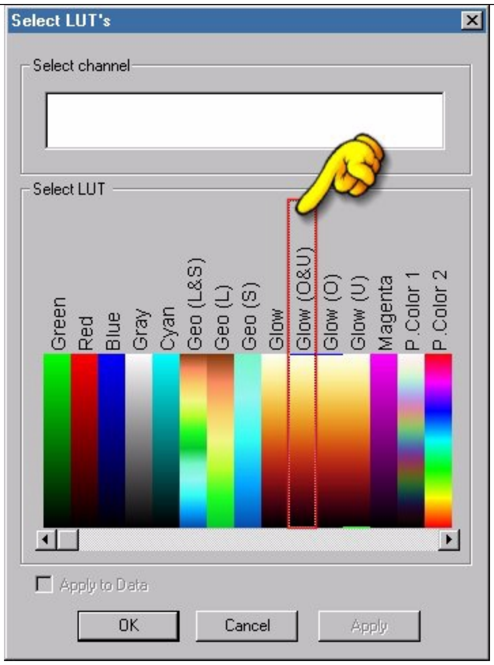
The vt value determines the interpretation of the data of the experiment as follows:

Value	**Description**
VT_EMPTY	No value was entered
VT_UI1	An unnamed 1-byte character was saved in *bVal*.
VT_I2	A 2-byte integer value was saved in *iVal*.
VT_I4	A 4-byte integer value was saved in *lVal*.
VT_R4	An IEEE 4-byte real value was saved in *fltVal*.
VT_R8	An IEEE 8-byte real value was saved in *dblVal*.
VT_CY	A fixed point value is indicated. It consists of 15 digits before the point and 4 digits after the point. The value was saved in *cyVal*.
VT_BSTR	Exceptions for files in LEI format: A string was saved in the variable TCHAR
VT_NULL	A floating zero value was indicated. Here, the floating zero is not a NULL pointer. This value is required for a 3-state logic, for example, in the SQL database query language.
VT_ERROR	An SCODE error was indicated. The error type is specified *scodee*.
VT_BOOL	A Boolean value (true / false) was indicated. The value 0xFFFF (all bits=1) means "true," the value 0 (all bits=0) means "false."
VT_DATE	A value corresponding to a date was indicated. A date is saved as a number in double-precision format. All data is saved as differential dates in reference to January 1, 1900. For example, the entry "25" corresponds to 01-25-1900.

Procedures and exercises for selected processes

Setting instrument parameters for the first image recording

1	Click on the «**Acquire**»arrow symbol and then on the «Beam» button to open the «Beam Path Setting» dialog window.  Acquire Beam
2	Double-click on one of the **Instrument Parameter Settings (IPS)**, in the list box in the upper right corner of the dialog window to load important hardware settings.  L FITC-TRITC L FITC L FITCwide L GFP L REFLECTION L Transmission L TRITC L TRITCwide L YFP U User **What is an instrument parameter setting?** An **i**nstrument **p**arameter **s**etting (IPS) consists of a file, in which all hardware settings are stored which are specific to a certain recording method. The designation «FITC-TRITC», for example, refers to the settings for a two-channel recording with the two fluorescent dyes FITC and TRITC. An instrument parameter setting enables the user to store optimum hardware settings in a file and to load them again with a simple double-click. Instrument parameter settings labeled with the letter «**L**» are predefined by Leica and cannot be changed. User-defined, modifiable instrument parameter settings are stored below «**U**» in the list box. ***How to create user-defined instrument parameter settings (IPS)***
3	Assign the «Glow(O&U)» color look-up table to every active **«PMT» detection channel**.  [nm] 400 500 600 PMT 1 <None> Green ☑ Active ☐ Ext. Dyn. PMT 2 <None> Red ☐ Active ☐ Ext. Dyn. PMT 3 <None> Blue ☐ Active ☐ Ext. Dyn. ▶Click on the symbol for the color look-up table.


Select LUT's
Select channel
Select LUT
Green
Red
Blue
Gray
Cyan
Geo (L&S)
Geo (L)
Geo (S)
Glow
Glow (O&U)
Glow (O)
Glow (U)
Magenta
P.Color 1
P.Color 2
Apply to Data
OK
Cancel
Apply

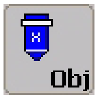
▶Click on «Glow (O&U)» and then on «OK».

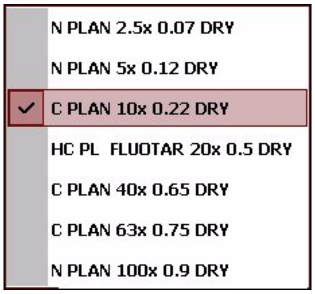
Note on image quality

The «Glow (O&U)» color look-up table visualizes maximum values in blue and minimum values in green. Thus, it is best suited for optimizing image quality. In step 12 you will find a description of how you can use this color look-up table to optimize the intensity value range of a recording for display on the monitor.

4 Before you start to optimize image quality, check the default setting of the instrument parameters listed in the following steps 5 through 9, which cannot be changed during image recording.

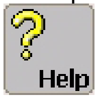
5 Click on the «Objective» button and select a microscope objective in the list.




N PLAN 2.5x 0.07 DRY
N PLAN 5x 0.12 DRY
✓ C PLAN 10x 0.22 DRY
HC PL FLUOTAR 20x 0.5 DRY
C PLAN 40x 0.65 DRY
C PLAN 63x 0.75 DRY
N PLAN 100x 0.9 DRY

If no entries appear in the list, the microscope objectives have to be assigned to the slots of the objective nosepiece.

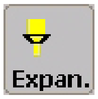
▶To learn how to do this, read the help description of the «Objective» button. To open this description, first click on the «Help» button and then on the «Objective» button.



Note on image quality

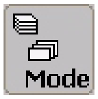
The numerical aperture of the microscope objective determines the resolution of the image, i.e. the minimum distance between two points in the object, which can still be displayed as distinct points in the image. Objectives with greater magnification also have a higher resolving power.

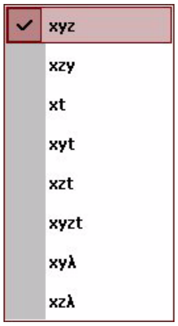
Using the beam expander lens («Beam Expander» button), an optimum adaptation of the diameter of the laser beam and the objective lens can be achieved.



In most cases the standard setting «Beam Exp 6» is best. However, in experiments in which weak lasers and high-resolution objectives are used, setting the beam expander to a smaller value can improve the illumination of the objective lens.

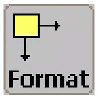
6 Click on the «Mode» button and select a scan mode.

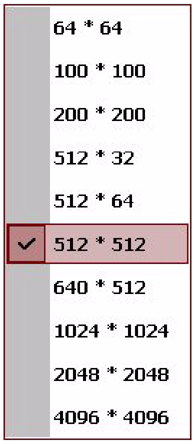




Using the scan mode, you define the dimensionality of the image recording. You can select the spatial image series «xyz, xzy», the time image series «xt, xyt, xzt, xyzt» and the spectral image series «xyλ, xzλ».

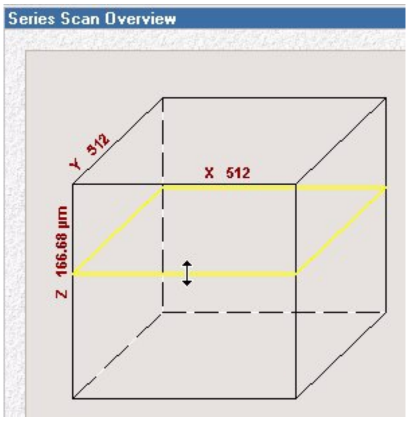
7 Click on the «Format» button and select the scan format.

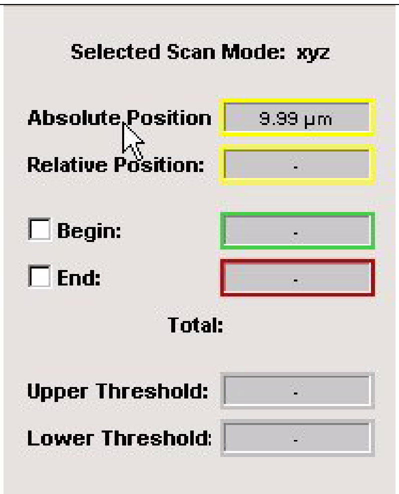




Note on image quality

The scan format determines the number of points that the scanner uses to sample the specimen and thus the number of pixels of the image. The higher the scan format at a given scan field size, the smaller is the distance between the sampling points and the better the resolution of the image.

8	Click on the «Speed» button and select the scan speed. Speed Using the scan speed, you determine how many image lines per second are sampled by the scanner. **Note on image quality** Changing the scan speed also has an impact on image quality. The lower the scan speed at a given scan format, the better the signal-to-noise ratio of the image. You can double the scan speed, if you activate bidirectional scanning by clicking the «Scan» button. ->Scan When bidirectional scanning is active, not only the forward sweep but also the reverse sweep of the scanner is used for sampling.
9	Click the «Bit» button and select the bit depth. Bit With a bit depth of 8 bit it is possible to resolve 256 different intensity values, with a bit depth of 12 bit the number is 4096.
10	Next, start the continuous scanning mode with the «Continuous» button to optimize image quality during image recording. Continuous
11	Click on the «Series» button and select a suitable focal plane (z-position) for image recording; in general this would be the brightest image plane in the specimen. Series  ▶Drag the yellow square to the desired z-position **or**



Selected Scan Mode: xyz

Absolute Position: 9.99 µm
Relative Position: -

☐ Begin: -
☐ End: -

Total:

Upper Threshold: -
Lower Threshold: -

▶Double-click on «Absolute Position» and enter a numerical value.

Note on finding the focal plane

If you have difficulty finding the focal plane, this can be due to a wrong setting of the signal amplification of the detectors.

Signal

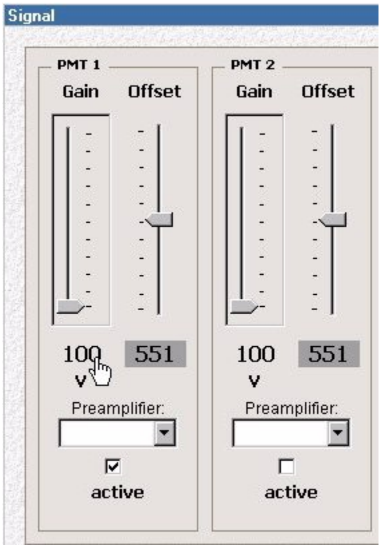
▶Click on the «Signal» button and set the «Gain» value of all active detection channels to the maximum voltage.

You can adjust many instrument parameters more quickly and conveniently by using the control panel instead of the software buttons.

How to adjust instrument parameters with the control panel

12 Click on the «Signal» button and adjust the signal amplification (Gain) and set a threshold value (Offset) for each of the active detectors (PMT) separately.

Signal



Signal

PMT 1: Gain 100 V, Offset 551, Preamplifier: , ☑ active

PMT 2: Gain 100 V, Offset 551, Preamplifier: , ☐ active

▶Move the slider or double-click on the number and enter a value.

▶Adjust the «Gain» value so that the brightest points in the image (structure) are displayed in blue.

▶Adjust the «Offset» value so that the dark points in the image (background) are displayed in green. As a rule of thumb, approximately half of the background should be displayed in green.

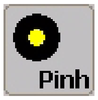
Note on image quality

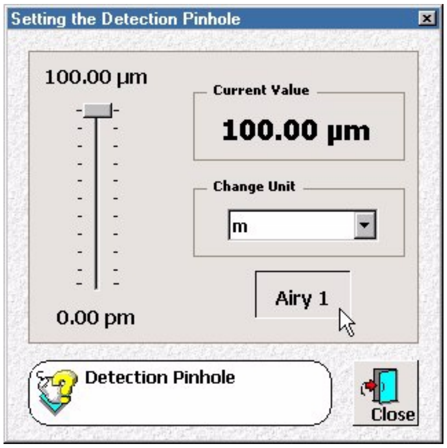
Adapting the detection parameters to the specimen ensures that the intensity value range of the image data set is optimally assigned to the digital value range and thus best visualized.

You can adjust many instrument parameters more quickly and conveniently by using the control panel instead of the software buttons.

How to adjust instrument parameters with the control panel

13 Click on the «Pinhole» button and set the detection pinhole to one Airy unit.





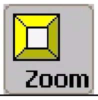
Note on image quality

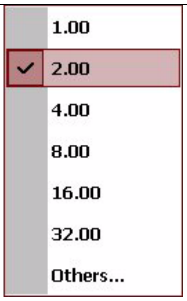
The detection pinhole is essential for the resolution of the image. Setting the diameter of the detection pinhole to one Airy unit ensures that the thickness of an optical section is minimum and thus maximum resolution is attained in the z-dimension of the image.

You can adjust many instrument parameters more quickly and conveniently by using the control panel instead of the software buttons.

How to adjust instrument parameters with the control panel

14 Click on the «Electronic Zoom» button and zoom into the area of the image that you want to record.





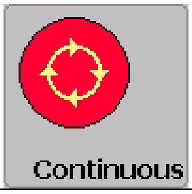
Note on image quality

Using the electronic zoom, you can improve the resolution of the image. If for example the zoom factor 2.00 is selected, only one fourth of the maximum scan field size is sampled. Consequently, at a given scan format, the distance between the sampling points is reduced and resolution is improved.

You can adjust many instrument parameters more quickly and conveniently by using the control panel instead of the software buttons.

How to adjust instrument parameters with the control panel

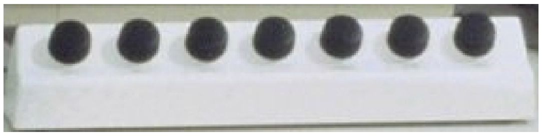
15 Click on the «Continuous» button to stop the continuous scanning mode.



l Now that all essential instrument parameters are set, you can continue recording an image series.

Setting instrument parameters using the control panel

1 You can set many instrument parameters more quickly and conveniently when you use the **control panel** instead of the software buttons.

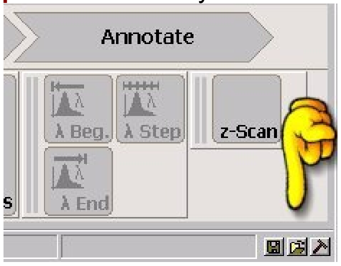


The seven rotary knobs of the control panel can be assigned to different instrument parameters, which can then be controlled by means of the control panel.

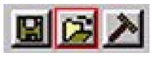
Note

The status bar of the control panel is displayed at the bottom margin of the user interface. If this is not the case, select «View → Status Bars → Control Panel Status Bar».

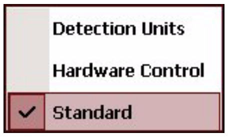
2 Load a **predefined function template** for the rotary knobs of the control panel.



▶You find the three small buttons used to configure the control panel in the right corner of the control panel status bar.



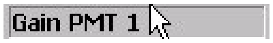
▶Click on the button in the middle.



▶Select one of the predefined function templates, e.g. «Standard».

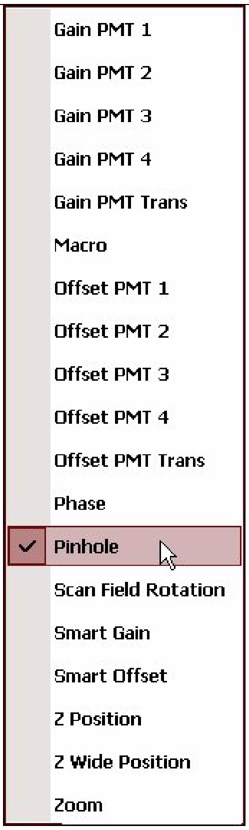
▶The instrument parameters assigned to the control panel are displayed in seven fields in the status bar. The order of these fields corresponds to the order of the rotary knobs on the control panel.

3 You can change the **assignment** of the rotary knobs of the control panel according to your needs.

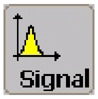


▶In the control panel status bar click on the field (rotary knob) that you want to assign to another instrument parameter.

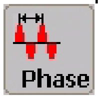
▶In the list box, select the new instrument parameter which you want to assign to the corresponding rotary knob of the control panel.


Gain PMT 1
Gain PMT 2
Gain PMT 3
Gain PMT 4
Gain PMT Trans
Macro
Offset PMT 1
Offset PMT 2
Offset PMT 3
Offset PMT 4
Offset PMT Trans
Phase
✓ Pinhole
Scan Field Rotation
Smart Gain
Smart Offset
Z Position
Z Wide Position
Zoom

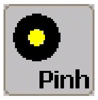
▶Select «Gain PMT 1..» or «Offset PMT 1...» if you want to adjust the signal amplification and the threshold value for the corresponding detector. It corresponds to this button in the software:



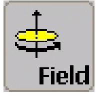
▶Select «Phase» to correct a possible phase shift when using the bidirectional scanning mode. It corresponds to this button in the software:



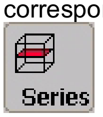
▶Select «Pinhole» if you want to set the diameter of the detection pinhole. It corresponds to this button in the software:

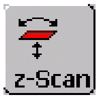


▶Select «Scan Field Rotation» if you want to adjust the degree of rotation of the scan field. It corresponds to this button in the software:

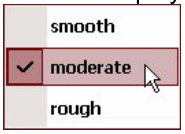
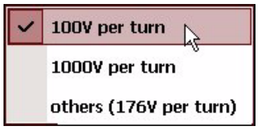
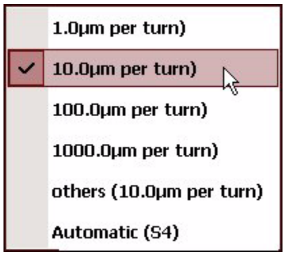


▶Select «Z Position» and «Z Wide Position» if you want to set the focal plane (z-position). It corresponds to these buttons in the software:

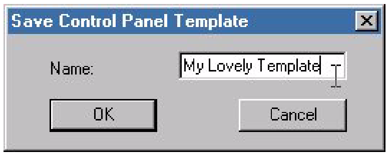




▶Select «Zoom» if you want to set the zoom factor of the electronic zoom. It corresponds to this button in the software:

	Zoom ▶Use «Smart Gain» and «Smart Offset» to set the signal amplification and threshold value of the detection channel, which you previously selected in the Viewer window. ▶Select «Macro» to save the currently executed command as a macro.
4	You can vary the **response sensitivity** of the rotary knobs. For this purpose, click with the right mouse button on an instrument parameter that is displayed in the status bar of the control panel.  ▶You can select from three sensitivity steps when you click on the instrument parameters «Phase», «Pinhole», «Scan Field Rotation» or «Zoom».  ▶For the recording parameters «Gain», «Offset», «Smart Gain» and «Smart Offset», the number of volts can be adjusted by rotating the radio button. ▶Select «others...» to enter a user-defined value.  1.0µm per turn) ✓ 10.0µm per turn) 100.0µm per turn) 1000.0µm per turn) others (10.0µm per turn) Automatic (S4) ▶For the recording parameters «Z Position» and «Z Wide Position», the number of micrometers can be adjusted by rotating the radio button. ▶Select «others...» to enter a user-defined value. ▶When you click on the «Z Wide Position» instrument parameter, you will find the additional default setting «Automatic». **Default setting «Automatic» with «Z Wide Position»** With the default setting «Automatic», the response sensitivity of the rotary knob is set automatically depending on the magnification factor of the objective currently in use. ▶For objectives with a magnification of m < 10, the response sensitivity is set to: SC = 150 µ m per turn. ▶For objectives with a magnification of 10 < m < 20, the response sensitivity is set to: S3 = 15 µ m per turn. ▶For objectives with a magnification of 20 < m < 30, the response sensitivity is set to: S2 = 7 µ m per turn. ▶For objectives with a magnification of 30 < m < 60, the response sensitivity is set to: S1 = 1 µ m per turn. ▶For objectives with a magnification of m > 60, the response sensitivity is set to: S0 = 0.5 µ m per turn.
5	Save the **new assignment** as a user-defined function template.

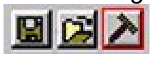
▶Click on the left button.


Save Control Panel Template
OK
Cancel

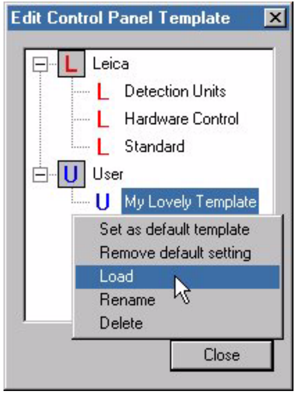
Name: My Lovely Template

▶Give the new function template a name and click on OK.

6 When you highlight the **newly defined function template** and open the context menu with the right mouse button, you can select one of the following optional commands:



▶Click on the right button.


Edit Control Panel Template
L Leica
L Detection Units
L Hardware Control
L Standard
U User
U My Lovely Template
Set as default template
Remove default setting
Load
Rename
Delete
Close

The new function template of the control panel appears in the list box below «**U**». Whenever you double-click on «My Lovely Template», the corresponding function template is loaded.

▶Select «Set as default setting», if you want the template to be loaded automatically when the software is started.

▶Select «Remove default setting», if you want to undo the definition of a template as default setting.

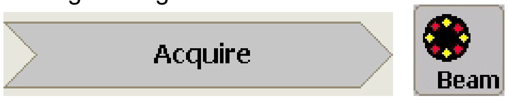
▶Select «Load» to activate the highlighted template.

▶Select «Rename» to give the template a new name.

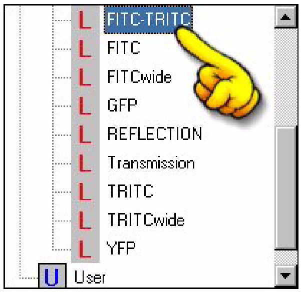
▶Select «Delete» to remove the template from the list.

Creating user-defined instrument parameter settings (IPS)

1 Click on the «**Acquire**» arrow symbol and then on the «Beam» button to open the «Beam Path Setting» dialog window.


Acquire
Beam

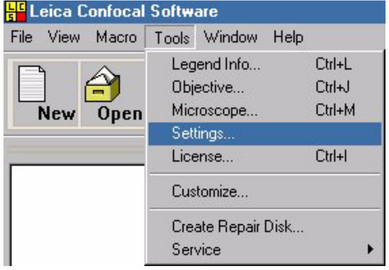
2 The instrument parameter settings are displayed in the **list box in the upper right corner** in this dialog window.


L FITC-TRITC
L FITC
L FITCwide
L GFP
L REFLECTION
L Transmission
L TRITC
L TRITCwide
L YFP
U User

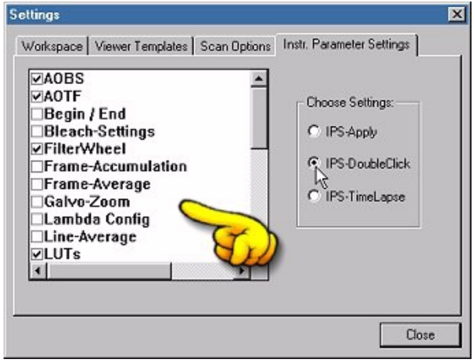
What is an instrument parameter setting?

An **i**nstrument **p**arameter **s**etting (IPS) consists of a file, in which all hardware settings are stored which are specific to a certain recording method. The designation «FITC-TRITC», for example, refers to the settings for a two-channel recording with the two fluorescent dyes FITC and TRITC. An instrument parameter setting enables the user to store optimum hardware settings in a file and to automatically load them again with a simple double-click. Instrument parameter settings labeled with the letter «**L**» are predefined by Leica and cannot be changed. User-defined, modifiable instrument parameter settings are stored below «**U**» in the list box.

3 Select **Tools** → **Settings** → **Instr. Parameter Settings**» and specify the parameters, which are to be retrieved when an instrument parameter setting is loaded.


Leica Confocal Software
File
View
Macro
Tools
Window
Help
New
Open
Legend Info... Ctrl+L
Objective... Ctrl+J
Microscope... Ctrl+M
Settings...
License... Ctrl+I
Customize...
Create Repair Disk...
Service

▶Select «Tools → Settings → Instr. Parameter Settings».


Settings
Workspace
Viewer Templates
Scan Options
Instr. Parameter Settings
Close

☑AOBS
☑AOTF
☐Begin / End
☐Bleach-Settings
☑FilterWheel
☐Frame-Accumulation
☐Frame-Average
☐Galvo-Zoom
☐Lambda Config
☐Line-Average
☑LUTs

Choose Settings:
○ IPS-Apply
● IPS-DoubleClick
○ IPS-TimeLapse

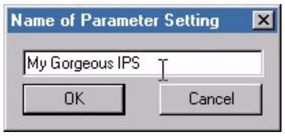
▶Select the «IPS-DoubleClick» option and click on the check boxes of the instrument parameters you want to include.

4 To adapt instrument parameters to the currently used specimen during image recording, click on the «Continuous» button and start the continuous scanning mode.

Continuous

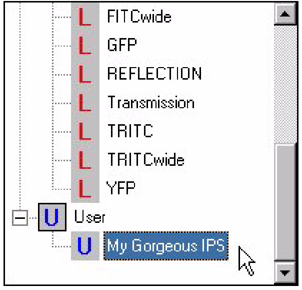
Setting instrument parameters for the first image recording

5 In the «Beam Path Setting» dialog window, click on the «Save» button to save the modified parameters as an instrument parameter setting.



My Gorgeous IPS

▶Give the new instrument parameter setting a name and click on OK.



The new instrument parameter setting appears in the list box below «**U**». Whenever you double-click on «My Gorgeous IPS», all hardware settings saved with this instrument parameter setting are loaded.

Where are instrument parameter settings stored?

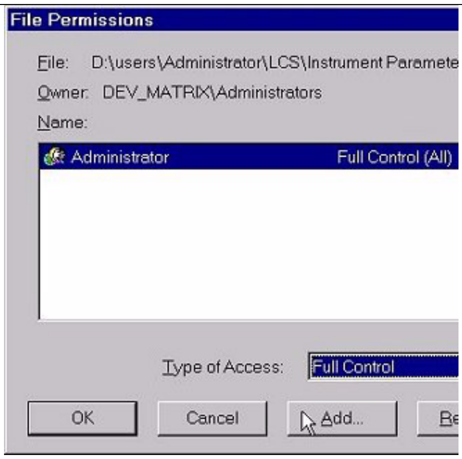
User-defined instrument parameter settings are stored in the user directory of the user who is currently logged onto Windows NT. As default setting the directory of users is located at: D:\users\... Consequently, access to a specific instrument parameter setting is only given to the user who produced it. If several users should have access to a user-defined parameter setting, the user who produced it or the administrator has to unblock it for other users.

6 You can give other users **access** to your instrument parameter setting.

▶Open the Windows Explorer and select the directory where the instrument parameter settings are stored. As default setting you will find this directory, for example of a user logged onto the system as administrator, at: D:\ users\ Administrator\ Lcs\ Instrument Parameter Settings\...

▶Highlight the instrument parameter setting, click the right mouse button to open the context menu and select «Properties».

▶In this dialog window, click on the «Security» tab and then on «Permissions».



File Permissions

File: D:\users\Administrator\LCS\Instrument Paramete

Owner: DEV_MATRIX\Administrators

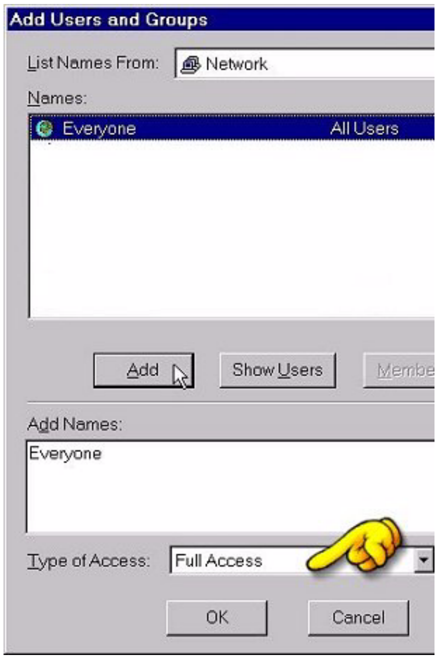
Name:

Administrator — Full Control (All)

Type of Access: Full Control

OK
Cancel
Add...
Re

▶Click on the «Add» button in the «File Permissions» dialog window to select the users who will receive permission to use the file.



Add Users and Groups

List Names From: Network

Names:

Everyone — All Users

Add Names: Everyone

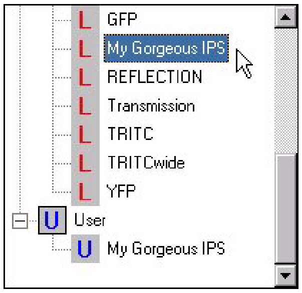
Type of Access: Full Access

Add
Show Users
Membe
OK
Cancel

▶Select the users in the «Names» field (e.g. «Everyone») and click on «Add».

▶Assign the «Full Access» permission status to the new user in the «Type of Access» list box and close the dialog window with «OK».

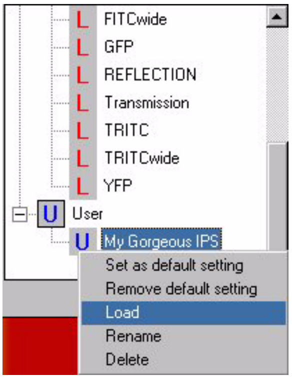
▶In Windows Explorer, copy the file of the instrument parameter setting to the directory: C:\Program files\ Leica\ Lcs\ Instrument Parameter Settings\... **Important: Use the «Copy» and not the «Cut» command to do this!**



L GFP
L My Gorgeous IPS
L REFLECTION
L Transmission
L TRITC
L TRITCwide
L YFP
U User
U My Gorgeous IPS

The next time you open the «Beam Path Setting» dialog window, the instrument parameter setting will appear in the list labeled with «L» and can be used by all users.

7 When you highlight the **newly defined instrument parameter setting** and open the context menu with the right mouse button, you can select one of the following optional commands:


FITCwide
GFP
REFLECTION
Transmission
TRITC
TRITCwide
YFP
User
My Gorgeous IPS
Set as default setting
Remove default setting
Load
Rename
Delete

▶Select «Set as default setting», if you want the parameter setting to be loaded automatically when the software is started.

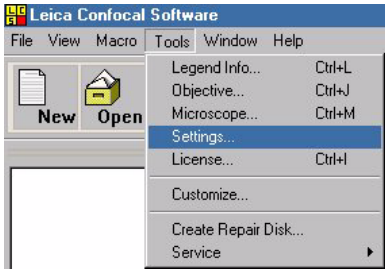
▶Select «Remove default setting», if you want to undo the definition of an instrument parameter setting as default setting.

▶Select «Load» to activate the highlighted parameter setting.

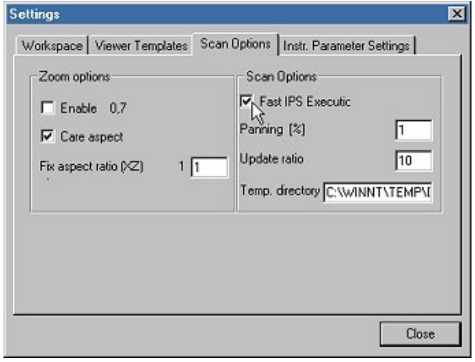
▶Select «Rename» to give the parameter setting a new name.

▶Select «Delete» to remove the parameter setting from the list.

8 If the **loading speed** of an instrument parameter setting is too slow, you can activate a fast mode.


Leica Confocal Software
File View Macro Tools Window Help
New
Open
Legend Info... Ctrl+L
Objective... Ctrl+J
Microscope... Ctrl+M
Settings...
License... Ctrl+I
Customize...
Create Repair Disk...
Service

▶Select «Tools ⟶ Settings ⟶ Scan Options».


Settings
Workspace
Viewer Templates
Scan Options
Instr. Parameter Settings
Zoom options
Scan Options
Close

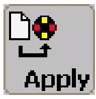
☐ Enable 0,7
☑ Care aspect
Fix aspect ratio (XZ) 1 1
☑ Fast IPS Executic
Panning [%] 1
Update ratio 10
Temp. directory C:\WINNT\TEMP\

▶Click on the «Fast IPS Execution» check box to activate the fast mode.

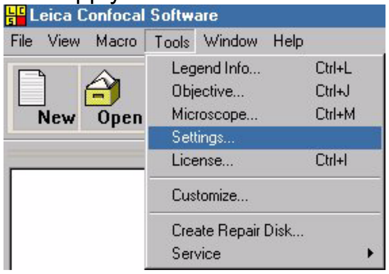
Note

If you open instrument parameter settings which have not been created with the version of the Leica Confocal Software that you have currently in use, it is possible that the fast mode cannot be carried out. In this case, an error message will be displayed which prompts you to restart the system.

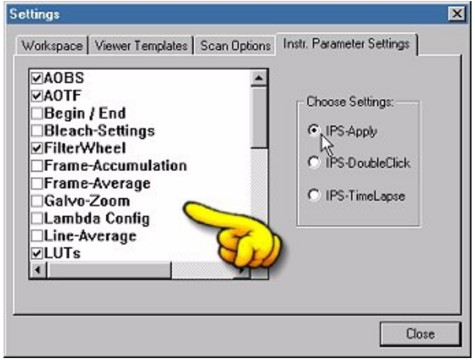
9 If you want to reuse the instrument parameter settings of an existing experiment for a new image recording, open the experiment and click on the «Apply» button.



▶Select «Tools → Settings → Instr. Parameter Settings» and specify the instrument parameters to be loaded when the «Apply» button is clicked.


Leica Confocal Software
File
View
Macro
Tools
Window
Help
New
Open
Legend Info... Ctrl+L
Objective... Ctrl+J
Microscope... Ctrl+M
Settings...
License... Ctrl+I
Customize...
Create Repair Disk...
Service

▶Select «Tools/ Settings/ Instr. Parameter Settings».


Settings
Workspace
Viewer Templates
Scan Options
Instr. Parameter Settings
Choose Settings:
Close

☑AOBS
☑AOTF
☐Begin / End
☐Bleach-Settings
☑FilterWheel
☐Frame-Accumulation
☐Frame-Average
☐Galvo-Zoom
☐Lambda Config
☐Line-Average
☑LUTs

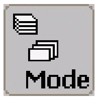
● IPS-Apply
○ IPS-DoubleClick
○ IPS-TimeLapse

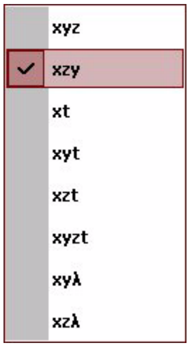
▶Select the «IPS-Apply» option and click on the check boxes of the instrument parameters you want to include.

Recording spatial image series

<table>
<tr>
<td>1</td>
<td>

Click on the «**Acquire**» arrow symbol and then on the «Mode» button to select the «xyz» or «xzy» scan mode.



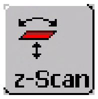

xyz
✓ xzy
xt
xyt
xzt
xyzt
xyλ
xzλ

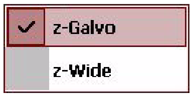
▶Select the «xyz» scan mode if you want to record a stack of horizontal images (orthogonal to the optical axis).

▶Select the «xzy» scan mode if you want to record a stack of vertical images (parallel to the optical axis).

Which scan modes are available?

The number of available scan modes depends on the selected z-actuator type. Click on the «z-Scan» button to choose the z-actuator.



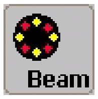

✓ z-Galvo
z-Wide

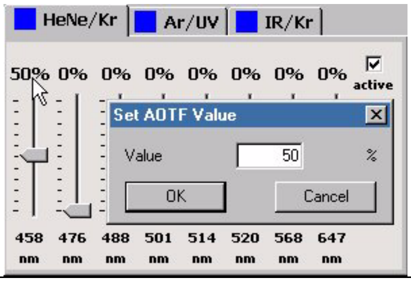
All scan modes are available when you use the galvanometer-driven z-specimen stage. For this stage select the «z-Galvo» option in the list.

There are only the scan modes «xyz, xt, xyt, xyzt and xyλ» available when you use the electromotor-driven z-specimen stage (or the motorized objective nosepiece with inverted microscopes). For this stage select the «z-Wide» option in the list.

</td>
</tr>
<tr>
<td>2</td>
<td>

Click on the «Beam» button and open the «Beam Path Setting» dialog window to choose the excitation wavelength and set the laser intensity.



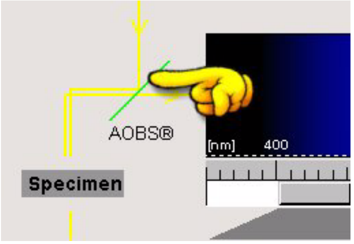

HeNe/Kr
Ar/UV
IR/Kr
50% 0% 0% 0% 0% 0% 0% 0%
☑ active
Set AOTF Value
OK
Cancel
458 nm
476 nm
488 nm
501 nm
514 nm
520 nm
568 nm
647 nm

Value 50 %

</td>
</tr>
</table>

▶Move the slider or double-click on the number and enter a value for the laser intensity.

3 Select a suitable **excitation beam splitter**:



If your system is equipped with an acousto-optical beam splitter (AOBS), the beam path is optimized automatically for the wavelength you selected.
Otherwise double-click on the green line and select a beam splitter:

▶Select the dichroic RSP 500 for image recordings of FITC and GFP with an excitation wavelength of 488 nm and a detection bandwidth of 500 to 600 nm.

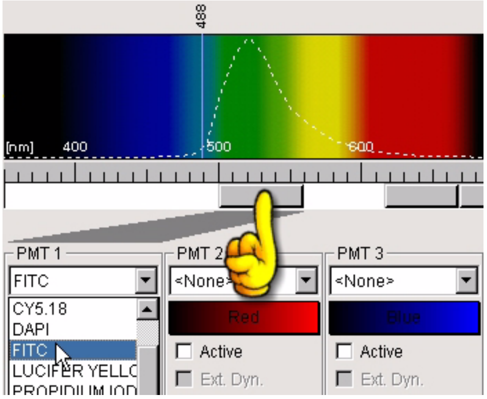
▶Select the dichroic RSP 465 for image recordings of CFP with an excitation wavelength of 458 nm and a detection bandwidth of 465 to 525 nm.

▶Select the double dichroic DD 488 / 543 for image recordings of FITC and TRITC or GFP and DsRED with an excitation wavelength of 488 and 543 nm and a detection bandwidth of 500 to 530 nm and 555 to 700 nm, respectively.

▶Select the triple dichroic TD 488 / 543 / 633 for image recordings of FITC, TRITC and CY5 with an excitation wavelength of 488, 543 and 633 nm and a detection bandwidth of 500 to 535 nm, 555 to 620 nm and 650 to 750 nm, respectively.

▶Select the neutral filter RT 30/70 for recordings of reflected light.

4 Select one or several **«PMT» detection channels** and set the fluorescence dye and detection bandwidth.

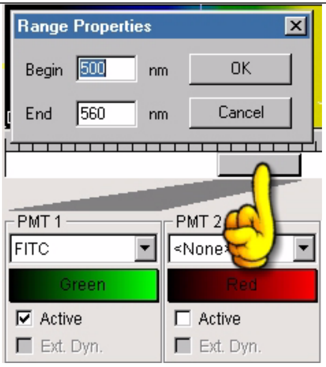


▶Click on the list box of a detection channel and select the fluorescence dye you want to use. The emission curve of the fluorescence dye is displayed in the spectrum.

▶Position the slider below the emission curve.

Note on positioning the detection band

When recording fluorescence light, take care that you do not position the detection band directly below the laser line, since thus reflected light will also be recorded. The distance between laser line and detection band should be at least 5 nm in the blue spectral range (excitation line 488 nm), 10 nm in the green spectral range (excitation line 543 nm) and 15 nm in the red spectral range (excitation line 633 nm).



Range Properties

Begin 500 nm

End 560 nm

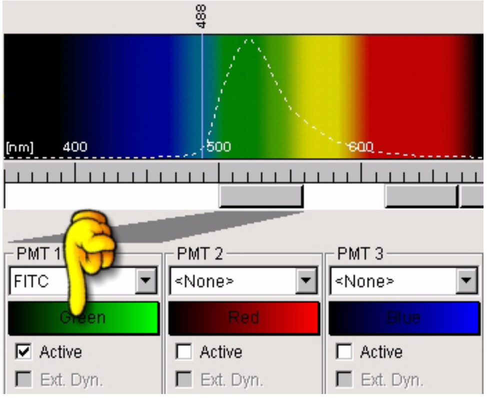
PMT 1: FITC, ☑ Active, ☐ Ext. Dyn.

PMT 2: <None>, ☐ Active, ☐ Ext. Dyn.

OK
Cancel
Green
Red

▶Double-click on the slider of the selected detection channel.

▶In the «Range Properties» dialog window enter a wavelength for the beginning and the end of the detection band.



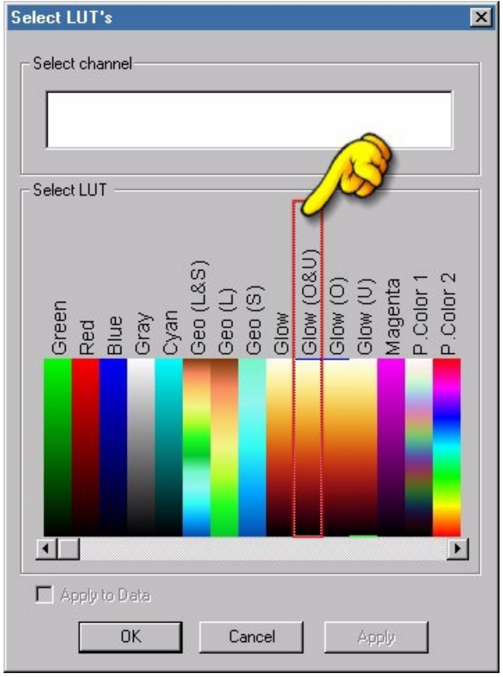
PMT 1: FITC, ☑ Active, ☐ Ext. Dyn.

PMT 2: <None>, ☐ Active, ☐ Ext. Dyn.

PMT 3: <None>, ☐ Active, ☐ Ext. Dyn.

488
[nm]
400
500
600
Green
Red
Blue

▶Click on the symbol for the color look-up table.



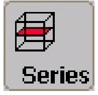
Select LUT's
Select channel
Select LUT
Green
Red
Blue
Gray
Cyan
Geo (L&S)
Geo (L)
Geo (S)
Glow
Glow (O&U)
Glow (O)
Glow (U)
Magenta
P.Color 1
P.Color 2
Apply to Data
OK
Cancel
Apply

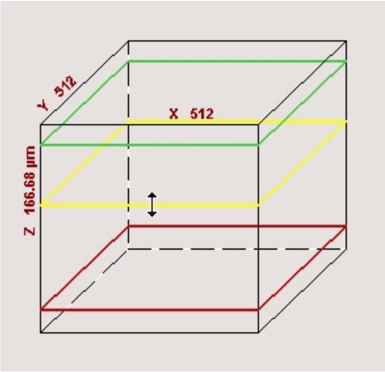
▶Click on the color look-up table you want to use and on «OK».

For the first image recording it is recommendable to use the «Glow(O&U)» color look-up table.

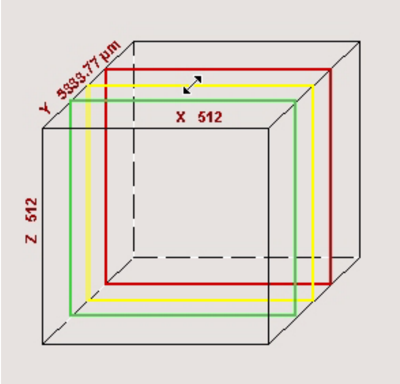
Setting instrument parameters for the first image recording

5 Click on the «Series» button and open the «Series Scan Overview» dialog window to define begin point and end point of the spatial image series.





Scan mode: xyz

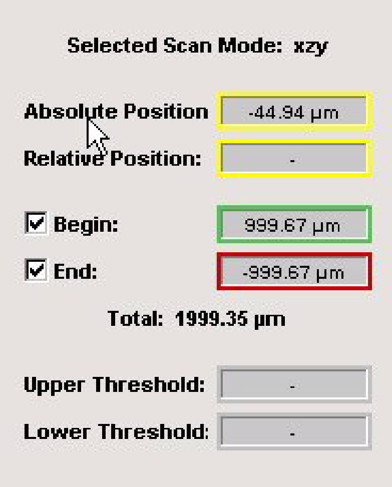


Scan mode: xzy

▶To set the begin point, move the yellow square to the desired z-position (xyz scan mode) or y-position (xzy scan mode) and click the «Begin» check box.

▶To set the end point, move the yellow square to the desired z-position (xyz scan mode) or y-position (xzy scan mode) and click the «End» check box.

or



Selected Scan Mode: xzy

Absolute Position: -44.94 µm

Relative Position: -

☑ Begin: 999.67 µm

☑ End: -999.67 µm

Total: 1999.35 µm

Upper Threshold: -

Lower Threshold: -

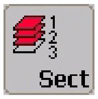
▶Double-click on «Absolute Position», enter a position value and click the «Begin» check box.

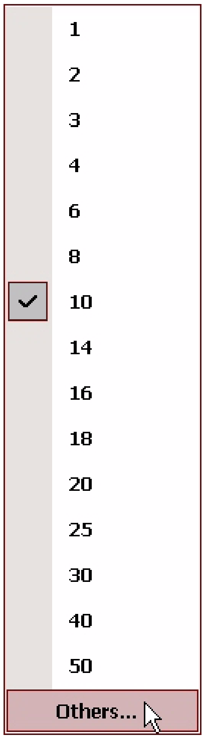
▶Double-click on «Absolute Position», enter a position value and click the «End» check box.

Relative z-positioning

If you use the electromotor-driven z-actuator and you have selected the «z-Wide» option with the «z-Scan» button, you can set a relative z-position. The relative z-position is calculated in reference to an «Upper Threshold» which has to be set in the software of the microscope stand. Relative z-positioning is only available with microscope stands of the following types: DM RXA, DM RXA2, DM IRE2 and DM LFSA (as well as with older IRBE stands with EPROM version 2.40). The «Upper Threshold» and «Lower Threshold» values are displayed in the «Series Overview» dialog window.

6 Click on the «Sections» button and determine the number of recordings between begin point and end point.





▶Select one of the values in the list or «Others».

When you select «Others», the «**Z/Y-Configuration**» dialog window appears where you can calculate the number of recordings «#» or the distance between the recordings «Step size» depending on the total height of the image stack «Image dim.z».

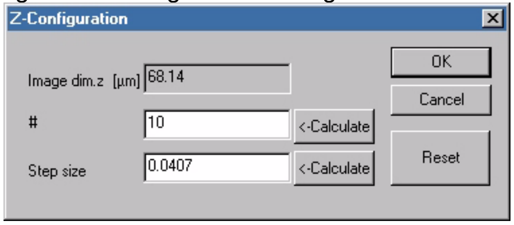


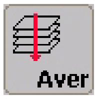
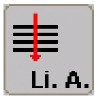
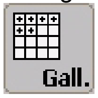
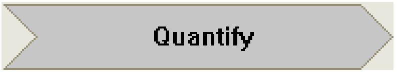
Image dim.z [µm] 68.14

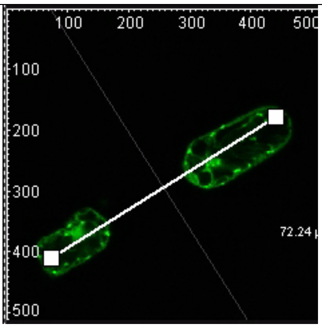
# 10

Step size 0.0407

▶When you change the number of recordings and click on «Calculate» next to «#», the step size is calculated with the priority of leaving the height of the image stack unchanged.

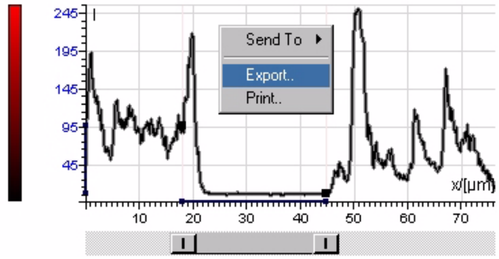
▶When you change the number of recordings and click on «Calculate» next to «Step size»,

	the step size is calculated with the priority of leaving the number of recordings unchanged. ▶When you change the step size and click on «Calculate» next to «Step size», the number of recordings is calculated with the priority of leaving the height of the image stack unchanged. ▶When you change the step size and click on «Calculate» next to «#», the number of recordings is calculated with the priority of leaving the step size unchanged.
7	Start the continuous scanning mode with the «Continuous» button to optimize image quality during image recording. Continuous ▶Set the focal plane (z-position), the detector parameters and the detection pinhole. For more details, read steps 7 through 15 in the tutorial «Setting instrument parameters for the first image recording». ***Setting instrument parameters for the first image recording***
8	Click on the «Series» button to start recording the spatial image series. ▶To improve the signal-to-noise ratio click on the «Average» button. Define how often the recording of an image is to be repeated. The average value is displayed in the result image.  ▶To improve the signal-to-noise ratio click on the «Line Average» button. Define how often the recording of a line is to be repeated. The average value is displayed in the result image.  ▶Click on the «Gallery» button to display the single images in the Viewer window already during image recording.  **Note** It has a negative impact on the system performance when you record large image data sets and use the «Gallery» function.
9	For a statistical evaluation of the spatial image series click on the «**Quantify**» arrow symbol and then, for example, on the «Profile» button.  Clicking this button opens the «Profile» dialog window and displays a measurement section on the image.


100
200
300
400
500
100
200
300
400
500
72.24

▶The measurement section can be positioned anywhere on the image.

10 In the «Profile» dialog window where an **evaluation graph** and various statistical data are displayed, you have access to the following additional commands:


245
195
145
95
45
Send To
Export..
Print..
x/[µm]
10
20
30
40
50
60
70

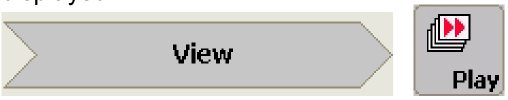
▶Hold the mouse pointer over the diagram and click the right mouse button to open the context menu.

▶Select «Export» to save the evaluation data in an ASCII text file.

▶Select «Send to» and «Document» to save a snapshot of the evaluation graph as a new file in the currently active experiment.

▶Select «Print» to print out the evaluation graph.

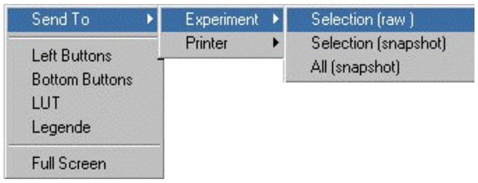
▶Click on the «View» arrow symbol and the «Play» button to run the spatial image series as a movie. Simultaneously, the evaluation graph and evaluation data of each single image are displayed.



11 You can save an **image of a single detection channel** as a new data set in the currently active experiment.

▶Click on the image in the selected detection channel.

▶Hold the mouse pointer over the Viewer window and click the right mouse button to open the context menu.

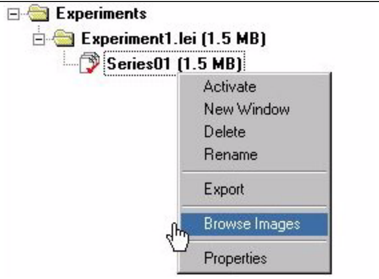

Send To
Left Buttons
Bottom Buttons
LUT
Legende
Full Screen
Experiment
Printer
Selection (raw)
Selection (snapshot)
All (snapshot)

▶Choose «Selection (raw)» to save the image data of the selected image in a new file.

▶Choose «Selection (snapshot)» to save a snapshot of the selected image in a new file.

▶Choose «All (snapshot)» to save a snapshot of all images in the Viewer window in a new file.

The new files are displayed in the «Experiment Overview» window where you have access to the following commands in the context menu:


Experiments
Experiment1.lei (1.5 MB)
Series01 (1.5 MB)
Activate
New Window
Delete
Rename
Export
Browse Images
Properties

▶Highlight a file of your choice and click the right mouse button to open the context menu.

▶Select «Activate» to display the image data of the file in the Viewer window.

▶Select «New Window» to open the currently displayed recording in a second Viewer window. This is useful, for example, when you want to visualize the same image data with another color look-up table.

▶Select «Delete» to remove the file from the list.

▶Select «Rename» to give the file a new name.

▶Select «Export» to save a recording in the *.avi file format.

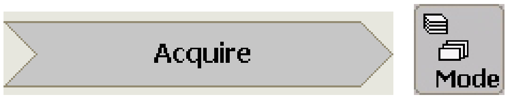
▶Select «Browse Images» to display all images of an experiment as thumbnails images on the user interface.

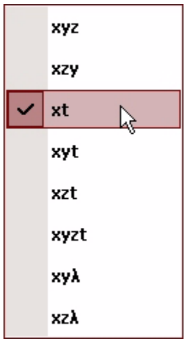
▶Select «Properties» to document important properties of the recording in a dialog window.

▶To move single files from one experiment to the other (drag and drop) click on the file, keep the left mouse button pressed and drag the file to the target folder.

Recording time image series

1 Click on the «**Acquire**» arrow symbol and then on the «Mode» button to select the «xt», «xyt», «xzt» or «xyzt» scan mode.





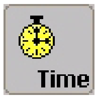
▶Select the «xt» scan mode if you want to record a line at regular time intervals.

▶Select the «xyt» scan mode if you want to record horizontal images (orthogonal to the optical axis) at regular time intervals.

▶Select the «xzt» scan mode if you want to record vertical images (parallel to the optical axis) at regular time intervals.

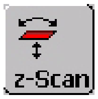
▶Select the «xyzt» scan mode if you want to record a stack of horizontal images (orthogonal to the optical axis) at regular time intervals.

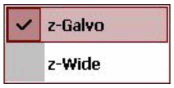
The selection of one of these scan modes automatically activates the «Time» button which is required for configuring a time series.



Which scan modes are available?

The number of available scan modes depends on the selected z-actuator type. Click on the «z-Scan» button to choose the z-actuator.

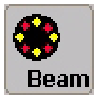


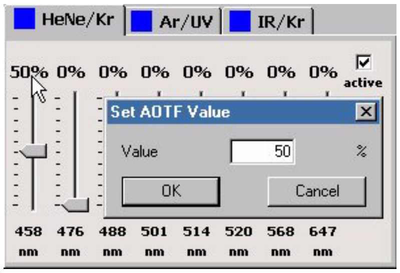


All scan modes are available when you use the galvanometer-driven z-specimen stage. For this stage select the «z-Galvo» option in the list.

There are only the scan modes «xyz, xt, xyt, xyzt and xyλ» available when you use the electromotor-driven z-specimen stage (or the motorized objective nosepiece with inverted microscopes). For this stage select the «z-Wide» option in the list.

2 Click on the «Beam» button and open the «Beam Path Setting» dialog window to choose the excitation wavelength and set the laser intensity.

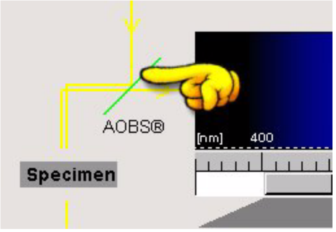




Value 50 %

▶Move the slider or double-click on the number and enter a value for the laser intensity.

3 Select a suitable **excitation beam splitter**:



If your system is equipped with an acousto-optical beam splitter (AOBS), the beam path is optimized automatically for the wavelength you selected.

Otherwise double-click on the green line and select a beam splitter:

▶Select the dichroic RSP 500 for image recordings of FITC and GFP with an excitation wavelength of 488 nm and a detection bandwidth of 500 to 600 nm.

▶Select the dichroic RSP 465 for image recordings of CFP with an excitation wavelength of 458 nm and a detection bandwidth of 465 to 525 nm.

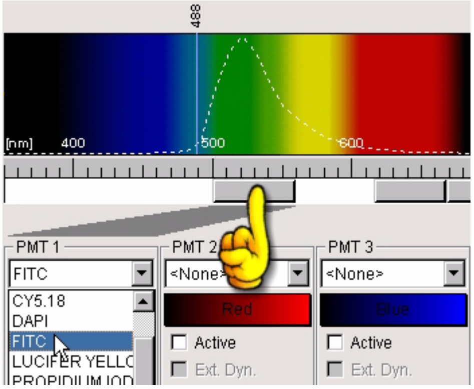
▶Select the double dichroic DD 488 / 543 for image recordings of FITC and TRITC or GFP and DsRED with an excitation wavelength of 488 and 543 nm and a detection bandwidth of 500 to

530 nm and 555 to 700 nm, respectively.

▶Select the triple dichroic TD 488 / 543 / 633 for image recordings of FITC, TRITC and CY5 with an excitation wavelength of 488, 543 and 633 nm and a detection bandwidth of 500 to 535 nm, 555 to 620 nm and 650 to 750 nm, respectively.

▶Select the neutral filter RT 30/70 for recordings of reflected light.

4 Select one or several **«PMT» detection channels** and set the fluorescence dye and detection bandwidth.

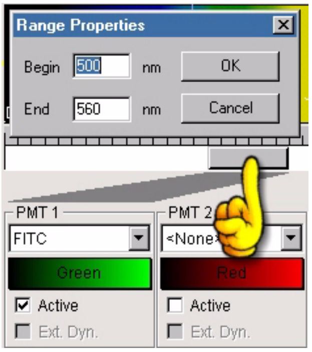

488
[nm]
400
500
600
PMT 1
FITC
CY5.18
DAPI
FITC
LUCIFER YELLO
PROPIDIUM IOD
PMT 2
<None>
Red
Active
Ext. Dyn.
PMT 3
<None>
Blue
Active
Ext. Dyn.

▶Click on the list box of a detection channel and select the fluorescence dye you want to use. The emission curve of the fluorescence dye is displayed in the spectrum.

▶Position the slider below the emission curve.

Note on positioning the detection band

When recording fluorescence light, take care that you do not position the detection band directly below the laser line, since thus reflected light will also be recorded. The distance between laser line and detection band should be at least 5 nm in the blue spectral range (excitation line 488 nm), 10 nm in the green spectral range (excitation line 543 nm) and 15 nm in the red spectral range (excitation line 633 nm).


Range Properties

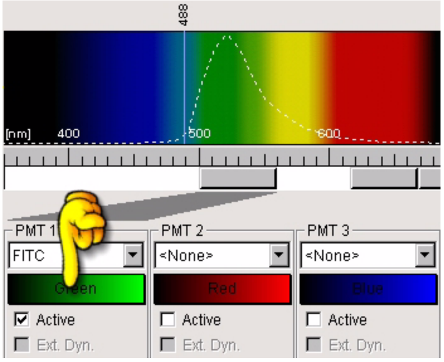
Begin 500 nm

End 560 nm

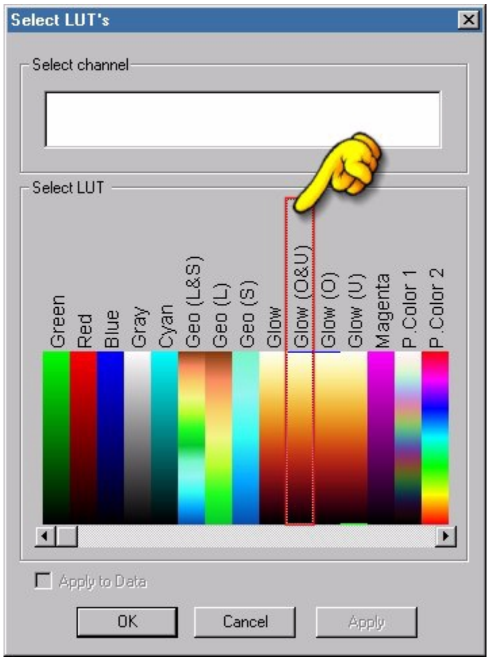
OK
Cancel
PMT 1
FITC
Green
☑ Active
☐ Ext. Dyn.
PMT 2
<None>
Red
☐ Active
☐ Ext. Dyn.

▶Double-click on the slider of the selected detection channel.

▶In the «Range Properties» dialog window enter a wavelength for the beginning and the end of the detection band.


488
[nm]
400
500
600
PMT 1
FITC
Green
☑ Active
☐ Ext. Dyn.
PMT 2
<None>
Red
☐ Active
☐ Ext. Dyn.
PMT 3
<None>
Blue
☐ Active
☐ Ext. Dyn.

▶**Click on the symbol for the color look-up table.**


Select LUT's
Select channel
Select LUT
Green
Red
Blue
Gray
Cyan
Geo (L&S)
Geo (L)
Geo (S)
Glow
Glow (O&U)
Glow (O)
Glow (U)
Magenta
P.Color 1
P.Color 2
☐ Apply to Data
OK
Cancel
Apply

▶**Click on the color look-up table you want to use and on «OK».**

For the first image recording it is recommendable to use the «Glow(O&U)» color look-up table.

Setting instrument parameters for the first image recording

5 Click on the «Time» button and set all parameters required for the time image series.

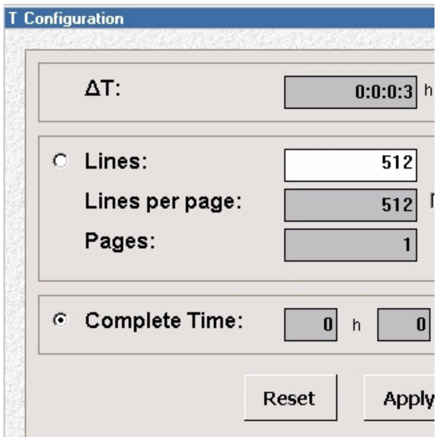
Time

The different scan modes for a time image series also have different parameters that have to be set.

Functioning of the «Time Configuration» dialog window

Each parameter can be calculated in reference to the other parameters. Click on the parameter to be calculated. The corresponding entry field is grayed out. Now enter the values for the other parameter. Click on «Apply» to calculate the parameter. Click on «Reset» to display the Most recently saved values.

When you choose the «xt» scan mode, the following parameters have to be set:



ΔT: 0:0:0:3 h
○ Lines: 512
Lines per page: 512
Pages: 1
● Complete Time: 0 h 0

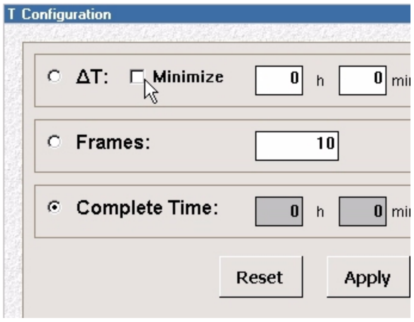
▶In the «Lines» field, enter the number of recordings of the line.

☑ Maximize (default)

▶Deselect the «Maximize» check box. In the «Lines per page» field, you can now enter the number of lines to be recorded before the data is transferred to the computer. As default setting a recording is carried out continuously until the memory of the scan head can store no additional data.

▶In the «Complete Time» field, enter the total time that you want the recording to take.

When you choose the «xyt» or «xzt» scan mode, the following parameters have to be set:



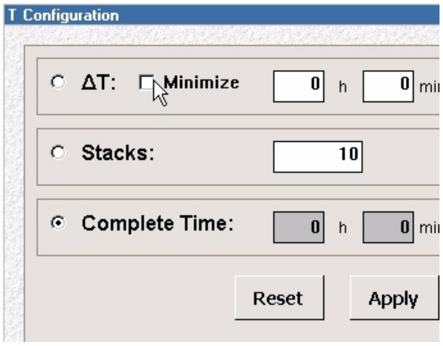
○ ΔT: ☐ Minimize 0 h 0 mi
○ Frames: 10
● Complete Time: 0 h 0 mi

▶In the «Frames» field, enter the number of recordings of the image.

▶In the «Δ T» field, you define the time interval between the recording of two single images. When you select the «Minimize» check box, the minimum interval is set, i.e. the time needed for recording one single image (which depends on scan parameters such as scan format or scan speed) plus a pause interval required for positioning the scan mirror.

▶In the «Complete Time» field, enter the total time that you want the recording to take.

When you choose the «xyzt» scan mode, the following parameters have to be set:



T Configuration

○ ΔT: ☐ Minimize 0 h 0 mi

○ Stacks: 10

● Complete Time: 0 h 0 mi

▶In the «Stacks» field, enter the number of recordings of the image stack.

▶In the «Δ T» field, you define the time interval between the recording of two image stacks.When you select the «Minimize» check box, the minimum interval is set, i.e. the time needed for recording one image stack (which depends on scan parameters such as scan format or scan speed) plus a pause interval required for positioning the scan mirror.

▶In the «Complete Time» field, enter the total time that you want the recording to take.

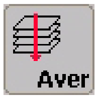
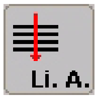
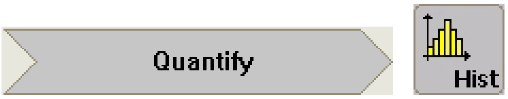
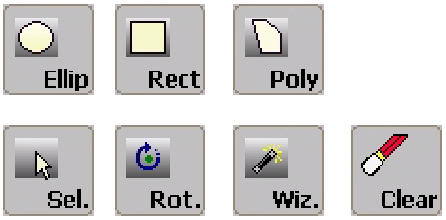
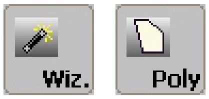
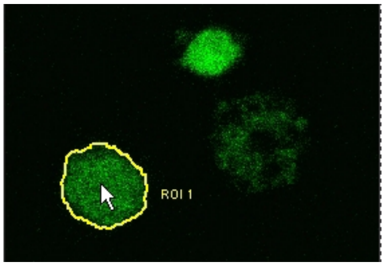
6 Start the continuous scanning mode with the «Continuous» button to optimize image quality during image recording.

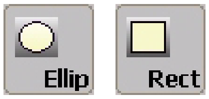
Continuous

▶Set the focal plane (z-position), the detector parameters and the detection pinhole. For more details, read steps 7 through 15 in the tutorial «Setting instrument parameters for the first image recording».

Setting instrument parameters for the first image recording

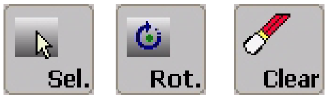
7 Click on the «Series» button to start recording the time image series.

	▶To improve the signal-to-noise ratio click on the «Average» button. Define how often the recording of an image is to be repeated. The average value is displayed in the result image.  ▶To improve the signal-to-noise ratio click on the «Line Average» button. Define how often the recording of a line is to be repeated. The average value is displayed in the result image. 
8	For a statistical evaluation of the time image series, click on the «**Quantify**» arrow symbol and then, for example, on the «Histogram» button.  When you click on this button, the «Histogram» dialog window opens and the ROI buttons needed for the next steps are activated automatically. 
9	To draw a region of interest (ROI) on the image click, for example, on the «Wizard» button and then on the «Polygon» button.   ▶In the image double-click on the structure which you want to evaluate. The structure is automatically defined as a region of interest. ▶Select other geometrical shapes with the «Ellipse» button or «Rectangle» button and mark further regions of interest in the image.



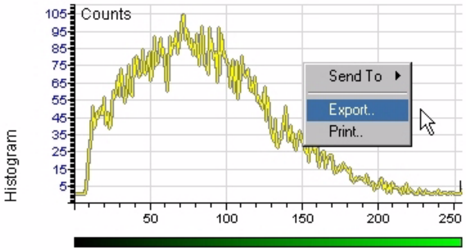
▶Deactivate the «Wizard» button, when you want to draw the regions of interest without the aid of the wizard function.

▶Using the «Select» button and the «Rotate» button, you can move, rotate and change the size of the regions of interest. With the «Clear» button you remove all regions of interest.



10

In the «Histogram» dialog window where an **evaluation graph** and various statistical data are displayed you have access to the following additional commands:



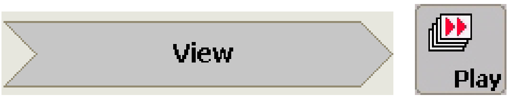
▶Hold the mouse pointer over the diagram and click the right mouse button to open the context menu.

▶Select «Export» to save the evaluation data in an ASCII text file.

▶Select «Send to» and «Document» to save a snapshot of the evaluation graph as a new file in the currently active experiment.

▶Select «Print» to print out the evaluation graph.

▶Click on the «**View**» arrow symbol and the «Play» button to run the time image series as a movie. Simultaneously, the evaluation graph and evaluation data of each single image are displayed.

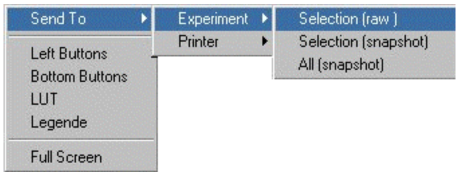


11

You can save an **image of a single detection channel** as a new data set in the currently active experiment.

▶Click on the image in the selected detection channel.

▶Hold the mouse pointer over the Viewer window and click the right mouse button to open the context menu.

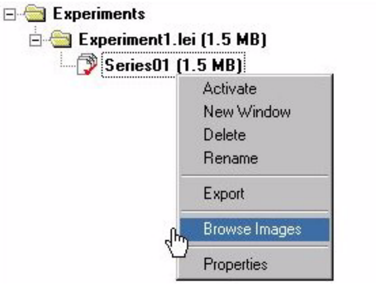

Send To
Left Buttons
Bottom Buttons
LUT
Legende
Full Screen
Experiment
Printer
Selection (raw)
Selection (snapshot)
All (snapshot)

▶Choose «Selection (raw)» to save the image data of the selected image in a new file.

▶Choose «Selection (snapshot)» to save a snapshot of the selected image in a new file.

▶Choose «All (snapshot)» to save a snapshot of all images in the Viewer window in a new file.

The new files are displayed in the «Experiment Overview» window where you have access to the following commands in the context menu:


Experiments
Experiment1.lei (1.5 MB)
Series01 (1.5 MB)
Activate
New Window
Delete
Rename
Export
Browse Images
Properties

▶Highlight a file of your choice and click the right mouse button to open the context menu.

▶Select «Activate» to display the image data of the file in the Viewer window.

▶Select «New Window» to open the currently displayed recording in a second Viewer window. This is useful, for example, when you want to visualize the same image data with another color look-up table.

▶Select «Delete» to remove the file from the list.

▶Select «Rename» to give the file a new name.

▶Select «Export» to save a recording in the *.avi file format.

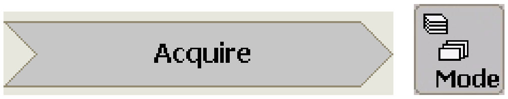
▶Select «Browse Images» to display all images of an experiment as thumbnails images on the user interface.

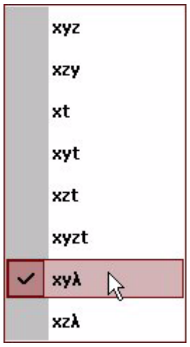
▶Select «Properties» to document important properties of the recording in a dialog window.

▶To move single files from one experiment to the other (drag and drop) click on the file, keep the left mouse button pressed and drag the file to the target folder.

Recording lambda series (spectral image series)

1 Click on the «**Acquire**» arrow symbol and on the «Mode» button to select the «xyλ» or «xzλ» scan mode.

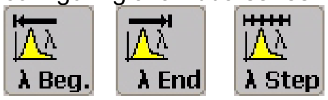




▶Select the «λxy» scan mode if you want to record horizontal images (orthogonal to the optical axis), each at a specific wavelength.

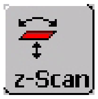
▶Select the «xzλ» scan mode if you want to record vertical images (parallel to the optical axis), each at a specific wavelength.

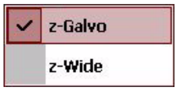
The selection of one of these scan modes automatically activates the buttons required for configuring a lambda series:



Which scan modes are available?

The number of available scan modes depends on the selected z-actuator type. Click on the «z-Scan» button to choose the z-actuator.



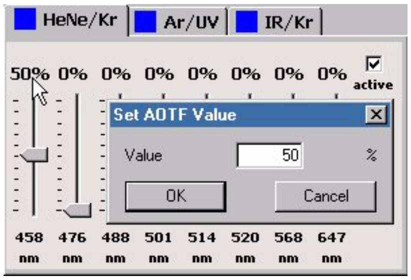


All scan modes are available when you use the galvanometer-driven z-specimen stage. For this stage select the «z-Galvo» option in the list.

There are only the scan modes «xyz, xt, xyt, xyzt and xyλ» available when you use the electromotor-driven z-specimen stage (or the motorized objective nosepiece with inverted microscopes). For this stage select the «z-Wide» option in the list.

Furthermore, the scan modes for recording a lambda series, namely «xyλ» and «λxz», are only available if only one detection channel is active.

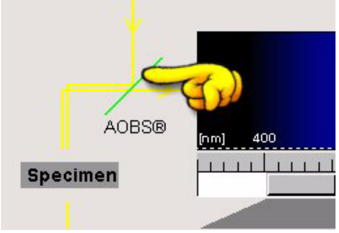
2 Click on the «Beam» button and open the «Beam Path Setting» dialog window to choose the excitation wavelength and set the laser intensity.


HeNe/Kr
Ar/UV
IR/Kr
50% 0% 0% 0% 0% 0% 0% 0%
☑ active
Set AOTF Value
OK
Cancel
458 nm 476 nm 488 nm 501 nm 514 nm 520 nm 568 nm 647 nm

Value 50 %

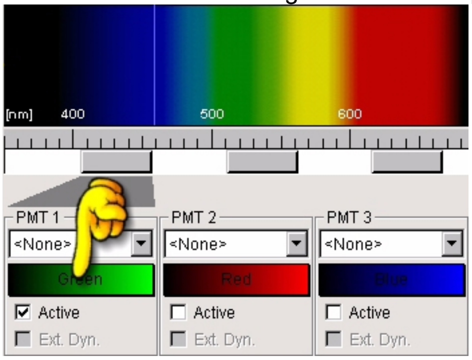
▶Move the slider or double-click on the number and enter a value for the laser intensity.

3 Select a suitable **excitation beam splitter**:

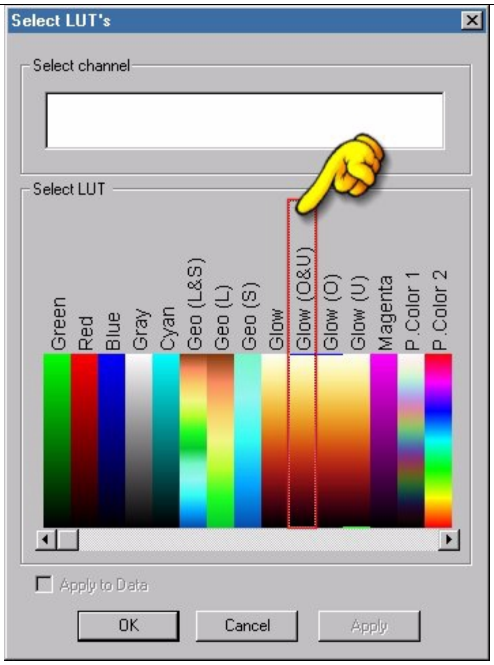

AOBS®
[nm] 400
Specimen

If your system is equipped with an acousto-optical beam splitter (AOBS), the beam path is optimized automatically for the wavelength you selected.
Otherwise double-click on the green line and select the neutral filter «RT 30/70», which is best suitable for lambda series.

4 Select only one **«PMT» detection channel** and assign a color look-up table to it.


[nm] 400 500 600
PMT 1
<None>
Green
☑ Active
☐ Ext. Dyn.
PMT 2
<None>
Red
☐ Active
☐ Ext. Dyn.
PMT 3
<None>
Blue
☐ Active
☐ Ext. Dyn.

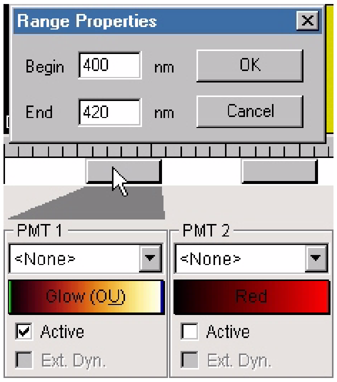
▶Click on the symbol for the color look-up table.


Select LUT's
Select channel
Select LUT
Green
Red
Blue
Gray
Cyan
Geo (L&S)
Geo (L)
Geo (S)
Glow
Glow (O&U)
Glow (O)
Glow (U)
Magenta
P.Color 1
P.Color 2
☐ Apply to Data
OK
Cancel
Apply

▶Click on the color look-up table you want to use and on «OK».
For the first image recording it is recommendable to use the «Glow(O&U)» color look-up table.

Setting instrument parameters for the first image recording

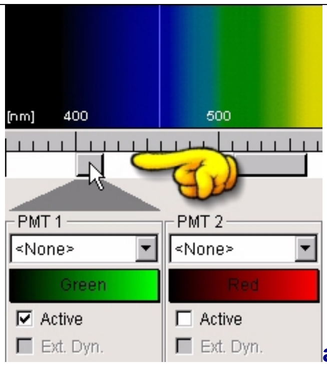
5 Define the **detection bandwidth** for the selected detection channel.


Range Properties
OK
Cancel
PMT 1
<None>
Glow (OU)
☑ Active
☐ Ext. Dyn.
PMT 2
<None>
Red
☐ Active
☐ Ext. Dyn.

Begin 400 nm
End 420 nm

▶Double-click on the slider of the selected detection channel.
▶In the «Range Properties» dialog window enter a wavelength for the beginning and the end of the detection band.

6 Define the **begin point and end point** of the lambda series.

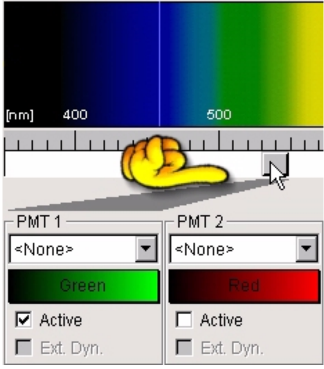


PMT 1
<None>
Green
☑ Active
☐ Ext. Dyn.

PMT 2
<None>
Red
☐ Active
☐ Ext. Dyn.

and λ Beg.

▶Move the slider to the desired position in the spectrum and click on the «Lambda Begin» button to save the position as begin point.



PMT 1
<None>
Green
☑ Active
☐ Ext. Dyn.

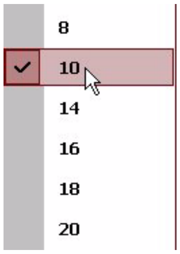
PMT 2
<None>
Red
☐ Active
☐ Ext. Dyn.

and λ End

▶Move the slider to the desired position in the spectrum and click on the «Lambda End» button to save the position as end point.

7 Click on the «Lambda Steps» button and define the number of recordings.

λ Step



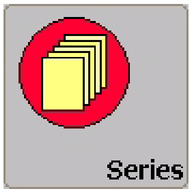
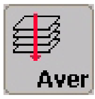
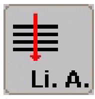
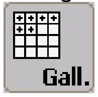
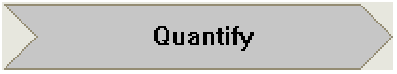
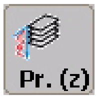
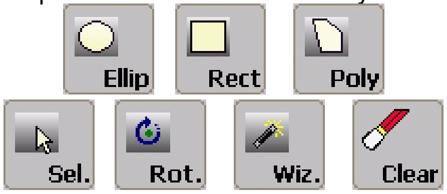
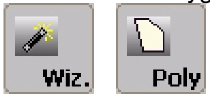
8
✓ 10
14
16
18
20

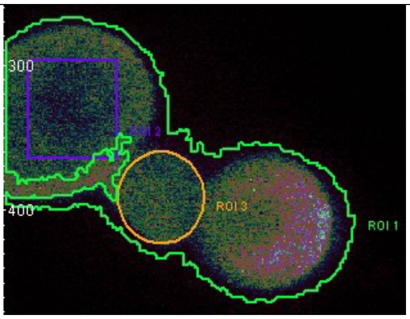
The selected number of recordings will be carried out at uniform step widths within the range delimited by the begin point and end point of the lambda series.

8 Start the continuous scanning mode with the «Continuous» button to optimize image quality during image recording.

Continuous

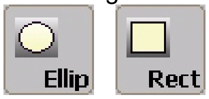
▶Set the focal plane (z-position), the detector parameters and the detection pinhole. For more details, read steps 7 through 15 in the tutorial «Setting instrument parameters for the first image recording».

	Setting instrument parameters for the first image recording
9	Click on the «Series» button to start recording the lambda series.  ▶To improve the signal-to-noise ratio click on the «Average» button. Define how often the recording of an image is to be repeated. The average value is displayed in the result image.  ▶To improve the signal-to-noise ratio click on the «Line Average» button. Define how often the recording of a line is to be repeated. The average value is displayed in the result image.  ▶Click on the «Gallery» button to display the single images in the Viewer window already during image recording.  **Note** It has a negative impact on the system performance when you record large image data sets and use the «Gallery» function.
10	For a statistical evaluation of the lambda series, click on the «**Quantify**» arrow symbol and then, for example, on the «Pr.(z)» button.   When you click on this button, the «Profile through Stack» dialog window opens and the ROI buttons needed for the next steps are activated automatically. 
11	To draw a region of interest (ROI) on the image click, for example, on the «Wizard» button and then on the «Polygon» button. 



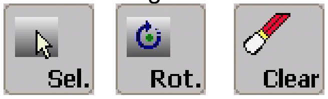
▶In the image double-click on the structure which you want to evaluate. The structure is automatically defined as a region of interest.

▶Select other geometrical shapes with the «Ellipse» button or «Rectangle» button and mark further regions of interest in the image.

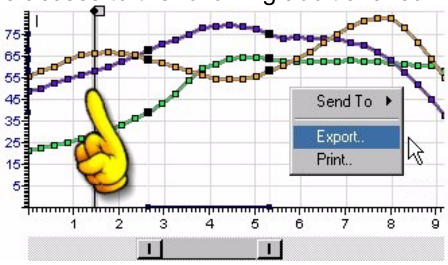


▶Deactivate the «Wizard» button, when you want to draw the regions of interest without the aid of the wizard function.

▶Using the «Select» button and the «Rotate» button, you can move, rotate and change the size of the regions of interest. With the «Clear» button you remove all regions of interest.



12 In the «Profile through Stack» dialog window where an **evaluation graph** and various statistical data are displayed, you have access to the following additional commands:



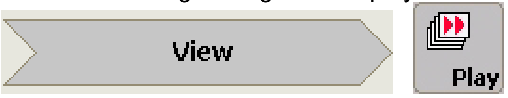
▶Hold the mouse pointer over the diagram and click the right mouse button to open the context menu.

▶Select «Export» to save the evaluation data in an ASCII text file.

▶Select «Send to» and «Document» to save a snapshot of the evaluation graph as a new file in the currently active experiment.

▶Select «Print» to print out the evaluation graph.

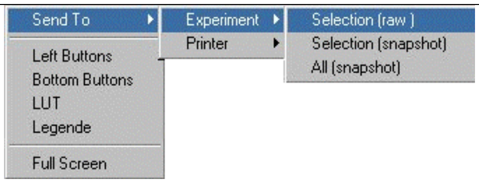
▶Click on the «**View**» arrow symbol and the «Play» button to run the lambda image series as a movie. Using a vertical subsidiary line, the evaluation graph and the corresponding evaluation data of each single image are displayed at the same time.



12 You can save an **image of a single detection channel** as a new data set in the currently active experiment.

▶Click on the image in the selected detection channel.

▶Hold the mouse pointer over the Viewer window and click the right mouse button to open the context menu.

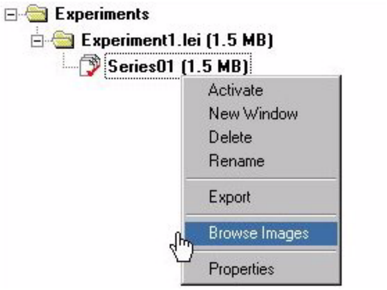

Send To
Experiment
Selection (raw)
Printer
Selection (snapshot)
All (snapshot)
Left Buttons
Bottom Buttons
LUT
Legende
Full Screen

▶Choose «Selection (raw)» to save the image data of the selected image in a new file.

▶Choose «Selection (snapshot)» to save a snapshot of the selected image in a new file.

▶Choose «All (snapshot)» to save a snapshot of all images in the Viewer window in a new file.

The new files are displayed in the «Experiment Overview» window where you have access to the following commands in the context menu:


Experiments
Experiment1.lei (1.5 MB)
Series01 (1.5 MB)
Activate
New Window
Delete
Rename
Export
Browse Images
Properties

▶Highlight a file of your choice and click the right mouse button to open the context menu.

▶Select «Activate» to display the image data of the file in the Viewer window.

▶Select «New Window» to open the currently displayed recording in a second Viewer window. This is useful, for example, when you want to visualize the same image data with another color look-up table.

▶Select «Delete» to remove the file from the list.

▶Select «Rename» to give the file a new name.

▶Select «Export» to save a recording in the *.avi file format.

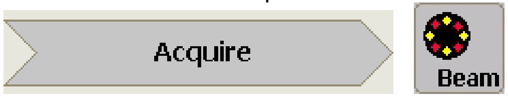
▶Select «Browse Images» to display all images of an experiment as thumbnails images on the user interface.

▶Select «Properties» to document important properties of the recording in a dialog window.

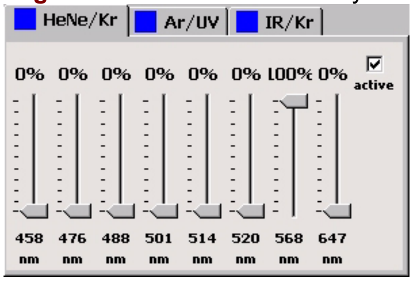
▶To move single files from one experiment to the other (drag and drop) click on the file, keep the left mouse button pressed and drag the file to the target folder.

Recording sequential image series

1 First, you have to set all parameters for the first recording method and save the settings as an instrument parameter setting. For this purpose, click on the «**Acquire**» arrow symbol and on the «Beam» button to open the «Beam Path Setting» dialog window.

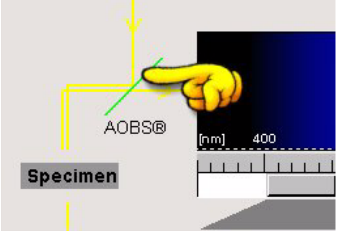


2 Choose the **excitation wavelength** and set the laser intensity.



▶Move the slider or double-click on the number and enter a value for the laser intensity.

3 Select a suitable **excitation beam splitter**:



If your system is equipped with an acousto-optical beam splitter (AOBS), the beam path is optimized automatically for the wavelength you selected.
Otherwise double-click on the green line and select a beam splitter:

▶Select the dichroic RSP 500 for image recordings of FITC and GFP with an excitation wavelength of 488 nm and a detection bandwidth of 500 to 600 nm.

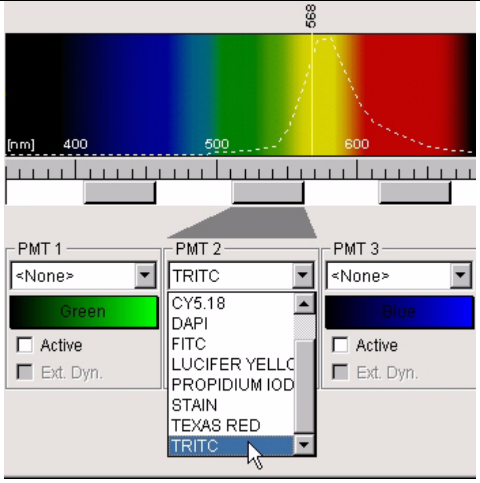
▶Select the dichroic RSP 465 for image recordings of CFP with an excitation wavelength of 458 nm and a detection bandwidth of 465 to 525 nm.

▶Select the double dichroic DD 488 / 543 for image recordings of FITC and TRITC or GFP and DsRED with an excitation wavelength of 488 and 543 nm and a detection bandwidth of 500 to 530 nm and 555 to 700 nm, respectively.

▶Select the triple dichroic TD 488 / 543 / 633 for image recordings of FITC, TRITC and CY5 with an excitation wavelength of 488, 543 and 633 nm and a detection bandwidth of 500 to 535 nm, 555 to 620 nm and 650 to 750 nm, respectively.

▶Select the neutral filter RT 30/70 for recordings of reflected light.

4 Select a **«PMT» detection channel** and set fluorescence dye and detection bandwidth.

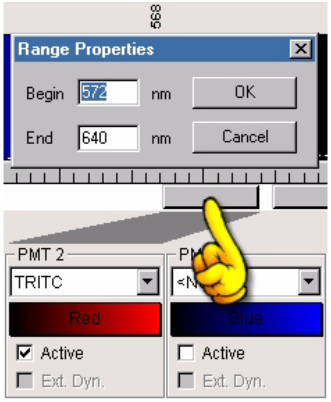

568
[nm]
400
500
600
PMT 1
<None>
Green
Active
Ext. Dyn.
PMT 2
TRITC
CY5.18
DAPI
FITC
LUCIFER YELLO
PROPIDIUM IOD
STAIN
TEXAS RED
TRITC
PMT 3
<None>
Blue
Active
Ext. Dyn.

▶Click on the list box of a detection channel, for example «PMT2», and select the fluorescence dye to be used, for example «TRITC». The emission curve of the fluorescence dye is displayed in the spectrum.

▶Position the slider below the emission curve.

Note on positioning the detection band

When recording fluorescence light, take care that you do not position the detection band directly below the laser line, since thus reflected light will also be recorded. The distance between laser line and detection band should be at least 5 nm in the blue spectral range (excitation line 488 nm), 10 nm in the green spectral range (excitation line 543 nm) and 15 nm in the red spectral range (excitation line 633 nm).

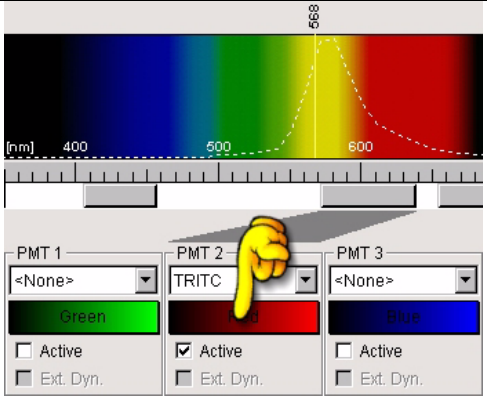
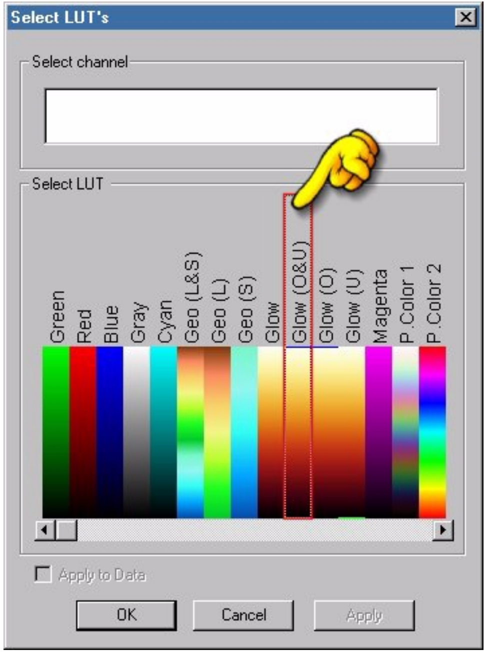
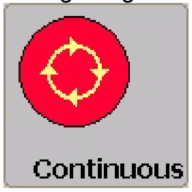

568
Range Properties
OK
Cancel
PMT 2
TRITC
Red
☑ Active
☐ Ext. Dyn.
PM
<N
Blue
☐ Active
☐ Ext. Dyn.

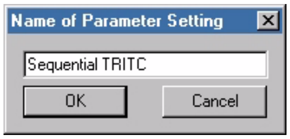
Begin 572 nm

End 640 nm

▶Double-click on the slider of the selected detection channel.

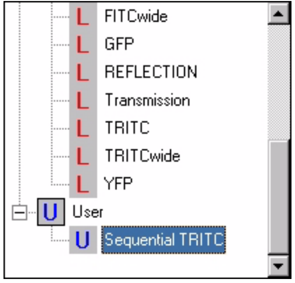
▶In the «Range Properties» dialog window enter a wavelength for the beginning and the end of the detection band.

	 568 [nm] 400 500 600 PMT 1 <None> Green ☐ Active ☐ Ext. Dyn. PMT 2 TRITC Red ☑ Active ☐ Ext. Dyn. PMT 3 <None> Blue ☐ Active ☐ Ext. Dyn. ▶Click on the symbol for the color look-up table.  Select LUT's Select channel Select LUT Green Red Blue Gray Cyan Geo (L&S) Geo (L) Geo (S) Glow Glow (O&U) Glow (O) Glow (U) Magenta P. Color 1 P. Color 2 ☐ Apply to Data OK Cancel Apply ▶Click on the color look-up table you want to use and on «OK». For the first image recording it is recommendable to use the «Glow(O&U)» color look-up table. *Setting instrument parameters for the first image recording*
5	Start the continuous scanning mode with the «Continuous» button to optimize image quality during image recording.  Continuous ▶Set the focal plane (z-position), the detector parameters and the detection pinhole. For more details, read steps 7 through 15 in the tutorial «Setting instrument parameters for the first image recording». *Setting instrument parameters for the first image recording*
6	In the «Beam Path Setting» dialog window, click on the «Save» button to save the settings of the first recording method as an instrument parameter setting.


Name of Parameter Setting
OK
Cancel

Sequential TRITC

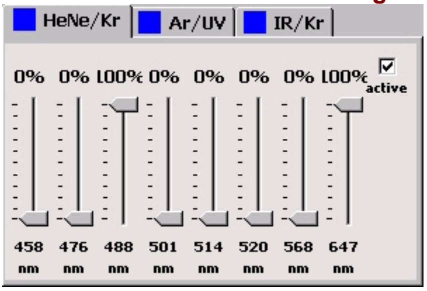
▶Give the instrument parameter setting a name and click on OK.


L FITCwide
L GFP
L REFLECTION
L Transmission
L TRITC
L TRITCwide
L YFP
U User
U Sequential TRITC

The new instrument parameter setting appears in the list box below «**U**».

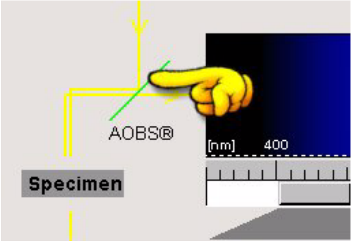
How to create user-defined instrument parameter settings (IPS)

7 Next, all instrument parameters for the second recording method are set and saved as an instrument parameter setting. Choose the **excitation wavelength** and set the laser intensity.


HeNe/Kr
Ar/UV
IR/Kr
0% 0% 100% 0% 0% 0% 0% 100%
☑ active
458 nm
476 nm
488 nm
501 nm
514 nm
520 nm
568 nm
647 nm

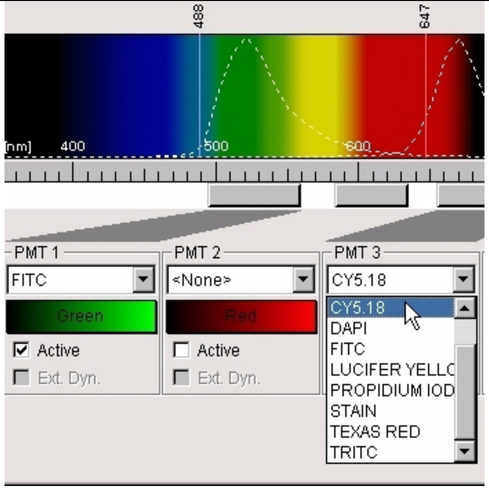
▶Move the slider or double-click on the number and enter a value for the laser intensity.

8 Select a suitable **excitation beam splitter**:


AOBS®
[nm] 400
Specimen

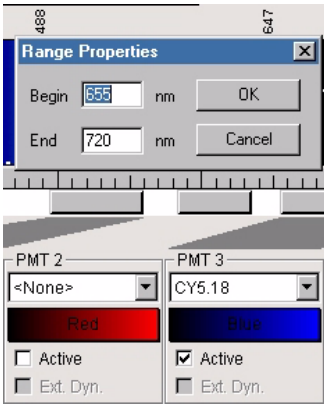
▶For a detailed description of the available beam splitters see step 3 in this tutorial.

9 Select a **«PMT» detection channel** and set fluorescence dye and detection bandwidth.


488
647
[nm] 400
500
600
PMT 1
FITC
Green
☑ Active
☐ Ext. Dyn.
PMT 2
<None>
Red
☐ Active
☐ Ext. Dyn.
PMT 3
CY5.18
CY5.18
DAPI
FITC
LUCIFER YELLO
PROPIDIUM IOD
STAIN
TEXAS RED
TRITC

▶Click on the list boxes of the detection channels, for example «PMT1» and «PMT3», and select the fluorescence dye to be used in each channel, for example «FITC» and «CY5». The emission curves of the fluorescence dyes are displayed in the spectrum.

▶Position the sliders below the respective emission curves.

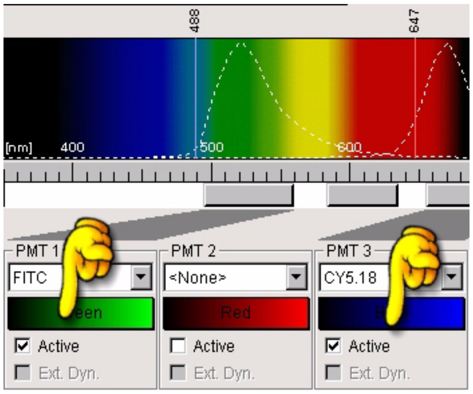

488
647
Range Properties
OK
Cancel
PMT 2
<None>
Red
☐ Active
☐ Ext. Dyn.
PMT 3
CY5.18
Blue
☑ Active
☐ Ext. Dyn.

Begin 655 nm

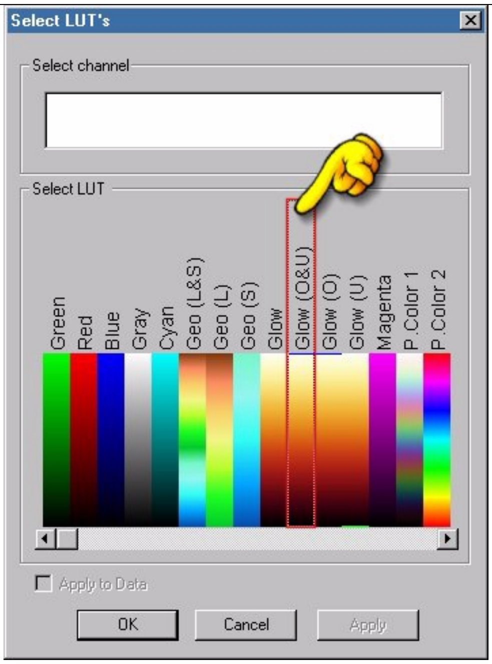
End 720 nm

▶Double-click on the slider of the selected detection channel.

▶In the «Range Properties» dialog window, enter a value for the beginning and the end of the detection band.

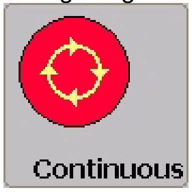

488
647
[nm] 400
500
600
PMT 1
FITC
een
☑ Active
☐ Ext. Dyn.
PMT 2
<None>
Red
☐ Active
☐ Ext. Dyn.
PMT 3
CY5.18
☑ Active
☐ Ext. Dyn.

▶Click on the symbol for the color look-up table.


Select LUT's
Select channel
Select LUT
Green
Red
Blue
Gray
Cyan
Geo (L&S)
Geo (L)
Geo (S)
Glow
Glow (O&U)
Glow (O)
Glow (U)
Magenta
P.Color 1
P.Color 2
☐ Apply to Data
OK
Cancel
Apply

▶Click on the color look-up table you want to use and on «OK».
For the first image recording it is recommendable to use the «Glow(O&U)» color look-up table.

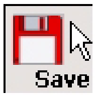
10 Start the continuous scanning mode with the «Continuous» button to optimize image quality during image recording.

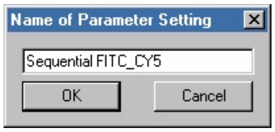

Continuous

▶Set the focal plane (z-position), the detector parameters and the detection pinhole. For more details, read steps 7 through 15 in the tutorial «Setting instrument parameters for the first image recording».

Setting instrument parameters for the first image recording

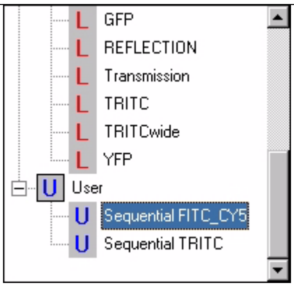
11 In the «Beam Path Setting» dialog window, click on the «Save» button to save the settings for the second recording method as an instrument parameter setting.




Name of Parameter Setting
OK
Cancel

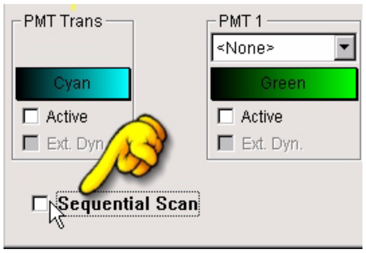
Sequential FITC_CY5

▶Give the instrument parameter setting a name and click on OK.


L GFP
L REFLECTION
L Transmission
L TRITC
L TRITCwide
L YFP
U User
U Sequential FITC_CY5
U Sequential TRITC

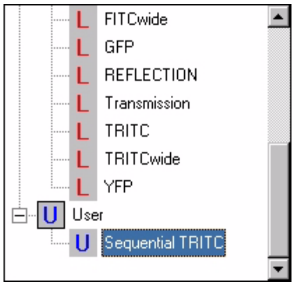
The new instrument parameter setting appears in the list box below «**U**».

12 In the «Beam Path Setting» dialog window, click on the check box labeled «**Sequential Scan**» in the bottom left corner.

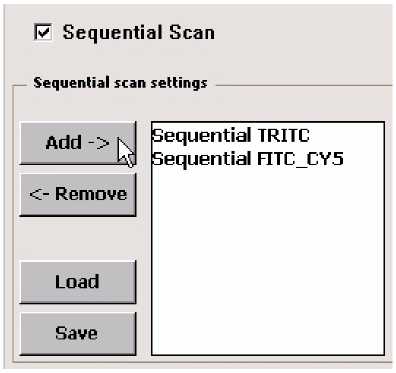

PMT Trans
Cyan
☐ Active
☐ Ext. Dyn
PMT 1
<None>
Green
☐ Active
☐ Ext. Dyn.
☐ Sequential Scan

The dialog elements needed for configuring a sequential image recording appear.

13 In the «Beam Path Setting» dialog window, copy the instrument parameter settings into the «**Sequential scan settings**» list box.


L FITCwide
L GFP
L REFLECTION
L Transmission
L TRITC
L TRITCwide
L YFP
U User
U Sequential TRITC

▶Highlight the instrument parameter setting in the list box in the upper right corner of the dialog window.


☑ Sequential Scan
Sequential scan settings
Add ->
<- Remove
Load
Save
Sequential TRITC
Sequential FITC_CY5

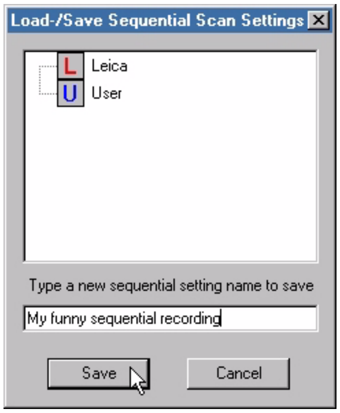
▶Click on the «Add» button in the «Sequential scan settings» field in the lower left corner of the dialog window.

or

▶Click on the instrument parameter setting, keep the left mouse button pressed and move the IPS to the «Sequential scan settings» list box (drag and drop).

▶Click on the «Remove» button to remove the instrument parameter setting from the list.

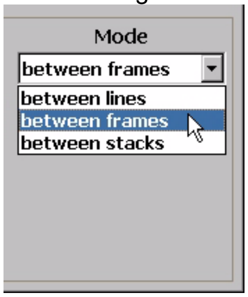
▶Click on the «Save» button to save the instrument parameter settings together as a sequential recording method.



Type a new sequential setting name to save: My funny sequential recording

▶Use the «Load» button to load this sequential recording method again.

14 In the **«Mode» list box**, select one of the three sequential scan modes. This selection defines when the sequential recording methods are changed.



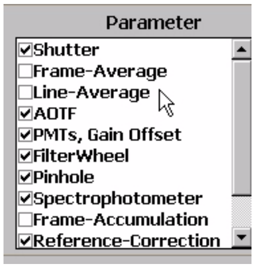
Mode: between frames

▶Select «between lines» if you want to change the sequential recording method after a line has been recorded.

▶Select «between frames» if you want to change the sequential recording method after an image has been recorded.

▶Select «between stacks» if you want to change the sequential recording method after an image stack has been recorded.

15 In the **«Parameter» list box**, check the parameters which will be used for the recording of all sequential recording methods.

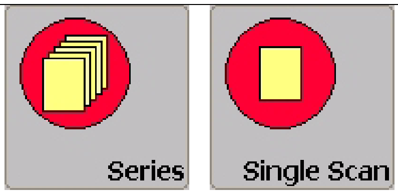


Parameter
- ☑ Shutter
- ☐ Frame-Average
- ☐ Line-Average
- ☑ AOTF
- ☑ PMTs, Gain Offset
- ☑ FilterWheel
- ☑ Pinhole
- ☑ Spectrophotometer
- ☐ Frame-Accumulation
- ☑ Reference-Correction

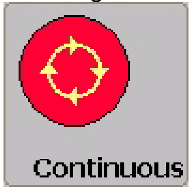
▶Click on the check box of the desired parameters.

16 Next, adjust all other instrument parameters which differ depending on the type of recording: spatial image series, time image series or spectral image series.

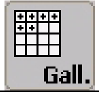
17 Click on the «Series» button or on the «Single Scan» button to start the sequential image recording.


Series
Single Scan

- If you have selected the sequential mode «between lines», you can also use the «Continuous» button to start the sequential recording. The advantage of the continuous scanning mode is that you can modify many parameters during image recording.

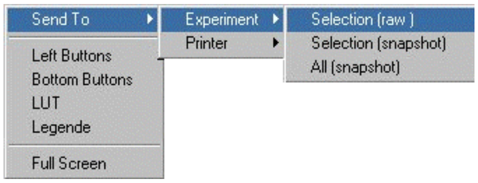

Continuous

- Click on the «Gallery» button to display the single images in the Viewer window already during image recording.



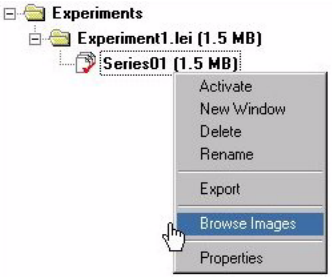
18 You can save an **image of a single detection channel** as a new data set in the currently active experiment.

- Click on the image in the selected detection channel.
- Hold the mouse pointer over the Viewer window and click the right mouse button to open the context menu.


Send To
Left Buttons
Bottom Buttons
LUT
Legende
Full Screen
Experiment
Printer
Selection (raw)
Selection (snapshot)
All (snapshot)

- Choose «Selection (raw)» to save the image data of the selected image in a new file.
- Choose «Selection (snapshot)» to save a snapshot of the selected image in a new file.
- Choose «All (snapshot)» to save a snapshot of all images in the Viewer window in a new file.

The new files are displayed in the «Experiment Overview» window where you have access to the following commands in the context menu:


Experiments
Experiment1.lei (1.5 MB)
Series01 (1.5 MB)
Activate
New Window
Delete
Rename
Export
Browse Images
Properties

- Highlight a file of your choice and click the right mouse button to open the context menu.
- Select «Activate» to display the image data of the file in the Viewer window.
- Select «New Window» to open the currently displayed recording in a second Viewer window. This is useful, for example, when you want to visualize the same image data with another color look-up table.
- Select «Delete» to remove the file from the list.
- Select «Rename» to give the file a new name.
- Select «Export» to save a recording in the *.avi file format.

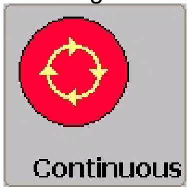
▶Select «Browse Images» to display all images of an experiment as thumbnails images on the user interface.

▶Select «Properties» to document important properties of the recording in a dialog window.

▶To move single files from one experiment to the other (drag and drop) click on the file, keep the left mouse button pressed and drag the file to the target folder.

Recording images of regions of interest (ROI scanning)

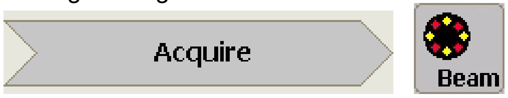
1 The first step is to optimize the instrument parameters for the recording of the overall image. To do this during image recording, click on the «Continuous» button to start the continuous scanning mode.



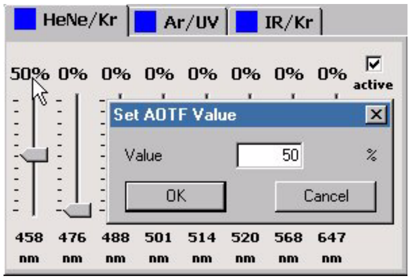
▶Set the focal plane (z-position), the detector parameters and the detection pinhole. For more details, read steps 7 through 15 in the tutorial «Setting instrument parameters for the first image recording».

Setting instrument parameters for the first image recording

2 Click on the «**Acquire**» arrow symbol and then on the «Beam» button to open the «Beam Path Setting» dialog window.



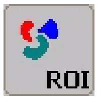
▶Select the excitation wavelength and set the laser intensity for the image background (overall image).



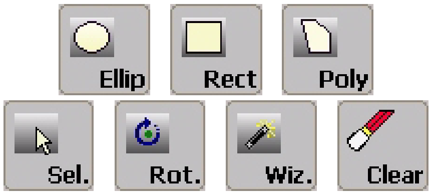
Value 50 %

▶Move the slider or double-click on the number and enter a value for the laser intensity.

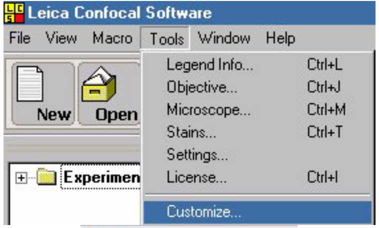
3 Click on the «ROI» button to save the laser setting for the image background.

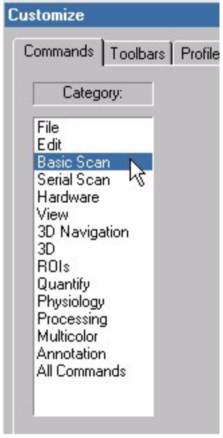


When you click on this button, the ROI buttons needed for the next steps are activated automatically.



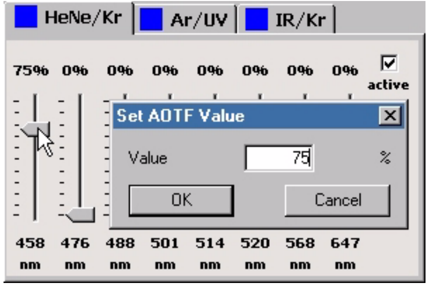
▶If you do not find the «ROI» button in the «Acquire» button bar of the user interface, select Tools → Customize → Commands → Category Basic Scan.


Leica Confocal Software
File
View
Macro
Tools
Window
Help
New
Open
Experimen
Legend Info... Ctrl+L
Objective... Ctrl+J
Microscope... Ctrl+M
Stains... Ctrl+T
Settings...
License... Ctrl+I
Customize...


Customize
Commands
Toolbars
Profile
Category:
File
Edit
Basic Scan
Serial Scan
Hardware
View
3D Navigation
3D
ROIs
Quantify
Physiology
Processing
Multicolor
Annotation
All Commands

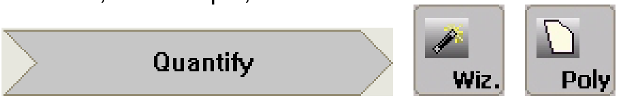
▶Click on the «ROI» button, keep the left mouse button pressed and drag it onto the user interface.

4 Choose the **excitation wavelength** and set the laser intensity for the region of interest (ROI) which will be defined in the next step.


HeNe/Kr
Ar/UV
IR/Kr
75% 0% 0% 0% 0% 0% 0% 0%
☑ active
Set AOTF Value
Value 75 %
OK
Cancel
458 nm 476 nm 488 nm 501 nm 514 nm 520 nm 568 nm 647 nm

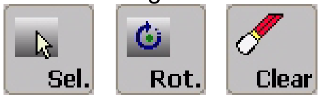
▶Move the slider or double-click on the number and enter a value for the laser intensity.

5 To draw the first region of interest on the overall image, click on the «**Quantify**» arrow symbol and then, for example, on the «Wizard» button and the «Polygon» button.

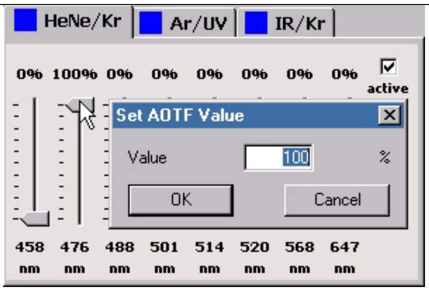
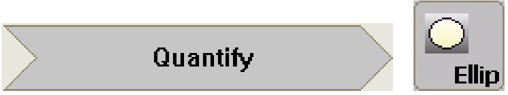
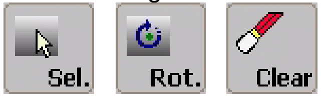
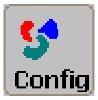
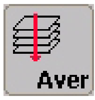
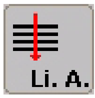
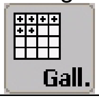

Quantify
Wiz.
Poly

▶In the image double-click on a structure of your choice to be defined as region of interest. The structure is automatically defined as a region of interest.

▶Using the «Select» button and the «Rotate» button, you can move, rotate and change the size of the regions of interest. With the «Clear» button you remove all regions of interest.


Sel.
Rot.
Clear

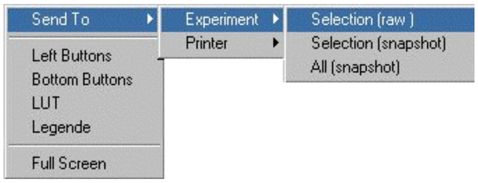
6 Choose the **excitation wavelength** and set the laser intensity for a second region of interest.

	 HeNe/Kr Ar/UV IR/Kr 0% 100% 0% 0% 0% 0% 0% 0% ☑ active Set AOTF Value Value 100 % OK Cancel 458 nm 476 nm 488 nm 501 nm 514 nm 520 nm 568 nm 647 nm ▶Move the slider or double-click on the number and enter a value for the laser intensity.
7	To draw the second region of interest onto the image, click on the «**Quantify**» arrow symbol and then, for example, on the «Ellipse» button.  Quantify Ellip ▶Deactivate the «Wizard» button, when you want to draw the regions of interest without the aid of the wizard function. ▶Using the «Select» button and the «Rotate» button, you can move, rotate and change the size of the regions of interest. With the «Clear» button you remove all regions of interest.  Sel. Rot. Clear
8	Repeat the procedure described in steps 4 through 7 of this tutorial for any additional region of interest to be defined in the image.
9	Then click on the «Configuration» button to get an overview of the defined regions of interest and the laser lines assigned to them.  ▶Here you can modify the assignment of laser line and region of interest, if required.
10	Click on the «Series» button to start the image recording. ▶To improve the signal-to-noise ratio click on the «Average» button. Define how often the recording of an image is to be repeated. The average value is displayed in the result image.  ▶To improve the signal-to-noise ratio click on the «Line Average» button. Define how often the recording of a line is to be repeated. The average value is displayed in the result image.  ▶Click on the «Gallery» button to display the single images in the Viewer window already during image recording. 

11 You can save an **image of a single detection channel** as a new data set in the currently active experiment.

▶Click on the image in the selected detection channel.

▶Hold the mouse pointer over the Viewer window and click the right mouse button to open the context menu.

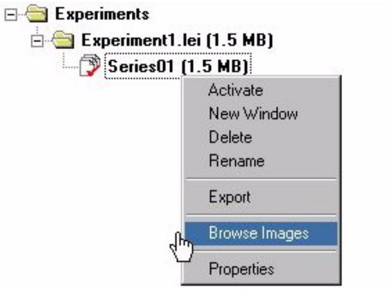

Send To
Experiment
Selection (raw)
Printer
Selection (snapshot)
All (snapshot)
Left Buttons
Bottom Buttons
LUT
Legende
Full Screen

▶Choose «Selection (raw)» to save the image data of the selected image in a new file.

▶Choose «Selection (snapshot)» to save a snapshot of the selected image in a new file.

▶Choose «All (snapshot)» to save a snapshot of all images in the Viewer window in a new file.

The new files are displayed in the «Experiment Overview» window where you have access to the following commands in the context menu:


Experiments
Experiment1.lei (1.5 MB)
Series01 (1.5 MB)
Activate
New Window
Delete
Rename
Export
Browse Images
Properties

▶Highlight a file of your choice and click the right mouse button to open the context menu.

▶Select «Activate» to display the image data of the file in the Viewer window.

▶Select «New Window» to open the currently displayed recording in a second Viewer window. This is useful, for example, when you want to visualize the same image data with another color look-up table.

▶Select «Delete» to remove the file from the list.

▶Select «Rename» to give the file a new name.

▶Select «Export» to save a recording in the *.avi file format.

▶Select «Browse Images» to display all images of an experiment as thumbnails images on the user interface.

▶Select «Properties» to document important properties of the recording in a dialog window.

▶To move single files from one experiment to the other (drag and drop) click on the file, keep the left mouse button pressed and drag the file to the target folder.

General

Introduction to the Leica Confocal Software Help

Three different help levels are available:

Quick Help
When you let the mouse pointer hover over a Leica Confocal Software button, a brief explanation of the function of this button is displayed. This so-called Help Banner automatically disappears when the mouse pointer is moved.

Context-sensitive Help
If you want to use the context-sensitive help function, click on the Help button:

The entire user interface is then frozen and a question mark appears beside the mouse pointer. Then, instead of triggering the button function, clicking a button opens an explanation of the button's function. If the Help button is not present on the user interface:

▶	Select the Customize option from the Tools menu. Here you will find all buttons arranged by categories.
▶	The Help button can be found in the File category.
▶	Click on it using the left mouse button and drag it to the desired window.

Contents of the Online Help
Select the Contents option from the Help menu to view the online help table of contents. which allows you to select any function in order to view information on it.

Keyword Search (Index)
Select the Index option from the Help menu to view an index of key words. Select a key word. View the corresponding content pages by double-clicking the key word or selecting it and then clicking the Display button.

Full-text Boolean Search
Select the Search option from the Help menu to launch the full-text search engine. Enter your search word in the input field. Click the triangle to the right of the input field to view the available logical operators. Select the desired operator. Enter the second search word you would like to associate with the first search word behind the operator:

Examples	**Result**
Pinhole AND Sections	This phrase finds help topics containing both the word "pinhole" and the word "sections".
Pinhole OR Sections	This phrase finds help topics containing either the word "pinhole" or the word "sections" or both.
Pinhole NEAR Sections	This phrase finds help topics containing the word "pinhole" and the word "sections" if they are located within a specific search radius. This method also looks for words that are similar in spelling to the words specified in the phrase.
Pinhole NOT Sections	This phrase finds help topics containing the word "pinhole" and not containing the word "sections".

Favorites
If you select Favorites in the dialog window of the online help tab, you can include the current help topic in a list, making them easily available for future use.

Opening the Context-Sensitive Help

Function
Click Help to open the context-sensitive online help function, which provides you with short explanations for the various buttons and functions of the Leica Confocal Software.

▶	Click the Help button.
▶	A question mark appears next to the mouse pointer. This temporarily disables the functions of all buttons.
▶	Use the mouse pointer to click the button that you want an explanation of.
▶	Online help opens directly to the description for the corresponding button.

Online help also provides you with an index of key words and a search function so that you can search for specific topics and buttons. Furthermore, you can print the individual descriptions.

You can also open the online help by selecting Contents, Search or Index option under the Help menu.

Data recording functions

Setting the Beam Path

Beam

Function
Open the Beam Path Setting dialog window using the Beam button, and set up the beam path and the detectors with the corresponding color look-up tables used to record the image.

Selecting the excitation wavelength
Above the spectrum in the dialog window you will find boxes to set the excitation wavelength using an AOTF:

▶	Click the check box next to the laser name to activate the laser.
▶	Set the output for the laser line by moving the slider on the corresponding scale to the desired value, or
▶	Double click the percentage value for the laser output. This opens a second dialog window where you can enter an exact value.
▶	The active laser line is shown as a line in the spectrum.

Loading and saving parameter settings
Also above the spectrum is a list box for loading and saving parameter settings. The manufacturer has predefined parameter settings for the most important fluorescent dyes. They are labeled with the letter **L** (for Leica) and can be loaded only, not modified.

You can also save the settings made for a specific image recording as user-defined parameter configuration so that you can load them any time with a single click:

▶	Click the Save button.
▶	This opens a dialog window for entering a name for the parameter configuration.
▶	The parameter configuration is stored in the list box under User and is labeled with the letter **U** (for User).

Furthermore, you can individually select the parameters that you want to save using the Save command:

▶	Select the Settings option from the Tools menu.
▶	In the Settings dialog window, click the Instrument Parameters tab.
▶	Select individual parameters by clicking the corresponding check boxes. The Select all and Deselect all buttons allow you to select all or no parameters quickly.

Click one of the user-defined or preset parameter configurations and then press the right mouse button to open a context menu which contains the following commands:

Command	**Function**
Set as default setting	The standard setting for the parameters is loaded when you start the software.
Remove default setting	This removes the default setting property from the selected configuration.
Load	Loads the parameter configuration.
Rename (only for **U**)	Use this to change the name of the parameter configuration.

Delete (only for **U**)	Deletes the parameter configuration.

Selecting the excitation beam splitter
The excitation beam splitter directs the excitation light of the laser to the specimen and separates the fluorescence light and reflected excitation light returned from the specimen.

If the confocal system is equipped with an AOBS (Acousto-Optical Beam Splitter), the AOBS is automatically adjusted after selecting one or several laser lines so that fluorescence light is transmitted at its maximum and the reflection light is suppressed at its maximum.

& see Adjusting the Acousto-Optical Beam Splitter (AOBS) (page 113)

If the confocal system is equipped with conventional excitation beam splitters, a suitable filter must be selected for the corresponding application. The symbol (green, tilted line) for the excitation beam splitter is located to the left of the spectrum. Click on the symbol and select the desired beam splitter:

▶	Neutral filters, such as the RT 30/70 filter, are used in reflection applications to lead the excitation light to the sample and the reflected light to the detection pin hole.
▶	Dichroic filters, such as the reflection short pass filter RSP 510, are used in fluorescence applications to split and detect fluorescent light of a particular wavelength range from the excitation light.
▶	Double dichroic filters are used for image recording, where the preparation is marked with two fluorescent dyes and two excitation wavelengths. The DD 488/ 568 double dichroic, for instance, is used to split excitation light with a 488 nm and 568 nm wavelength from the detection light. Depending on the system configuration, you can also select DD 488/ 543 and DD 458/ 514 type double dichroites.
▶	Triple dichroic filters are used for image recording, if the preparation is marked with three fluorescent dyes and three excitation wavelengths. The TD 488/568/633 triple dichroic, for instance, is used to split excitation light with a 488 nm, 568 nm, and 633 nm wavelength from the detection light. Depending on the system configuration, you can also select TD 488/ 568/ 647 and TD 488/ 543/ 633 type triple dichroites.

Setting detectors and color look-up tables (LUT)

Below the spectrum in the dialog window the boxes for the four detectors, PMT 1 to 4, as well as for the PMT Trans transmitted light detector are shown. Adjust the settings as follows:

▶	Activate each of the desired detectors by clicking on its corresponding Active check box below the color look-up table icon. A cast shadow now links the activated detector to the corresponding slide bar on the scale of the spectrum.
▶	Specify the wavelength range for the detection by dragging the two ends of the slider to the desired positions on the scale, or
▶	Double-click the slider bar to open an additional dialog window in which you can enter a precise value for the begin point and the end point of the wavelength range.
▶	For fluorescence applications, list fields with common fluorescence dyes have been set up for each detector. Select the desired fluorescence dye to display its emission curve in the spectrum.
▶	Select a color look-up table by clicking on the corresponding icon.

& see Selecting Color Look-Up Tables (page 159)

Adjusting the Acousto-Optical Beam Splitter (AOBS) (optional)

This page contains the following topics

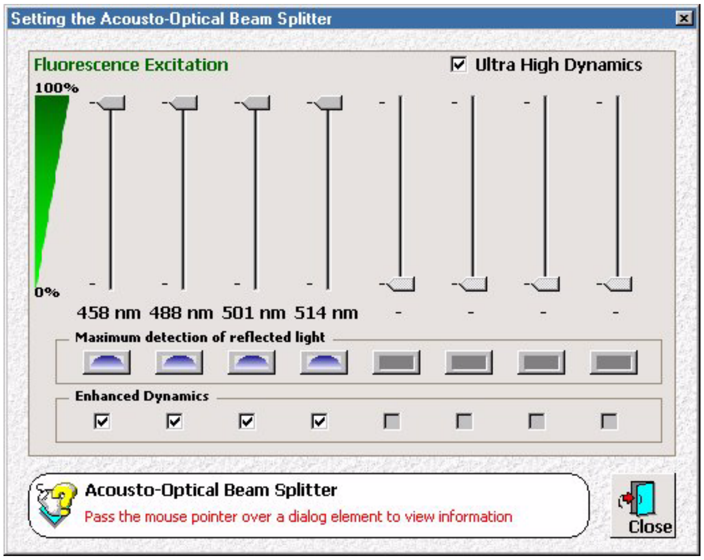
- Function of the acousto-optical beam splitter
- Use of the AOBS with fluorescence recordings
- Use of the AOBS for mixed reflection / fluorescence recordings

Function

The Acousto-Optical Beam Splitter (AOBS) directs the excitation light of the laser onto the specimen and separates the fluorescence light returned from the specimen and the reflected excitation light. In Leica confocal microscopes, the AOBS replaces and optimizes conventional excitation filters, such as double dichroite or triple dichroite.

The AOBS is a transparent optical crystal to which an ultrasonic field is applied. Light, which penetrates the optical crystal, is deflected depending on its wavelength and the wavelength of the ultrasonic field. By modulating the ultrasonic amplitude, the AOBS can be used not only to select a laser line, but also to control the amount of light as well as the beam splitter ratio of excitation and detection light for the selected laser line. The AOBS allows the simultaneous use of up to 8 excitation wavelengths.

Use of the AOBS with fluorescence recordings

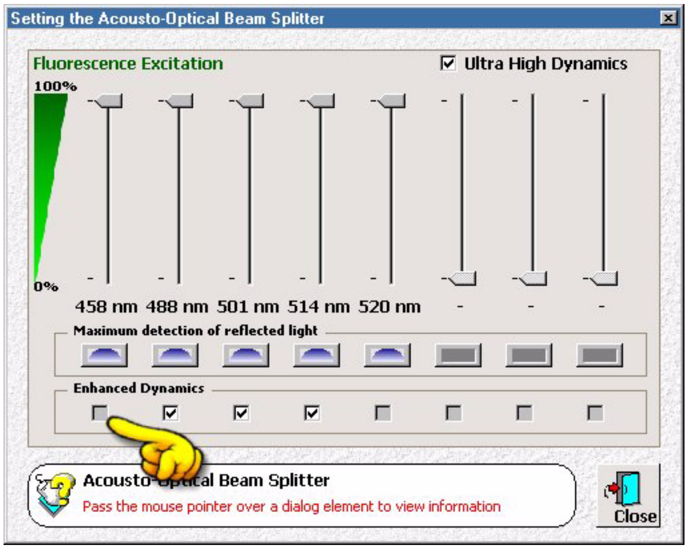

Setting the Acousto-Optical Beam Splitter
Fluorescence Excitation
100%
0%
458 nm
488 nm
501 nm
514 nm
Maximum detection of reflected light
Enhanced Dynamics
Close

☑ Ultra High Dynamics

Enhanced Dynamics: ☑ 458 nm ☑ 488 nm ☑ 501 nm ☑ 514 nm ☐ ☐ ☐ ☐

Acousto-Optical Beam Splitter
Pass the mouse pointer over a dialog element to view information

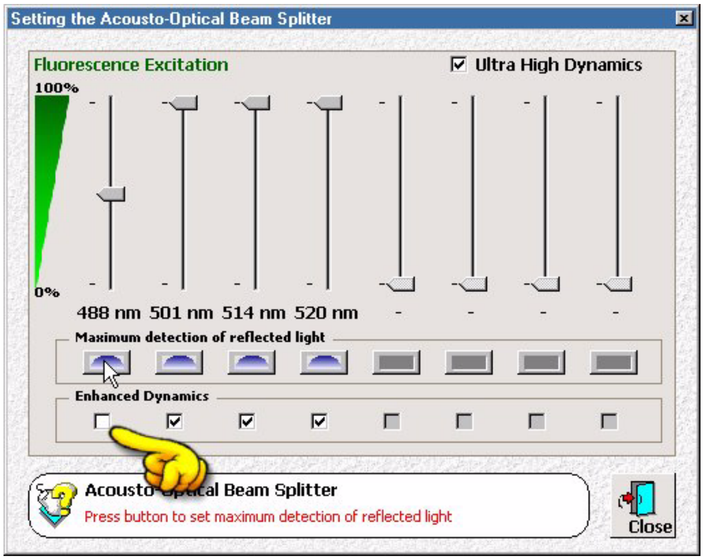
►	The standard setting is specified to fluorescence recordings with up to 4 excitation wavelengths. As soon as you activate a laser line with the slider in the Beam Path Setting dialog, the AOBS is automatically set so that the excitation light is optimally deflected to the specimen. For fluorescence light, the AOBS is transparent, independent of the slider. The slider in the AOBS dialog is set to 100%. & see Setting the Beam Path (page 111)
►	The two check boxes Enhanced Dynamics and Ultra-High Dynamics are activated as standard setting. This double dynamic setting ensures that reflection light, which returns through the beam path in reverse direction, is removed from the optical axis and, therefore, not detected.


Setting the Acousto-Optical Beam Splitter
Fluorescence Excitation
☑ Ultra High Dynamics
100%
0%
458 nm 488 nm 501 nm 514 nm 520 nm - - -
Maximum detection of reflected light
Enhanced Dynamics
☐ ☑ ☑ ☑ ☐ ☐ ☐ ☐
Close

Acousto-Optical Beam Splitter
Pass the mouse pointer over a dialog element to view information

▶	If more than 4 excitation wavelengths are used, the double dynamic setting is no longer applicable for all laser lines. In this case, the improved dynamic (Enhanced Dynamics check box) for the laser line with the shortest wavelength is automatically switched off. With each additional excitation wavelength, the improved dynamic is deactivated for an additional laser line.

Use of the AOBS for mixed fluorescence / reflection recordings


Setting the Acousto-Optical Beam Splitter
Fluorescence Excitation
☑ Ultra High Dynamics
100%
0%
488 nm 501 nm 514 nm 520 nm - - - -
Maximum detection of reflected light
Enhanced Dynamics
☐ ☑ ☑ ☑ ☐ ☐ ☐ ☐
Close

Acousto-Optical Beam Splitter
Press button to set maximum detection of reflected light

▶	If reflection light is to be recorded, too, it is usually sufficient to vary the portion of the reflection light by using the slider.
▶	If this is not sufficient, click the Maximum detection of reflected light button. The corresponding slider is automatically set to 50% and the improved dynamic (Enhanced Dynamics check box) is switched off.
▶	For true reflection light recordings, additionally deactivating the Ultra High Dynamics check box allows for increasing the reflection light for all laser lines to the maximum value.

Selecting an Objective

Function

Click the Objective button to open a list of objectives and make an appropriate selection. This list contains only the objectives that were previously assigned to one of the maximum of seven threaded ports on the objective nosepiece. You can assign objectives as follows:

- Select the Objectives option from the Tools menu. A dialog window opens containing an extensive list of objectives and the symbolic representation of the slots on the objective nosepiece.
- Find in the list the objective that you are using and select it with the mouse. Click and hold the left mouse button and drag the objective onto the symbol representing the slot in which the objective is installed.
- The assignment is saved in the software and the objective appears in the selection list, which you can open by pressing the Objective button.
- Repeat this procedure for every objective that you have installed in the objective revolver.

You can use the Add, Remove and Edit buttons in this dialog window to add new objectives, remove objectives or edit existing objective labels.

The microscope models DM RXA, DM RXE, DM IRBE, and DM IRBE2 use a software program to control the objective nosepiece. The program automatically rotates the selected objective into the beam path whenever you select an objective by using the button or Objective dialog window. All other microscope models require not only software adjustment of the objective, but also manual rotation of the objective into the beam path.

Additional information

When selecting the correct objective for a specific application, the objective's correction class (achromatic, apochromatic, fluorite objectives and plane objectives) and especially the magnification factor and the numerical aperture are of great significance. The numerical aperture determines the resolution capacity of an objective and is deduced from the flare angle of the light cone recorded by the objective and the refraction index of the medium between objective and specimen: NA = n*sin α.

Objectives with greater magnification generally have larger numerical apertures but smaller entrance pupils and therefore can record light only from a relatively small scan field. Objectives with larger apertures permit higher resolutions but allow less free working distance. The following table illustrates this relationship:

Objective	Resolution (xy)	Resolution Air (z)	Resolution Water (z)	Resolution Oil (z)	Scan field size (xy)
HC PL FLUOTAR 5x 0.15	1301	19410	25879	29559	3000
HC PL FLUOTAR 10x 0.30	651	4768	6407	7335	1500
N PLAN 20x 0.40	488	2630	3566	4093	750
N PLAN 50x 0.75	260	649	948	1108	300
PL APO 100x 1.40	139	319	209	236	150
	Values in nm at wavelength λ 488 nm				Values in µm

Typical Applications

Material scientists commonly use dry objectives to study surface structures. Immersion Objectives are the best choice for producing images of layered structures in which material layers with varying refraction indexes come into contact with each other. When working with biological specimens, the choice between an oil immersion objective and water immersion objective depends on the specimen itself and its embedding medium. You will achieve the best resolution when the refraction indexes of the embedding medium and/or the specimen are matched to the refraction index of the objective medium.

Setting the detectors

Function

Use the Signal button to open a dialog window for adjusting the detectors so that the entire range of intensities of a color look-up table is assigned and displayed in the image. For this purpose, a Gain value and an Offset value can be set for each detector. The gain value modifies the amplification of the detected signal, thus changing the brightness and contrast of the image. The offset value defines a threshold value. Only those signals that lie above the threshold value are detected and displayed in the image.

There are two methods for setting the gain and offset values:

▶	Use the mouse to move the slide on the scale. The corresponding value is shown below the corresponding scale.
▶	Double-click the numerical value that is displayed below the scale. This opens a second dialog window where you can enter an exact value.

Best suited to optimization of gain value and offset value are the color look-up tables *Glow Over*, *Glow Under* and *Glow Over and Under*.

& see Selecting Color Look-Up Tables (page 159)

You can also set the gain value and offset value of the detectors using the corresponding radio buttons of the control panel.

& see Controlling Functions using the Control Panel (page 229)

A detector is not enabled unless its corresponding Active check box is checked in the Signal or Beam Path Setting dialog windows.

The Electronic Zoom

Zoom

Function
In confocal microscopy, the magnification of an image is determined both by the objective and the electronic zoom. The objective generates a first image whose magnification is based on the objective's magnification factor. Additional magnification is provided by the electronic zoom. A zoom factor of 1 scans the maximum scan field size with a specific number of dots. If the zoom factor is set to 2, the same number of dots is used to scan a scan field with half the page length of the maximum scan field (1/4 the original scan field). This achieves stronger magnification and thus better image resolution too, because a smaller scan field is scanned with the same frequency, which results in a higher density of data.

In the dialog window that was opened by pressing the Zoom button, you can select one of the preset zoom factors. If you click on the Others button, you can set a different zoom factor in two ways:

▶	Use the mouse to move the slide of the scale. The corresponding value is shown in the middle of the dialog window.
▶	Double-click the boldface numerical value located in the middle of the dialog window. This opens a second dialog window where you can enter an exact value.

You can also set the zoom factor using the corresponding rotary knob on the control panel.

& see Controlling Functions using the Control Panel (page 229)

Additional information
While zoom factors from 1 to 32 can be set, the useful magnification of the electronic zoom can not be increased infinitely. The limit is achieved using the smallest optically resolvable distance, which is determined by the resolution capacity of the objective. According to the Nyquist theorem, this smallest optically resolvable distance can only be mapped without information loss if it is scanned with about 2 to 3 raster points. If the scan frequency is exceeded at a relatively high zoom factor and the specified scan format, further magnification is no longer useful because no more optical details can be resolved (zero-order magnification).

& see Selecting a Scan Format (page 120)
& see Selecting an Objective (page 115)

With bleach-sensitive specimens it is advisable to restrict the use of the electronic zoom to short time intervals. Since you are recording a segment of the specimen with a relatively higher scan frequency in the case of high zoom factors, the specimen is exposed to a stronger impact of light. This can lead to photomechanical destruction of the specimen (optical bleaching).

Optimal Zoom Factors

The following table shows which zoom factors (marked red)—depending on the scan field size and scan format specified by the objective—can be used to scan the specimen without loss of data:

Objective			**Scan format**	**Zoom factors**	**Scan field size (µm)**	**Scan Frequency (nm)**	**Resolution/ Scan Frequency**
Magnification	**Numerical Aperture**	**Resolution at λ 488 nm**					
20	0.6	325	1024 x 1024	1	750	732	0.4
				2	375	366	0.8
				4	187	183	1.8
				6	125	122	**2.7**
			512 x 512	1	750	1465	0.2
				2	375	732	0.4
				4	187	365	0.9
				8	94	183	1.8
				10	75	146	**2.2**
			200 x 200	1	750	3750	0.1
				2	375	1875	0.2
				4	187	935	0.3
				8	94	470	0.7
				16	47	235	1.4
				32	23	115	**2.8**
40	1.25	156	1024 x 1024	1	375	366	0.4
				2	187	183	0.8
				4	94	92	1.7
				6	62	60	**2.6**
			512 x 512	1	375	732	0.2
				2	187	365	0.4
				4	94	183	0.8
				8	47	92	1.7
				10	37	72	**2.2**
			200 x 200	1	375	1875	0.1
				2	187	935	0.2
				4	94	470	0.3
				8	47	235	0.7
				16	23	115	1.3
				32	12	60	**2.6**
100	1.4	139	1024 x 1024	1	150	146	0.9
				2	75	73	1.9
				3	50	49	**2.8**
			512 x 512	1	150	293	0.5
				2	75	146	0.9
				4	37	72	1.9
				6	25	49	**2.8**
			200 x 200	1	150	750	0.2
				2	75	375	0.4
				4	37	185	0.7
				8	18	90	1.5
				12	12	60	**2.3**

> *The Leica Confocal Software features three distinguishing zoom functions: electronic zoom, 3D zoom and graphic zoom.*

& see Zooming the 3D View (page 178)
& see Viewer Options Dialog Window, Display Icon (page 147)

Enlarged Recording of a Frame

Function
The Zoom In function enables an enlarged recording of a freely selectable square frame of a specimen.

▶	Click the Zoom In button. At the same time, the Rectangle button is automatically activated. & see Defining the Region of Interest (ROI) as a Rectangle (page 220)
▶	Specify the frame to be enlarged by drawing a rectangular evaluation area (region of interest or ROI) into the image using the Rectangle button. & see Selecting and Moving the Region of Interest (page 221)
▶	Use the Select button to move the frame on the image.
▶	To start the image recording, press the Continuous Scan, Series Scan or Single Scan button.

The software automatically adapts the freely selected rectangular frame to the actual square form that can be implemented by the scanner. The dashed line shows the actual frame.

Additional information
The enlarging recording of a frame will only provide additional detailed information as long as the distance of the grid points remains larger than half the optical resolution. On the other hand, the resolution depends on the objective used, the adjusted scanning format, and the light wavelength used (empty magnification).

Typical Applications
All applications that deal with the optical resolution of very small structures and that can do without an overview image. While recording such specimens, care should be taken that an objective with a corresponding high optical resolution is used for image recording.

Setting the Detection Pinhole

Function
Clicking the Pinhole button opens a dialog window that you can use to set the diameter of the detection pinhole. In the upper right list field in the dialog window, select the units for displaying the diameter. You can select from µm, Airy units and digital values. There are two ways of specifying a value for the diameter:

▶	Use the mouse to move the slide on the scale that is displayed on the left in the dialog window. The corresponding value is shown in the middle of the dialog window.
▶	Double-click the boldface numerical value located in the middle of the dialog window. This opens a second dialog window where you can enter an exact value.

The diameter of the detection pinhole must be set to the currently used objective at all times. If you click the Airy 1 button, the detection pinhole is set automatically to the optimum value of 1 Airy unit depending on the objective in use.

In addition to the numerical aperture of the objective and the wavelength of the light, the detection pinhole also determines the thickness of the optical sections.

Additional information
The diameter of the pinhole has the optimum if it matches the diameter of the Airy disc. The Airy disc refers to the inner, light circle of the diffraction pattern of a point light source. The diameter of the Airy disc, in turn, is also dependent on several optical parameters and can be described for the Leica Confocal System as follows:

$$d_{Airy} = \frac{1{,}22 \cdot \lambda}{NA} \cdot M \cdot 3{,}5$$

To calculate the diameter of the Airy disc, you need the excitation wavelength λ (in the case of several wavelengths, a mean value should be used), the numerical aperture NA and the magnifying factor M of the objective. The factor of 3.6 refers to the magnification of other optical components belonging to the Leica Confocal microscope.

If the pinhole is set to the Airy disc, light from outside the focal plane is suppressed and the signal-to-noise ratio is high. These conditions allow the recording of optical sections of minimum thickness. The wider the diameter of the pinhole, the more light reaches the detector. The image becomes brighter but blurring from structures outside the focal plane will also appear in the image, making it increasingly unfocused.

Increasing the diameter of the pinhole above 1 Airy unit is recommended only for detecting very weak signals.

Generally when recording material scientific specimens, enough light is reflected into the detectors so that the detection pinhole can be closed completely (i.e. set to the minimum diameter).

Selecting a Scan Format

Format

Function
Clicking the Format button opens a dialog window containing a selection list of scan formats. Selecting the scan format selects the image raster that will be used for recording the images. An image raster is the amount of points scanning the specimen in the three directions in space. Besides the numerical aperture of the objective and the excitation wavelength, the scan format, together with the electronic zoom, determines in large part the spatial resolution of the recorded data.

Additional information
When selecting the scan format, observe the influences between the image raster and the resolution of the generated image. According to the Nyquist theorem (or sampling theorem), a structure can only be scanned without information loss if the smallest optically resolvable distance is scanned with about 2 to 3 raster points. This optically resolvable distance, called **lateral resolution**, depends on the numerical aperture of the objective and the wavelength of the applied excitation light:

$$resolution_{lateral} = \frac{0.4 \cdot \lambda}{NA}$$

The following example illustrates this correlation: For example, you selected the PL APO 100x objective with numeric diaphragm ND = 1.4 and a wavelength of λ = 488 nm. Based on these factors, the smallest optically resolvable distance is determined as follows:

$$\frac{0.4 \cdot 488}{1.4} = approx.140nm$$

Based on this rough formula, the distance of the raster points required to avoid information loss while recording is:

$$\frac{140nm}{3} = approx.47nm$$

If however you select a scan format of 1024x1024, a PL APO 100x objective, which has a maximum scan field size of 150 µm, would result in the following raster point distance:

$$\frac{150\mu m}{1024} = approx.146nm$$

To achieve the required 47 nm raster distance, you can either raise the scan format (to 2048x2048 for instance) or you can decrease the scan field size using the Electronic Zoom (Zoom=2).

The current raster distance is displayed in the Hardware legend. This value is referred to as Voxel Size and recalculated as soon change the scan format, the Electronic Zoom or the objective.

& see Electronic Zoom (page 117)
& see Selecting an Objective (page 115)

When the sampling rate of 2 to 3 points is exceeded, it is referred to as oversampling. In the case of oversampling, the structure is fully mapped but no additional information is gained. The disadvantage of oversampling is the long scanning time and the danger of destroying bleach-sensitive specimens. When the sampling rate is less than 2 to 3 points, it is referred to as undersampling. In this case, there is the danger that not all necessary information is sampled. The image might illustrate structures that are not actually present in the specimen. This effect is called aliasing.

Selecting a Scan Mode

Mode

Function

Clicking the Mode button opens a dialog window containing a list of available scan modes. The scan mode determines which optical levels of the specimen are to be scanned. Fundamentally, horizontal xy-sections or vertical xz-sections can be recorded. To generate a three-dimensional image of the specimen, the optical sections are continued in the corresponding third direction in space, thus recording a stack of individual images. In addition to this, it is possible to add the factor of time or wavelength while recording the images:

Mode	Function
xyz	An image stack is recorded from the xy-sections in the z direction.
xzy	An image stack is recorded from the xz-sections in the y direction.
xt	A line is recorded several times in succession.
xyt	A xy-section is recorded several times in succession.
xzt	A xz-section is recorded several times in succession.
xyzt	An image stack is recorded several times in succession from xy-sections in z-direction.

xyλ	An xy section is recorded at different wavelengths.
xzλ	An xz section is recorded at different wavelengths.

All scan modes (with the exception of xt) are composed of at least three dimensions. The third as well as other dimensions will be ignored by the device if you record images only from one optical plane (xy or xz) using the Continuous Scan function of the Single Scan function.

> *The xzy and xzt scan modes are only displayed in the list if you previously selected the selection point Galvo using the Z-Scan button. Likewise, the scan modes for an xyλ and xzλ wavelength will only be displayed if only* ***one*** *detector is activated in the Beam Path Setting dialog window.*

& see Setting the Beam Path (page 111)

Selecting a Scan Speed

⏩ Speed

Function
Clicking the Speed button opens a dialog window that you can use to select from four scan speeds.

Speed	
200	Image lines per second
400	Image lines per second
800	Image lines per second
1000	Image lines per second

The data recording speed be further increased in combination with the bidirectional scan.

& see Unidirectional or Bidirectional Scan (page 135)

Additional information
The higher the set scan speed, the shorter the dwell time of the laser point. Furthermore, the scan format, i.e. the number of sampling points in a row, needs to be taken into account. The higher the scan format at a constant speed is, the shorter the dwell time of the laser point over a sampling point.

The longer the light point of the laser beam dwells over the individual sampling points of the specimen, the more light is detected by the detector. So using a lower scan speed results in a better signal-to-noise ratio. The disadvantage of a lower scan speed is that the relatively long impact of the light on the specimen can bleach the specimen photochemically, making it unusable. This is especially important with fluorescence applications.

> *If the speed levels 800 or 1000 are set, technical limitations prevent the maximum scan field from being scanned. The system automatically switches to zoom factor 2 or zoom factor 4.*

Configuring a Time Series

Function

Click the Time button to open the Time Configuration dialog window for setting up a time series recording. The settings that can be changed in this dialog window depend on the selected scan mode. You can record a line (xt), a horizontal section (xyt), a vertical section (xzt), or a stack of horizontal sections (xyzt), interrupted by a particular time interval, many times in a row.

> *The Time button is not enabled unless you have selected a scan mode with time dimension using the Mode button.*

& see Selecting a Scan Mode (page 121)

Use the following parameters in the dialog window to configure a time series using the xt scan mode.

ΔT	The recording time for a line *(cannot be modified by the user)*
Lines	Number of line recordings
Lines per page	Number of lines per stored page
Pages	Number of stored pages *(calculated automatically)*
Maximize	Recording of the maximum possible number of lines per stored page
Complete Time	Total recording time, i.e. the product of ΔT and number of recordings

Using the Calculate button, you can calculate how many stored pages are needed for a particular number of lines per page.

Use the following parameters in the dialog window to configure a time series using the xyt or xzt scan mode.

ΔT	Recording time for an xy-section or xz-section plus pause interval
Minimize	The smallest possible interval (ΔT) is used.
Frames	Number of recordings of the xy-section or xz-section
Complete Time	Total recording time, i.e. the product of ΔT and number of recordings

Use the following parameters in the dialog window to configure a time series using the xyzt scan mode:

ΔT	Recording time for a stack of xy-sections plus pause interval
Minimize	The smallest possible interval (ΔT) is used.
Stacks	Number of recordings of the image stack
Complete Time	Total recording time, i.e. the product of ΔT and number of recordings

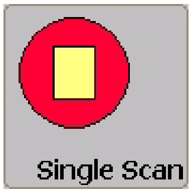
Each parameter can be calculated in relation to the other parameters. When entering the values, note how the dialog window operates. The entry field you click on will be activated:

▶	Click on the parameter to be calculated. The corresponding entry field is grayed out.
▶	Now enter the values for the other parameter.
▶	Click on Apply to calculate the parameter. By clicking on Reset, the last saved values are

	displayed.

When you have finished configuring these settings, press the Series Scan button.

Starting a Single Scan



Function
Use the Single Scan button to record a single image from a single optical level in the specimen.

Before recording an image using the single scan function, configure all required scan parameters using the Continuous Scan function to ensure optimal image quality.

& see Starting a Continuous Scan (page 124)

Typical Applications
The Single Scan function is designed for recording bleach-sensitive specimens. Use this function in the case of bleach-sensitive specimens not only for image recording, but also as an alternative to the continuous scan function for setting the scan parameters. The Single Scan function can also be used to check the image section by zooming in on the scan field.

Starting a Continuous Scan

Continuous

Function
Use the Continuous Scan button to permanently record images from a single optical level in the specimen. This does not generate image series, since the image being generated always replaces the previously generated image.

Press the button a second time to stop the continuous scan.

Additional information
The unit applies the last used scan parameters automatically. You can modify some of the parameters **while** the image is being recorded. Others have to be configured **before** you start the recording:

Before recording	**While Recording**
Selecting an objective	Setting the beam path
Selecting a scan format	Setting the detection pinhole
Selecting a scan mode	Adjusting the electronic zoom
Selecting a scan speed	Setting the detectors
Selecting a unidirectional or bidirectional scan	Adjusting the phase

The buttons of the functions that can not be changed during continuous scanning are disabled and appear gray.

Typical Applications
The continuous scan function is best used to optimize the image quality for the first scan of a specimen. While the specimen is being scanned continuously, you can modify the scan parameters listed above and check the results directly in the image.

> *With bleach-sensitive specimens it is advisable to restrict the use of the Continuous Scan function to short time intervals. Subjecting the specimen to the continuous light of the laser can destroy the specimen photochemically (optical bleaching), thus making it unusable.*

Series Scan Overview Dialog Window

Series

Function
This dialog window lets you define the begin and end points of an image series and trace the recording of the individual sections. The three-dimensional scan area is represented graphically as a cube. Within this cube, a yellow square represents the current z-position or y-position, a green one represents the begin point and a red one the end point. The corresponding position values are displayed to the right of the cube. Set the begin and end point as follows.

▶	Use the mouse pointer to drag the yellow square to the level in which the image series is to begin.
▶	Click in the white box for the begin point. The corresponding position value appears and is saved.
▶	Use the mouse pointer to drag the yellow square to the level in which the image series is to end.
▶	Click in the white box for the end point. The corresponding position value appears and is saved.
▶	The entire height of the image stack between the begin and end points is calculated and displayed (total).

Now click the Series Scan button. The dialog window stays open and you can follow the process of the image series being recorded.

> *The begin point and end point can also be defined using the control panel and the separate keys Begin and End.*

& see Defining the Begin Point for a Spatial Series (page 126)
& see Defining the End Point for a Spatial Series (page 127)

Additional information
The following additional parameters, which have to be set before recording an image series, are specified in the Series Scan Overview dialog window:

▶	Scan mode (top line to the right of the graphic image)
▶	Scan format (red digits at the upper edge of the cube)
▶	Number of optical sections (division between begin and end points)

The red number on the vertical edge of the cube represents maximum travel of the z-actuator that can not be changed.

& see Selecting a Scan Mode (page 121)
& see Selecting a Scan Format (page 120)
& see Defining the Number of Spatial Sections (page 128)

Defining the Begin Point for a Spatial Series

Begin

Function
Use the Begin button to define the begin point of a spatial image series. First, set the exact z-position or y-position in the Series Scan Overview dialog window or on the control panel using the corresponding radio button. Then click Begin. This saves the position value for the begin point. The end point is set in the same manner.

& see Series Scan Overview Dialog Window (page 125)
& see Controlling Functions using the Control Panel (page 229)

Defining the Begin Point for a Wavelength Series

λ Beg.

Function
For a wavelength series, this function records a stack of individual images, each of which are detected at a specific wavelength, from a single, optical level. Use the Lambda Scan Begin button to define the wavelength at which recording should begin:

> *The Lambda Scan Begin button is only active if you used the Mode button to select a scanning mode with the wavelength dimension.*

& see Selecting a Scan Mode (page 121)

▶	Press Beam to open the Beam Path Setting dialog window.
▶	Activate a detector by clicking its corresponding check box. A cast shadow now links the activated detector to the corresponding slide bar on the scale of the spectrum.
▶	Double-click this slide bar to open the Range Properties dialog window, where you can specify the end point of the series.
▶	Drag the slide bar on the scale of the spectrum to the desired start position. The left edge of the

	slide bar represents the wavelength at which the recording is to begin.
▶	Click Lambda Scan Begin to save the value.

The end point of a wavelength series is set in the same manner. In addition, you also have to set the number of wavelength steps.

& see Defining the End Point for a Wavelength Series (page 127)
& see Setting the Number of Wavelength Steps (page 129)

Typical Applications
A wavelength series can be applied to determine the maximum emission of a fluorochrome. This is useful because the Stokes shift of the emission curve of a fluorochrome is dependent on each specimen that is applied. This then allows you to set the detection range precisely for a specific application.

Defining the End Point for a Spatial Series

End

Function
Use the End button to define the end point of a spatial image series. First, set the exact z-position or y-position in the Series Scan Overview dialog window or on the control panel using the corresponding radio button. Then click End. This saves the position value for the end point. The begin point is set in the same manner.

& see Series Scan Overview Dialog Window (page 113)
& see Controlling Functions using the Control Panel (page 229)

Defining the End Point for a Wavelength Series

λ End

Function
For a wavelength series, this function records a stack of individual images, each of which are detected at a specific wavelength, from a single, optical level. Use the Lambda Scan End button to define the wavelength at which recording should end.

> *The Lambda Scan End button is only active if you used the Mode button to select a scanning mode with the wavelength dimension.*

& see Selecting a Scan Mode (page 121)

▶	Press Beam to open the Beam Path Setting dialog window.
▶	Activate a detector by clicking its corresponding check box. A cast shadow now links the activated detector to the corresponding slide bar on the scale of the spectrum.
▶	Double-click this slide bar to open the Range Properties dialog window, where you can specify the end point of the series.

▶	Drag the slide bar on the scale of the spectrum to the desired end position. The right edge of the slide bar represents the wavelength at which the recording is to end.
▶	Click Lambda Scan End to save the value.

The begin point of a wavelength series is set in the same manner. In addition, you also have to set the number of wavelength steps.

& see Defining the Begin Point for a Wavelength Series (page 126)
& see Setting the Number of Wavelength Steps (page 129)

Typical Applications
A wavelength series can be applied to determine the maximum emission of a fluorochrome. This is useful because the Stokes shift of the emission curve of a fluorochrome is dependent on each specimen that is applied. This then allows you to set the detection range precisely for a specific application.

Defining the Number of Spatial Sections

Sect

Function
Clicking the Sections button opens a dialog window that you can use to select the number of horizontal xy-sections and vertical xz-sections for recording an image series. If you want to specify a number of sections that is not listed, click the Others option. This opens the Z/Y Configuration dialog window, which contains the following data:

Parameter	**Description**
Image Dim. z/y (µm)	The height of the entire image stack between the begin and end points of the image series
# Sections	The number of configured sections
Step Size (µm)	The step size, i.e. the distance between two sections

In this dialog window, you can enter any value for the number of sections and the section width. The height of the image stack can not be changed, as this parameter is determined by the start and end point of the image series. As the section width always has to be a multiple of the minimum section width of the z-setting drive, certain combinations of values are only possible if either the height of the image stack or the number of sections is adapted. Depending on which of the two Calculate buttons you click, one of the two parameters is not changed.

If you want to calculate the number of sections with the priority of leaving the height of the image stack unchanged where possible:

▶	Enter the desired step size in the Step Size field.
▶	Then click the Calculate button next to the Step Size field.

If you want to calculate the number of sections with the priority of leaving the number of sections unchanged where possible:

▶	Enter the desired step size in the Step Size field.
▶	Then click the Calculate button next to the # Sections field.

If you want to calculate the step width with the priority of leaving the height of the image stack unchanged where possible:

▶	Enter the number of desired sections (whole numbers only) in the # Sections field.

▶	Then click the Calculate button next to the # Sections field.

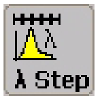
If you want to calculate the step width with the priority of leaving the number of sections unchanged where possible:

▶	Enter the number of desired sections (whole numbers only) in the # Sections field.
▶	Then click the Calculate button next to the Step Size field.

If you click the Reset button, the last shown values are displayed.

& see Starting a Series Scan (page 130)
& see Series Scan Overview Dialog Window (page 125)

Setting the Number of Wavelength Steps



Function
For a wavelength series, this function records a stack of individual images, each of which are detected at a specific wavelength, from a single, optical level. The images are recorded within a wavelength range, which is limited by the begin and end points. Use the Lambda Steps button to define the number of recordings that are to take place within this range.

> *The Lambda Steps button is only active if you used the Mode button to select a scanning mode with the wavelength dimension.*

& see Selecting a Scan Mode (page 120)
& see Defining the Begin Point for a Wavelength Series (page 126)
& see Defining the End Point for a Wavelength Series (page 127)

Typical Applications
A wavelength series can be applied to determine the maximum emission of a fluorochrome. This is useful because the Stokes shift of the emission curve of a fluorochrome is dependent on each specimen that is applied. This then allows you to set the detection range precisely for a specific application.

Starting a Series Scan

Function

Use the Series Scan button to create an image series. This creates a multidimensional image data block of the specimen. The available dimensions for recording an image series are the three directions in space (x, y, z) as well as the dimensions of time (t) and wavelength (λ). This allows you to record a three-dimensional, spatial image stack consisting of xy or xz-sections with the additional factor of time or wavelength.

> *Before recording an image series, configure all required scan parameters using the Continuous Scan function to ensure optimal image quality.*

& see Starting a Continuous Scan (page 124)

Before recording a spatial image series, configure the following settings:

▶	Select the scan mode using the Mode button. & see Selecting a Scan Mode (page 121)
▶	Select the scan format using the Size button. & see Selecting a Scan Format (page 120)
▶	Set the desired z-position or y-position in the Series Scan Overview dialog window or by means of the corresponding radio button on the control panel. & see Series Scan Overview Dialog Window (page 125) & see Controlling Functions using the Control Panel (page 229)
▶	Set and store the begin point for an image series using the Begin button or in the Series Scan Overview dialog window. & see Defining the Begin Point for a Spatial Series (page 126)
▶	Set the desired z-position or y-position in the Series Scan Overview dialog window or by means of the corresponding radio button on the control panel. & see Series Scan Overview Dialog Window (page 125) & see Controlling Functions using the Control Panel (page 229)
▶	Set and store the end point for an image series using the End button or in the Series Scan Overview dialog window. & see Defining the End Point for a Spatial Series (page 127)
▶	Select the number of spatial sections using the Sections button. & see Defining the Number of Spatial Sections (page 128)

When you have finished configuring these settings, press the Series Scan button.
You can track the recording process of the image stack in the Series Scan Overview dialog window.

Before recording a time series, configure the following settings:

▶	Select a scan mode with time dimension using the Mode button. & see Selecting a Scan Mode (page 121)
▶	Select the scan format using the Size button.

	& see Selecting a Scan Format (page 120)
▶	Set the desired z-position or y-position in the Series Scan Overview dialog window or by means of the corresponding radio button on the control panel. & see Series Scan Overview Dialog Window (page 125) & see Controlling Functions using the Control Panel (page 229)
▶	Set the number of recordings, the pause interval between the recordings, and the complete editing time. & see Setting a Time Series (page 123)

Before recording a wavelength series, configure the following settings:

▶	Select a scan mode with wavelength dimension using the Mode button. & see Selecting a Scan Mode (page 121)
▶	Select the scan format using the Size button. & see Selecting a Scan Format (page 120)
▶	Define the wavelength at which you want the wavelength series to begin. & see Defining the Begin Point for a Wavelength Series (page 126)
▶	Define the wavelength at which you want the wavelength series to end. & see Defining the End Point for a Wavelength Series (page 127)
▶	Select the desired number of recordings between the begin and end points of the wavelength series. & see Setting the Number of Wavelength Steps (page 129)

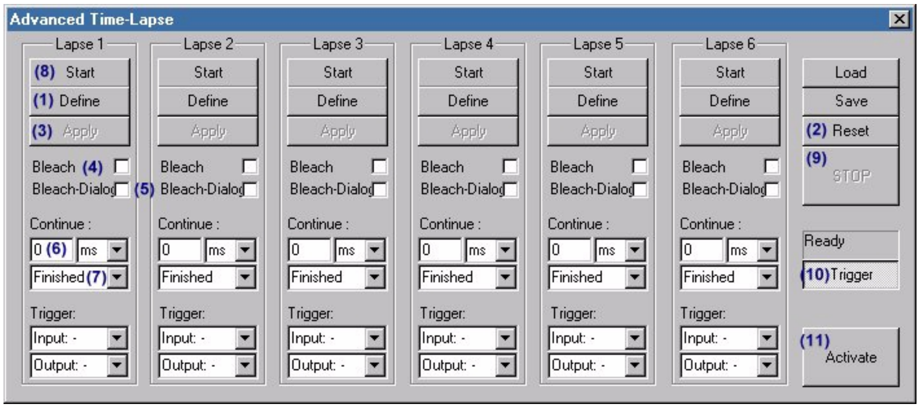
When you have finished configuring these settings, press the Series Scan button.

Configuring Recording Sequences

Time

Function

This function is used to combine up to six recording intervals with different hardware settings and their sequence into a recording sequence. Clicking on the Time Lapse button opens the following dialog window:



Configuration of Recording Sequence

▶	Using the Beam Path Setting dialog window and the buttons in the Acquire operating step (e.g. Pinhole, Series Scan Overview), adjust all required hardware parameters for the first recording interval (Lapse 1). & see Setting the Beam Path (page 111)

▶	Click on the Define **(1)** button to save these hardware settings for the recording interval. The settings of all scan functions are automatically saved. ℹ *Which hardware parameters are saved using Define, can be changed using the Setting dialog window. For this purpose, select the Tools menu, Settings option and Instrument Parameter Settings tab. In the Choose Settings field, click on IPS Time Lapse.*
▶	Repeat this process with different settings for each additional recording interval (Lapse 2...8). Reset **(2)** is used to undo the settings.
▶	Clicking Apply **(3)** transfers the settings defined for the recording interval to the current hardware settings. This button does **not** have to be used since the settings are automatically transferred at the start of the recording sequence. This function allows you to recheck the respective settings. & see Applying the Parameter Setting of an Experiment (page 137)
▶	If the specimen must be bleached prior to the start of a recording interval, activate the Bleach **(4)** check box. The bleaching pattern defined in the Bleach dialog window is used. This bleaching pattern is used for all recording intervals. Activate the Bleach Dialog **(5)** check box to open the Bleach dialog window and redefine the bleaching pattern. & see Definition of a Bleach Experiment (page 133)
▶	In the first list box under Continue **(6)**, set a pause interval between the processing of the current and the subsequent recording interval.
▶	In the second list box under Continue **(7)**, select the recording interval (Lapse 1...8) that is to be executed after processing the current interval. If you select Finished, the recording sequence ends with the current interval.
▶	Click on the Start **(8)** button to start the recording sequence. Click on Stop **(9)** to stop the recording sequence.

Triggering image recordings through external signals (requires special hardware equipment)

▶	If the recording interval is to be started through an external signal, click on the Trigger **(10)** button. The following settings for the trigger signals can be performed in the expanded dialog window:

List box	**Function**
Input	The channel for incoming trigger signals is selected here. If a trigger channel is selected, the recording interval is started upon receipt of a TTL trigger via the selected channel: & see Signal Connector of Trigger Electronics (page 134) **Input 1:** Pin 4 of the trigger electronics connector **Input 2:** Pin 12 of the trigger electronics connector **Input 3:** Pin 5 of the trigger electronics connector **Input 4:** Pin 13 of the trigger electronics connector **Input** –: An incoming trigger signal is not required to start the recording interval.
Output	The channel for outgoing trigger signals is selected here. If a trigger channel is selected, a TTL trigger is transmitted via the selected channel when the recording interval is started: & see Signal Connector of Trigger Electronics (page 134) **Output 1** Pin 7 of the trigger electronics connector. It is a line signal that is set to logical «1» for the duration of the actual data recording. **Output 2** Pin 15 of the connector of the trigger electronics. This is a free signal that can be used to trigger external devices in accordance with predefined events.

	Output –: No trigger signal is issued.

All inputs are decoupled through optocoupler. The maximum current that may be applied to the optocoupler is 2 mA. The minimum pulse duration for incoming trigger signals is 100 ms.

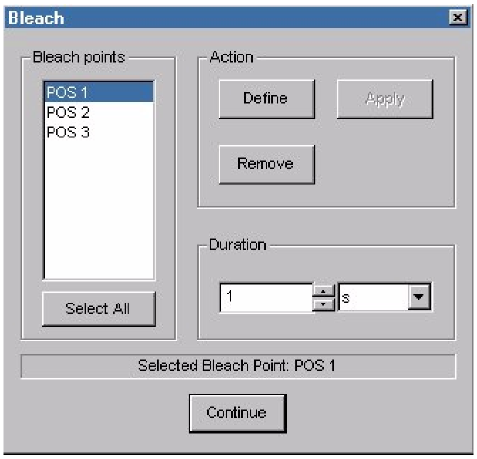
If a recording interval is started via incoming trigger signal, the processing of an already running recording is interrupted. A recording interval is only started by a trigger signal if the Activate ***(11)*** *button or Start is pressed.*

Definition of a Bleach Experiment

Function

Bleach points and a specific bleaching period can be defined and saved for each bleach point using this dialog window. This function can be used only in connection with an imaging sequence.

& see Configuring Recording Sequences (page 131)

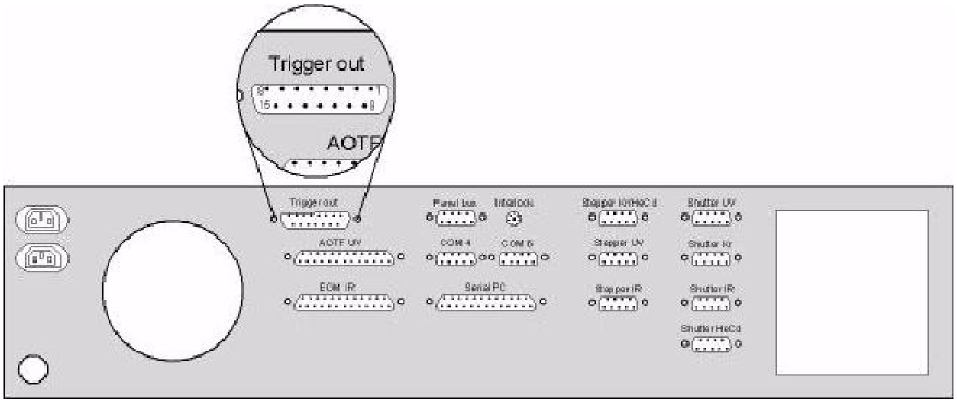


Selected Bleach Point: POS 1

▶	Hold the mouse pointer over the image in the Viewer display window when the Bleach dialog window is opened. The mouse pointer symbol changes to a cross.
▶	Select the bleach points in the image by clicking the corresponding positions in the image. The bleach points are entered in the Bleach Points list field with the designation POS 1..n. Use the Remove button to remove the bleach points.
▶	To set the bleaching duration for a single bleach point, mark the corresponding entry in the dialog window and select a time interval in the Duration field. To set the same bleaching duration for all bleach points, click the Select All button and then set the bleaching duration.
▶	Click the Define button. Besides the bleaching duration, this also automatically stores the laser

	intensity and the z-height of the selected bleach point. The laser intensity can be modified in the Beam Path Setting dialog window. & see Setting the Beam Path (page 111)
▶	Clicking Apply transfers the settings defined for the bleaching pattern to the current hardware settings.
▶	Click OK or Continue (if the dialog was opened via the Time Lapse dialog window).

The signal connector of the trigger electronics is located on the back of the supply unit



Signal connector assignment of the trigger electronics

Signal	Signal connector
Input 1	Pin 4 of the trigger electronics connector.
Input 2	Pin 12 of the trigger electronics connector.
Input 3	Pin 5 of the trigger electronics connector.
Input 4	Pin 13 of the trigger electronics connector.
Output 1	Pin 7 of the trigger electronics connector. This is a line signal. In contrast to the Output 3 (Pin 6) signal, it remains on a logical «1» for the duration of the actual data recording only. & see Configuring Recording Sequences (page 131)
Output 2	Pin 15 of the connector of the trigger electronics. This is a free signal that can be used within the Time Lapse function to trigger external devices in accordance with predefined events. & see Configuring Recording Sequences (page 131)
Output 3	Pin 6 of the connector of the trigger electronics. This is a line signal (line-clock). At each start of a new line, the signal features a rising edge. The signal remains on a logical «1» for the complete time in which the scanning beam is located on one line.
Output 4	Pin 14 of the connector of the trigger electronics. This is a frame signal (frame-clock). At each start of a new frame, the signal features a rising edge. The signal remains on a «1» for the complete time that is required for recording a frame.

Selecting a Unidirectional or Bidirectional Scan

->Scan

Function
If you click the Unidirectional/ Bidirectional Scan button, bidirectional scan mode is enabled. If this button is not clicked, unidirectional scan mode is automatically set.

In unidirectional scan mode, each line is scanned from left to right. No data is recorded while the laser beam is being guided to the starting point of the next line. In bidirectional scan mode, the first line is scanned from left to right and the second from right to left. In other words, the return run, or flyback, of the laser beam is implemented for recording data too, thus increasing the scan speed when using bidirectional scan mode.

If bidirectional scan mode is active, you can double the scan speed using the Speed button:

Unidirectional	**Bidirectional**	
200	Currently not available	Image lines per second
400	800	Image lines per second
800	1600	Image lines per second
1000	2000	Image lines per second

& see Selecting a Scan Speed (page 122)

In order to accurately align the pixels of the forward sweep and flyback, the phase between the forward sweep and the flyback can be adjusted. Use the Phase button for this purpose.

& see Setting the Phase (page 135)

ℹ

If the speed levels 800 or 1000 are set, technical limitations prevent the maximum scan field from being scanned. The system automatically switches to zoom factor 2 or zoom factor 4.

& see Electronic Zoom (page 117)

Adjusting the Phase

Phase

Function
During bi-directional image recording, there may be a phase displacement between the scanning beams moving forward and backwards. Click on the Phase button to open a dialog window that allows you to correct the displacement:

▶	Use the mouse pointer to move the slider on the scale until the pixel displacement disappears from the image.

It is also possible to adjust the phase with the respective knob on the control panel.

& see Controlling Functions using the Control Panel (page 229)

Additional information

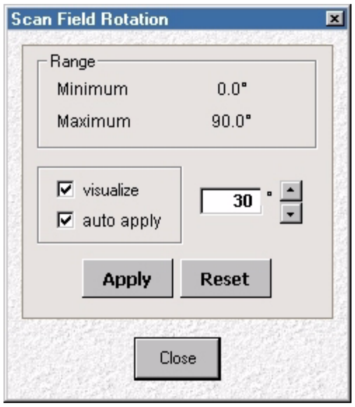
Before delivery of the instrument, the factory performs a phase adjustment for each zoom value and enters the corresponding values. Due to the temperature dependency of the mechanics and electronics, there may be a slight deviation from these default values during operation. Use this function to readjust these values.

Rotating the Scanning Field

Field

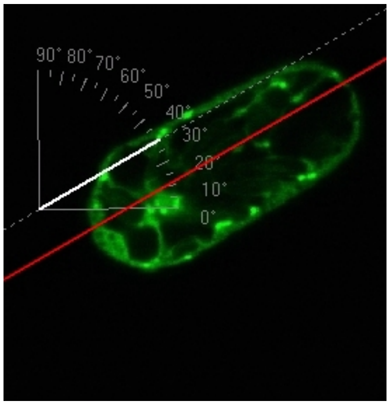
Function

Use the Scan Field Rotation function to rotate the scan field. This neither rotates the specimen nor the direction of the scanner, but rather the intermediate microscopic image.


Scan Field Rotation

Range
Minimum 0.0°
Maximum 90.0°

☑ visualize
☑ auto apply
30 °



▶	Select a rotating angle between 0 and 90 degrees in the Scan Field Rotation dialog window. The original position (0 degrees) is based on a predefined reference position in the scanning head. When the unit is switched on, it automatically relocates to this reference position.
▶	Click the Apply button to apply the specified rotation angle to the current image. Use Reset to return to the previously set value. If Auto Apply is selected, new settings are automatically applied to the image.
▶	Click the Visualize check box to display the rotation angle in the image. The rotation angle can be changed directly in the image by repositioning the white line using the mouse pointer.

Y-positioning

If the xzy, xzt or xzλ scan mode is specified, activating the Visualize check box will also display the y-level as a red line in the image. The y-position can now be modified in the Series Scan Overview dialog window and the positioning can be checked using the red line in the image.

& see Selecting a Scan Mode (page 121)
& see Series Scan Overview Dialog Window (page 125)

Typical Applications

This function is used to align textures on surfaces or long stretched-out biological structures relative to the viewer.

Applying the Parameter Setting of an Experiment

Apply

Function
Click the Apply button to use the hardware settings of a previous experiment for recording a new experiment. This lets you configure the settings for new image recordings with a single click with the scan parameters that have been optimally configured for a previous application.

▶	Activate the image data block that is configured with the settings you want to apply to the new experiment.
▶	Click the Apply button.

Recording an Image of a Line Using the Averaging Method

Li. A.

Function
The Line Average function launches an averaging method for the image recording. In this case, each single line is scanned several times. For every sampling point, the arithmetical average is calculated from the repeatedly measured intensity values in one line and represented in the result image. The next line of the specimen will not be scanned until after the set number of averaging steps has completed. The method used here determines a consecutive average. This means that every line recorded after the first line is averaged with the previously displayed line and is displayed in the result image (dynamic average).

Clicking the Line Average button opens a dialog window that you can use to set how often a line is to be scanned. You can select from 1 to 8 scan repeats.

Using a parallel function, it is possible to record complete images using the averaging method.

& see Recording an Image Using the Averaging Method (page 138)

Typical Applications
This function is used predominantly for recording live specimens.

> *Recording an image using the averaging method is not recommended for bleach-sensitive specimens. Repeated recordings and the resulting length of subjection to light can lead to the destruction of the specimen.*

Recording an Image Using Burst Mode

Burst

Function
If the workstation must process large amounts of data during the recording of images, it may lead to a delay of the scanning process. This is because the recorded image is first displayed on the monitor before additional images can be scanned by the scanner. The Burst function allows for decoupling the scan process of the laser and the refreshing of image data on the screen. For this purpose, the transmission of image data from the program memory to the monitor is delayed, but the scanning process is not. By clicking on the Burst button, you can select operating modes with different delay times:

Mode	**Description**
No Burst	Image data are recorded and simultaneously continuously displayed on the monitor.
Frame Burst	Image data are not displayed on the monitor until a single image has been recorded.
Complete Burst	Image data are not displayed on the monitor until an image series has been recorded.
Automatic	The software automatically sets the optimum operating mode for a specific application.

> *The following parameters can increase the amount of data to be processed to the extent that it results in a delay of the data display on the screen: scan format, scan speed, bidirectional scan, number of active detection channels, calculation of an overlay image and size of the Viewer window.*

Recording an Image Using the Averaging Method

Aver

Function
The Average function launches an averaging method for the image recording. In this case, every individual image, i.e. every xy-section or xz-section, is scanned several times. For every sampling point, the arithmetical average is calculated from the repeatedly measured intensity values and represented in the result image. The method used here determines a consecutive average. This means every image recorded after the first image is calculated with the results of the previously displayed image and is displayed in the result image (dynamic average).

Clicking the Average button opens a dialog window that you can use to set how often a section is to be scanned. You can select from 1 to 64 scan repeats.

Typical Applications
Recording an image using the averaging method is primarily useful for suppressing noise. In fluorescence microscopy, weak fluorescent specimens result in little light reaching the detector. The resulting low photon count leads to noisy images. In such cases, you can improve the signal-to-noise ratio by carrying out multiple recordings and determining averages of the image.

> *Recording an image using the averaging method is not recommended for bleach-sensitive specimens. Repeated recordings and the resulting length of subjection to light can lead to the destruction of the specimen.*

Image Recording with Accumulation Method

Accu

Function

The Accumulation button is used to apply an accumulation method for the image recording. In this case, every individual image, i.e. every xy-section or xz-section, is scanned several times. The repeatedly measured intensity values are added for each scanning point and the sum is displayed in the resulting image.

Clicking the Accumulation button opens a dialog window that you can use to set how often a section is to be scanned. You can select from 1 to 16 scan repeats.

Typical Applications

The accumulation method is intended to improve the image quality during image recordings when only weak fluorescence signals can be detected.

Image Recording With a Digital Resolution of 8 Bits or 12 Bits

Bit

Function

This function reads in the analog intensity values measured by the detector by means of an A/D converter, either as 8 bit signal or 12 bit signal.

Additional information

While digitizing the analog intensity signal with a digital resolution of 8 bit, 256 different intensity values can be displayed. Taking the natural statistical variations of the typical intensity value range of fluorescence specimen into account, a digitization with 8 bit is completely sufficient in most cases for an image recording without loss of information. For specimens with a higher intensity dynamics (e.g., for specimen with very intensity-weak and intensity-strong areas), an image recording with 12 bit digitization is recommended. In a 12 bit digitization, 4096 different intensity values can be resolved. However, the data capacity to be saved doubles.

Typical Applications

For image recording with very high intensity dynamics (mostly material specimens with high reflectivity of the surface and occurrences of very dark areas at the same time).

Setting the UV Lens Wheel (Option)

Function

For each objective, this function reduces the focus offset between visible and UV excitation light to a value below the optical resolving capacity. In order to achieve this, a suitable correction lens is brought into the beam path for each objective suitable for use with UV excitation light. To use this corrective feature, proceed as follows:

▶	Click the Objective button (standard procedure in Acquire step). & see Selecting an Objective (page 115)
▶	Select a UV-capable objective from the list (see indication on list).

In this case, the corrective lens for the selected objective is automatically moved into the beam path.

> *The possible occurrence of a focus offset between visible light and UV light has physical reasons and is not a malfunction of the device.*

Other Requirements

In order to achieve an automatic correction of the physically based color length fault between visible and UV light, it is not sufficient to manually rotate the objective into the beam path.

Selecting the Laser Line for the Image Background (ROI Scan)

Function

This function is required for the recording of regions of interest (ROI Scan). In this recording process, the image background is recorded with a different excitation wavelength than the selected regions of interest. The ROI button saves the laser settings for the image background before you separately specify the excitation wavelength for each region of interest with the Configuration button.

> *Additional information about this topic can be found in the chapter on "Procedures and exercises for selected processes*
>
> *",page 56.*

Selecting Laser Lines for Individual Regions of Interest (ROI Scan)

Config

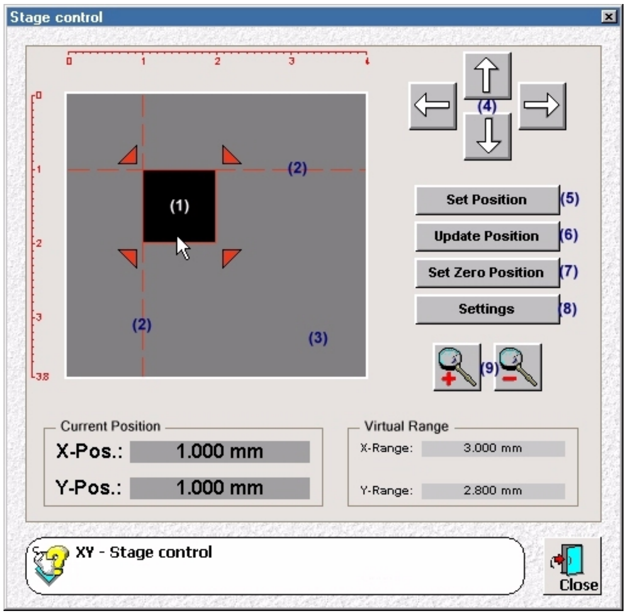
Function
This function is required for the recording of regions of interest (ROI Scan). In this recording process, the image background is recorded with a different excitation wavelength than the selected regions of interest. The Configuration button opens a dialog window which allows you to assign different laser lines to the individual regions of interest.

> *Additional information about this topic can be found in the chapter on „Procedures and exercises for selected processes*
>
> ",*page 56.*

Controlling the X/Y Specimen Stage

Stage

Function of dialog elements
The XY-Stage Control dialog window features the following dialog elements to control the «Märzhäuser» X/Y specimen stage:



Current Position
X-Pos.: 1.000 mm
Y-Pos.: 1.000 mm

Virtual Range
X-Range: 3.000 mm
Y-Range: 2.800 mm

The symbol of the scan field **(1)** in the dialog window changes its size in relation to the parameters that are also physically determining the size of the scan field. These parameters are: objective magnification, beam divergence, scan format and electronic zoom. The intersection of the guide lines

(2) at the top left corner of the scan field serves as reference point for positioning the specimen stage. The coordinates of this reference point are displayed in the Current Position field.

Positioning the X/Y specimen stage
The X/Y specimen stage can be positioned using the Set Position button **(5)**, the mouse pointer, the arrow keys in the dialog window **(4)**, the arrow keys of the keyboard or the joystick.

Positioning via button

▶	Click the Set Position button **(5)**. A dialog window displays in which you can enter the x- and y-coordinate of the reference point with a precision of several decimal points.

Positioning via mouse pointer

▶	Click the scan field in the dialog window, hold the left mouse button pressed and drag the scan field to the desired position. The stage will not be moved until the mouse button is released.
▶	To ensure that the specimen stage is not moved until the desired position is reached, you can specify a delay time between releasing the mouse button and moving the specimen stage. Click the Settings button **(8)** and set the delay time on the Delay tab.

Positioning via arrow keys

▶	Operate the four arrow keys in the dialog window **(4)** or the arrow keys of the keyboard. ℹ *Positioning the specimen stage with the arrow keys on the keyboard moves the scan field exactly by the length of the side of the scan field so that a specimen, which is larger than the scan field, can seamlessly be scanned.*
▶	To ensure that the specimen stage is not moved until the desired position is reached, you can specify a delay time between pressing the keys and moving the specimen stage. Click the Settings button **(8)** and set the delay time on the Delay tab.

Positioning via joystick

▶	If you position the specimen stage using the joystick, the coordinates in the Current Position field are not updated. Click the Update Position button **(6)** to display the current position.

Adjusting the positioning speed
You can adjust the speed at which the specimen stage is moved, and the time available for this action.

▶	Click the Settings button **(8)** and define the minimum and maximum positioning speed on the Speed tab by dragging the slider or entering the corresponding values in the entry fields.
▶	The positioning time is defined in the Positioning Time entry field, i.e. the time in which the specimen stage must reach its position. Please note that a high speed requires a short positioning time and a low speed requires a long positioning time.

Setting the zero position

▶	Click the Set Zero Position button **(7)** to define the current position of the specimen stage (intersection of guide lines in the dialog window) as the zero position. This value is also changed in the Hardware legend.

Changing the unit of measure of the positioning range
The measurements at the sides of the positioning range of the specimen stage **(3)** change according to the selected unit of measure. This unit can be set to nanometer (nm), micrometer (µm), millimeter (mm), centimeter (cm) or meter (m).

▶	Click the Settings button **(8)** and select the unit of measure on the Unit tab.

Enlarging the display of the positioning range
To be able to exactly position the specimen stage in the micrometer range, the display of the scan field and the positioning range can be enlarged in the dialog window.

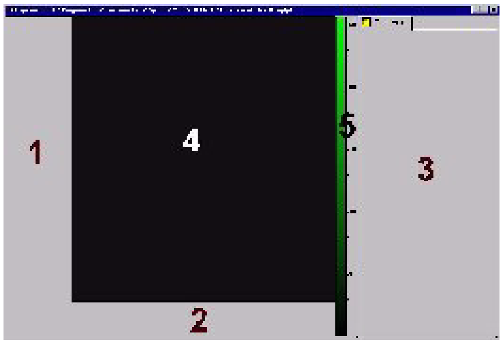
▶ Click the + and – buttons **(9)** and enlarge or reduce the display of the positioning range.

> *The position of the X/Y specimen stage can also be saved with recording parameter set. This allows the use of the X/Y specimen stage for recording sequences.*

& see Configuring Recording Sequences (page 131)

Data Viewing Functions

The Viewer Window



In the default setting, the Viewer window consists of three important areas. The image window (4), in which the recorded images are displayed, is located in the middle. Button pads (1) and (2) can be arranged to the left and below the image window and the Experiment legend (3) to the right of it. It is possible to select other default settings of the Viewer window and to save user-defined configurations as a template.

& see Saving Viewer Window Viewer as a Template (page 230)

Button pads (1) and (2)
By default, the button pad to the left of the image window contains the buttons for image viewing functions and the button pad below the image window contains the buttons used for scrolling through the individual images of an image series. Both button pads can be moved within the Viewer window or removed from it as separate windows. To do so, click on the double-lined edge of the button pad and drag it while keeping the mouse button depressed to the desired position.

Image window (4)
If you place the mouse pointer at any position within the image window and then press the right mouse button, a context menu containing the following commands opens:

Command	**Function**		
Send to	▸ Experiment	▸ Selection (raw)	This copies the raw data of a selected area in the image window as a new image in the current experiment. The new image can be processed.
		▸ Selection	This creates a new image in the current experiment

		(snapshot)	from the snapshot of a selected area in the image window. The new image can no longer be processed.
		▸ All (snapshot)	This creates a new image in the current experiment from the snapshot of the entire, current image window. The new image can no longer be processed.
	▸ Printer	▸ Selection	This prints the area selected in the image window.
		▸ All	This prints the entire, current image window.
Left buttons	This shows or hides the button pad to the left of the image window.		
Bottom buttons	This shows or hides the button pad located below the image window.		
LUT	This shows or hides the color look-up tables of the current image.		
Legend	This shows or hides the Experiment legend.		
Full screen	This enlarges the Viewer window to full screen.		
Viewer Options	This opens the Viewer Options dialog window.		

The **color look-up tables** **(5)** are displayed as color bars to the right of the image window. If you hold the mouse pointer over a color bar, grab points appear at the top and bottom of the color bar.You can use these grab points to limit the current color look-up table to a specific intensity range and to load a second color look-up table. This allows you to increase the contrast of the image graphically.

▶	Drag the top grab point down or the bottom grab point up.
▶	Double-click the area above or below the corresponding grab point.
▶	The Select LUT's dialog window opens so that you can select a second color look-up table.
▶	The upper and lower intensity ranges are represented in the colors of the second color look-up table.

& see Selecting Color Look-Up Tables (LUT) (page 159)

The Experiment Legend (3)

Various image parameters of an image recording are registered in the Experiment legend. To select the parameters that you want displayed, click any area of the legend. This opens a list of all available entries. Then select the desired entry. If you hold the mouse pointer over any area within the Experiment legend and press the right mouse button, a context menu containing the following commands opens:

Command	**Function**	
Experiment	▸ Edit	This opens the Edit Legend dialog window, where you can specify a (Title) for the legend, specify the (Number of legend entries) or (Clear all entries).
	▸ Activate	This displays the Experiment legend in the Viewer window.
	▸ Remove	This deletes the current Experiment legend.
Add Experiment tab	This creates a new Experiment legend in the Viewer window.	

The Hardware Legend

The hardware settings for image recording are registered in the Hardware legend. To open the legend, select the Hardware Legend option from the View menu. To select the entries that you want displayed in the legend, click the Edit button. This opens the Edit Legend Entries dialog window:

▶	The Available Entries list box shows all available entries. Select the entries that you want displayed in the legend. Then click Add to carry the selected entries over into the Show entries list box.
▶	The Show entries list box contains the entries that are to be displayed in the legend. Use the

	Remove button to remove entries from being displayed in the legend.
▶	Use the Move up and Move down buttons to shift one or more than one entry up or down in the list.
▶	You can use the Edit grid color and Edit background color buttons to modify the color of the frame and background of the legend.

The Hardware legend is aligned automatically along the right edge of the user interface. You can change however the size and position of the legend as desired:

▶	To change the width of the legend, use the mouse pointer to drag the edge to the position you desire.
▶	To change the position of the legend, double-click on the double-lined edge of the legend or click the ▲ icon once. This releases the legend from the user interface, turning it into a window. Now you can drag the legend to any position by pressing and holding the left mouse button.

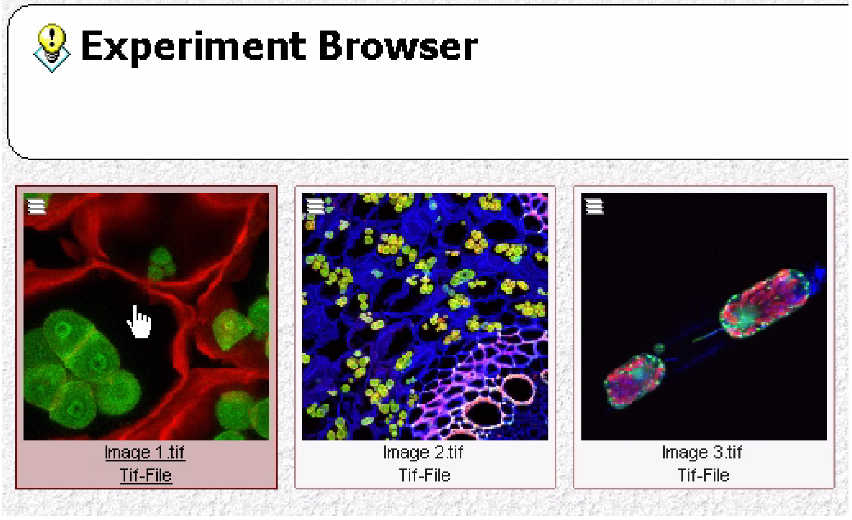
If the legend has been enlarged to cover the entire width of the user interface, you first have to shrink the height of it in order to drag it back to the edge of the user interface.

Option New Window
You can open a second Viewer window containing the same image as the currently open Viewer window by clicking the New Window option in the Window menu. This command does not create a new experiment, but rather opens a second view of the current image data. This feature is useful for viewing different representations of the same image data at the same time. Sequential numbers are added behind the file extension to distinguish between the resulting copies of the current Viewer window.

Displaying Recordings as Thumbnails in the Experiment Browser

Browse

Function
The Experiment Browser enables you to display all recordings of an experiment as thumbnails. This gives you an overview of the current image collection and quick access to individual recordings.



▶	Select an experiment in the Experiment Overview display window.
▶	Click the Browse button to open the Experiment Browser. All recordings of the experiment are displayed as thumbnails. Image series are displayed as maximum projection.
▶	Click a thumbnail to open the complete image data set in the Viewer window.
▶	Click the Browse button again to close the Experiment Browser.

Viewer Options Dialog Window

Viewer Options Dialog Window, 3D Icon

Function
To open the Viewer Options dialog window, select the Experiment Overview option in the View menu. The Experiment Overview display window appears on the left side of the user interface. The top part of this window displays the recorded images in a directory tree. The bottom part displays the Viewer Options dialog window. This dialog window is used to perform basic settings for different software functions. The left side shows the icons corresponding to the functions and the right side shows the related tabs. When you open the dialog window, it contains the icons of the functions that you are currently using. Click Show all to view all icons.

The tabs of the 3D icon can be used to perform optional settings for the following functions:

Rot Move Zoom

& see Rotating the 3D View (page 177)
& see Moving the 3D View (page 177)
& see Zooming the 3D View (page 178)

The **Navigation tab** displays the numerical values of the actions that are carried out using the Rotate, Move and Zoom buttons (and the mouse pointer).

Use the **Rotation** field to tilt a 3D view in all three spatial directions by modifying the angular degrees of the three axes. The 3D view is rotated around a fixed point, which is located in the center of the image. To best understand the rotation function, modify the angle of one axis at a time, while leaving the other two axes set to 0.

Rotation	Function
X from 0° to 45°	The 3D view is rotated 45° around the fixed point in the direction of the negative z-axis.
Y from 0° to 45°	The 3D view is rotated 45° around the fixed point in the direction of the negative y-axis.
Z from 0° to 45°	The 3D view is rotated 45° around the fixed point in the direction of the negative y-axis.

Use the **Translation field** to shift a 3D view to the right or left, up or down and enlarge or shrink it by modifying the coordinate values. (Users of all regional versions should be sure to use the dot as the decimal placeholder.)

Translation	Function
X	Positive values shift the 3D view to the right, negative to the left.
Y	Positive values enlarge the 3D view, negative shrink it.

Z	Positive values shift the 3D view up, negative down.

Use the **Predefined** field to change the viewing perspective of the image to Top view or Side view by clicking these buttons respectively.

In the **Display tab** click on one of the listed commands to show or hide the corresponding graphic element of the 3D view in the Viewer window:

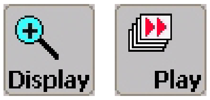
Graphic Elements	**Function**
Show LUT	Show the selected color look-up table on the z-axis.
Show scale	Show the scale.
Show bounding box	Show the bounding box, which limits the measurement volume.
Show axes	Show the coordinate axes.
Show data during 3D motion	Show the image data while the 3D view is being rotated, shifted, enlarged or shrunken.

Viewer Options Dialog Window, Display Icon

Function

To open the Viewer Options dialog window, select the Experiment Overview option in the View menu. The Experiment Overview display window appears on the left side of the user interface. The top part of this window displays the recorded images in a directory tree. The bottom part displays the Viewer Options dialog window. This dialog window is used to perform basic settings for different software functions. The left side shows the icons corresponding to the functions and the right side shows the related tabs. When you open the dialog window, it contains the icons of the functions that you are currently using. Click Show all to view all icons.

The tabs of the Display icon can be used to perform optional settings for the following functions:



& see Starting and Ending a Film (page 164)

On the **Settings tab**, use the graphic zoom to enlarge or reduce the image displayed in the Viewer window:

n-1	The image is reduced by displaying n pixel as 1 pixel in the resulting image.
Automatic	The image is displayed in the original image format.
1-n	The image is enlarged by displaying 1 pixel as n pixel in the resulting image.

ℹ

> *The Leica Confocal Software features three distinguishing zoom functions: graphic zoom, 3D zoom and electronic zoom.*

& see Zooming the 3D View (page 178)
& see Electronic Zoom (page 117)

Click on one of the selection points on the same tab to hide or display the respective graphic element in the Viewer window:

Coordinates	The z position of the current image is displayed.
Scale	A measurement bar with length indicators is displayed.
Grid	A grid is placed over the current image.

The length of the measurement bar and the grid width are calculated dependent on the objective, the electronic zoom and the beam divergence.

You can use the **Movie tab** to set the speed for running the film sequence of an image series. You can choose speeds from 6 images per minute to 25 images per second.

▶	Use the mouse to drag the slide on the scale to the desired value.
▶	Select the Ping-Pong mode if the film sequence is to run from the first to the last image and then in reverse order back to the first image. If this mode is not selected, the film sequence always starts with the first image again.

Viewer Options Dialog Window, Charts Icon

Function

To open the Viewer Options dialog window, select the Experiment Overview option in the View menu. The Experiment Overview display window appears on the left side of the user interface. The top part of this window displays the recorded images in a directory tree. The bottom part displays the Viewer Options dialog window. This dialog window is used to perform basic settings for different software functions. The left side shows the icons corresponding to the functions and the right side shows the related tabs. When you open the dialog window, it contains the icons of the functions that you are currently using. Click Show all to view all icons.

The tab of the Charts icon can be used to perform optional settings for the following functions:

Prof.	& see Measuring a Profile Along a Line Segment (page 214)
Pr. (z)	& see Measuring a Profile Within a Region of Interest (ROI) (page 213)
Mat.	& see Measuring Surfaces and Volumes (page 215)
R-Prof.	& see Measuring Roughness Along a Line Segment (page 231)

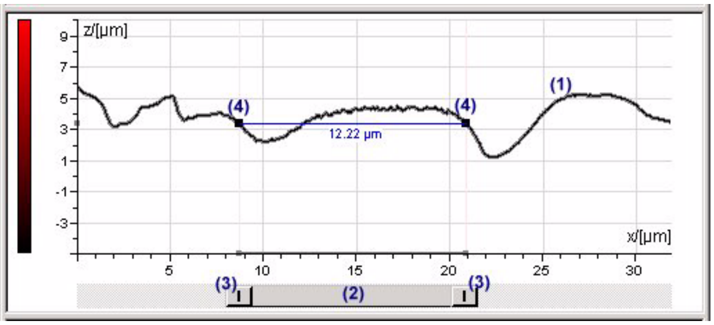
The options in **Charts tab** can only be selected if one of these buttons was used earlier to activate a quantification function.

Activating the two-point measurement

▶	Clink on 2 Point in the **Measurement field** to activate the two-point measurement.

Each of the buttons listed above opens a window that displays a measuring curve and statistical values.

If the two-point measurement is selected, the window shows a measurement slider (2) with two measurement points (3) below the measurement curve (1). The position of the two measurement points in the profile curve is symbolized by small black squares (4). This allows for very precise settings of the range to be evaluated within the measurement. In addition, the statistics that is displayed below the measurement curve is expanded by the following measurement values: position of measurement points, distance of measurement points, measurement values at the measurement points, difference of measured values between the measurement points.



Changing the position and length of the measurement slider

▶	To change the position, move the mouse over the slider, click and hold the left mouse button and drag it to the desired position.
▶	To change the length of the slider, grasp it at one end with the mouse and enlarge or reduce it while pressing the left mouse button.

Changing the scaling of the measurement curve

By default, the scaling of measurement curves is automatically adapted by the software to the intensity value range that is present in an image recording. However, the scaling can also be changed manually:

▶	Deselect the Auto check box to deactivate automatic scaling.
▶	You can now redefine the upper and lower limit value of the value range separately for each measurement curve (each detection channel).
▶	Check the results of the changed scaling directly in the window.

Viewer Options Dialog Window, Projections Icon

Function

To open the Viewer Options dialog window, select the Experiment Overview option in the View menu. The Experiment Overview display window appears on the left side of the user interface. The top part of this window displays the recorded images in a directory tree. The bottom part displays the Viewer Options dialog window. This dialog window is used to perform basic settings for different software functions. The left side shows the icons corresponding to the functions and the right side shows the related tabs. When you open the dialog window, it contains the icons of the functions that you are currently using. Click Show all to view all icons.

In the tabs of the Projections icon, you can make optional settings for the following functions:

fix Avg. fix Max. fix Trans. 3D SFP

& see Maximum Projection of an Image Stack with Invariable Projection Axis (page 169)
& see Average Projection of an Image Stack with Invariable Projection Axis (page 171)
& see Transparent Projection of an Image Stack with Invariable Projection Axis (page 173)
& see Creating an SFP Projection of an Image Stack (page 174)

The **Projections** tab contains various options for generating a projection image. Select whether the intensity maximums, the arithmetical average or a weighted average of the intensities are to be displayed in the projection image. Vary the weight factor for the transparent projection. Set a threshold value or scaling factor for the displayed intensities.

Type	**Function**
Maximum projection	From each of the examined columns of sampling points, the sampling point with the highest intensity is displayed in the projection image as the representative of all values within the column.
Average projection	From each of the examined columns of sampling points, the arithmetical average of the intensities measured in the column is displayed in the projection image.
Transparent projection	From each of the examined columns of sampling points, a weighted average of the intensities measured in the column is displayed in the projection image.

Transparent factor	**Function** (enabled only for transparent projection)
Drag the slide on the scale.	The higher the transparent factor α (0 < α < 1) setting, the stronger the intensity values flow from the lower levels in the image stack into the projection image.

Threshold	**Function** (enabled only for average and transparent projection)
Drag the slide on the scale.	Intensities that are below the specified threshold value are ignored in the calculation of the projection image.

Scaling	**Function** (enabled only for average and transparent projection)
Rescale to maximum	The measured intensity values are scaled to the maximum possible intensity values.

The **SFP tab** contains various options for generating an SFP projection image.

& see Principles and Types of Projections (page 166)

Field	**Function**
Light Direction	Here, enter a coordinate for x and y for the projection angle of the SFP projection. This angle symbolizes the angle of incidence of the laser beam onto the specimen. Click on the Apply button to create the projection with the new angle.
Absorption	The slider is used to vary the absorption coefficient α. The higher it is set, the smaller are the values calculated in the projection image. If the coefficient is set low, many of the pixels in the projection image reach the maximum value, making it increasingly difficult to distinguish between the structures.
Threshold	Use the slider to define a threshold value. The intensity values below this value are not taken into account for the creation of the projection image.
Rescale to maximum	The intensity values of the image are scaled to the maximum possible intensity values. This ensures that the calculated gray values of the projection image remain within the value range of, for example, 0 to 255 at 8 bits. This allows for brightening dark images.

Viewer Options Dialog Window, Overlay Icon

Structure of the Experiment Overview window
To open the Viewer Options dialog window, select the Experiment Overview option in the View menu. The Experiment Overview display window appears on the left side of the user interface. The top part of this window displays the recorded images in a directory tree. The bottom part displays the Viewer Options dialog window. This dialog window is used to perform basic settings for different software functions. The left side shows the icons corresponding to the functions and the right side shows the related tabs. When you open the dialog window, it contains the icons of the functions that you are currently using. Click Show all to view all icons.

The tab of the Overlay icon can be used to perform optional settings for the following functions:

Ovl Ovl rgb

& see Viewing an Overlay Image (page 162)

On the **Overlay tab** you can select among three types of creating an overlay image from raw data and the corresponding color look-up tables:

Color Merging	**Function**
True Color (add RGBs)	The color values of the pixels in the original images are averaged and displayed in the overlay image.
True Color (add RGBs) with dynamic adjustment	The color values of the pixels in the original images are averaged, scaled to the maximum possible color values and displayed in the overlay image.
Fast (bitwise "OR" RGB)	The color values of the pixels in the original images are mixed using a fast bit-by-bit calculation process and displayed in the overlay image. & see Boolean Operations (page 236)

Coloring	**Function**
Only Red/ Green/ Blue	The color look-up tables Red, Green and Blue are always used to create the overlay image, independent of the current color look-up tables of the original images.

Viewer Options Dialog Window, Scan Progress Icon

Function
To open the Viewer Options dialog window, select the Experiment Overview option in the View menu. The Experiment Overview display window appears on the left side of the user interface. The top part of this window displays the recorded images in a directory tree. The bottom part displays the Viewer Options dialog window. This dialog window is used to perform basic settings for different software functions. The left side shows the icons corresponding to the functions and the right side shows the related tabs. When you open the dialog window, it contains the icons of the functions that you are currently using. Click Show all to view all icons.

In the tab of the Scan Progress icon, you can make optional settings for the following functions:

Mode

& see Selecting a Scan Mode (page 121)

The currently set scan mode is shown in the **Scan Progress tab**. The status of an image recording can also be checked on the basis of the progress display.

Viewer Options Dialog Window, Surface View Icon

Function
To open the Viewer Options dialog window, select the Experiment Overview option in the View menu. The Experiment Overview display window appears on the left side of the user interface. The top part of this window displays the recorded images in a directory tree. The bottom part displays the Viewer Options dialog window. This dialog window is used to perform basic settings for different software functions. The left side shows the icons corresponding to the functions and the right side shows the related tabs. When you open the dialog window, it contains the icons of the functions that you are currently using. Click Show all to view all icons.

In the tab of the Surface View icon, you can make optional settings for the following functions:

3DView

& see Creating the 3D View (page 176)

In the **Visualization tab**, you can select whether the 3D view is to be generated as Surface, Wireframe or Isolines and specify the perspective in which the 3D view is displayed:

Render Mode	**Function**
Surface	The blank spaces between the pixels are filled in with surface.
Wireframe	All pixels are linked with lines, while the blank spaces remain free.
Isolines	Pixels that correspond to values of the same intensity are enclosed in a curve.

Projection Type	**Function**
Perspective	The 3D view is displayed in central perspective.
Parallel	The 3D view is displayed in parallel perspective.

The **Stretch height (factor) field** offers the ability to vary the scaling factor in the z-direction, thus stretching or shrinking the height of the 3D view.

Use the **Downsample rate field** to reduce the density of data of the 3D view to increase the speed of image processing. At a pixel density of 1:1, all measured intensity values are displayed in the image. At a pixel density of 1:2, only every second measured intensity value is used in the surface image.

Use the **Isoline interval field** to define a distance for separating the individual isolines in µm. This allows you to limit the number of isolines in the 3D view.

Use the **Isoline detail level field** to enter a limiting value, specifying that only isolines of a particular length are to be displayed in the 3D view. This limits the displayed isolines to those which correspond to an intensity value that has a a specific frequency of occurrence.

Viewer Options Dialog Window, Surface Measure Icon

Function

To open the Viewer Options dialog window, select the Experiment Overview option in the View menu. The Experiment Overview display window appears on the left side of the user interface. The top part of this window displays the recorded images in a directory tree. The bottom part displays the Viewer Options dialog window. This dialog window is used to perform basic settings for different software functions. The left side shows the icons corresponding to the functions and the right side shows the related tabs. When you open the dialog window, it contains the icons of the functions that you are currently using. Click Show all to view all icons.

In the tab of the Surface Measure icon, you can make optional settings for the following functions:

R-Prof.

& see Measuring Roughness Along a Line Segment (page 231)

In the **Leveling field**, there are options for an adaptation function to bring measurement curves of a roughness profile that show a trend into a horizontal position. For this purpose, a linear interpolation function (polynom) is fitted to the measurement curve.

& see Process Operating Step / Materials Folder (page)
& see Adaptation Function for Correcting Trend Curves

None	No adaptation function is applied to the measurement curve.
Automatic linear	The measurement curve is brought into a horizontal position using a linear adaptation function.
Interactive level	The position of the measurement curve is corrected using the linear adaptation function in such a way that the line segment between the two measurement points is on the horizontal plane.
Freeze	The newly positioned measurement curve is locked so that the item of the measurement points can be changed again without interactively modifying the measurement curve.

The **Multipoint Measurement field** provides the possibility of saving the two measurement points as well as the difference in height between the measurement points.

► Click on the Remember button to save the values. The Clear button deletes them again.

Viewer Options Dialog Window, Surface Calculation Icon

Function

To open the Viewer Options dialog window, select the Experiment Overview option in the View menu. The Experiment Overview display window appears on the left side of the user interface. The top part of this window displays the recorded images in a directory tree. The bottom part displays the Viewer Options dialog window. This dialog window is used to perform basic settings for different software functions. The left side shows the icons corresponding to the functions and the right side shows the related tabs. When you open the dialog window, it contains the icons of the functions that you are currently using. Click Show all to view all icons.

On the tab of the Surface Calculation icon, you can perform optional settings for the following functions:

Topo

& see Creating a Topographical Image

The **Topography tab** contains various options for generating a topographical image. Select whether the intensity maximums or centers of mass of the intensities are to be interpreted as height in the topographical image. Specify the type of visualization for height in the topographical image and set a threshold value for the displayed intensities.

Surface Reconstruction	**Function**
Search maximum intensity	From each of the examined columns of sampling points, the sampling point with the highest intensity is displayed in the topographical image as the representative of all values within the column.
Calculate center of mass of intensities	From each of the examined columns of sampling points, the center of mass of the surface, which is restricted by the curve of the measured intensity values, is displayed in the topographical image.

Topography Processing	**Function**
Invert height	The column of sampling points is examined in reversed direction. This reverses the height information. A negative of the topographical image is displayed.
Level	The horizontal position of a recording is corrected using a linear interpolation function (polynom). & see Process Operating Step / Materials Folder (page 202)

Threshold	**Function**
Drag the slider on the scale or enter a numerical value.	Intensities that are below the specified threshold value are ignored in the calculation of the topographical image.

Viewing the Detection Channels

Viewing Detection Channel 1

Ch 1

Function

Clicking the Channel 1 button displays the image data that has been recorded in detection channel 1 in the Viewer window. You can assign any of several other color look-up tables to the detection channel. This setting can be changed both in the result image and while the image is being recorded. To do so, open the Select Look-up Tables dialog window. There are two ways of opening the dialog window.

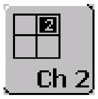
▶	Click the Select Look-up Tables button.
▶	Hold the mouse pointer over any position in the Viewer window. Click the right mouse button. Click on the LUT item in the context menu that appears. The color bars for the active detection channels appear in the Viewer window. Double-click the corresponding color bar.

& see Selecting Color Look-Up Tables (LUT) (page 159)

This method of assigning color look-up tables only influences the currently displayed image. As soon as you start a new scan, the color look-up tables configured in the Beam Path Setting dialog window are applied again.

& see Setting the Beam Path (page 111)

Viewing Detection Channel 2



Function

Clicking the Channel 2 button displays the image data that has been recorded in detection channel 2 in the Viewer window. You can assign any of several other color look-up tables to the detection channel. This setting can be changed both in the result image and while the image is being recorded. To do so, open the Select Look-up Tables dialog window. There are two ways of opening the dialog window:

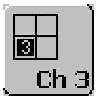
▶	Click the Select Look-up Tables button.
▶	Hold the mouse pointer over any position in the Viewer window. Click the right mouse button. Click on the LUT item in the context menu that appears. The color bars for the active detection channels appear in the Viewer window. Double-click the corresponding color bar.

& see Selecting Color Look-Up Tables (LUT) (page 159)

This method of assigning color look-up tables only influences the currently displayed image. As soon as you start a new scan, the color look-up tables configured in the Beam Path Setting dialog window are applied again.

& see Setting the Beam Path (page 111)

Viewing Detection Channel 3



Function

Clicking the Channel 3 button displays the image data that has been recorded in detection channel 3 in the Viewer window. You can assign any of several other color look-up tables to the detection channel. This setting can be changed both in the result image and while the image is being recorded. To do so, open the Select Look-up Tables dialog window. There are two ways of opening the dialog window:

▶	Click the Select Look-up Tables button.
▶	Hold the mouse pointer over any position in the Viewer window. Click the right mouse button. Click on the LUT item in the context menu that appears. The color bars for the active detection channels appear in the Viewer window. Double-click the corresponding color bar.

& see Selecting Color Look-Up Tables (LUT) (page 159)

ℹ

> *This method of assigning color look-up tables only influences the currently displayed image. As soon as you start a new scan, the color look-up tables configured in the Beam Path Setting dialog window are applied again.*

& see Setting the Beam Path (page 111)

Viewing Detection Channel 4

Ch 4

Function

Clicking the Channel 4 button displays the image data that has been recorded in detection channel 4 in the Viewer window. You can assign any of several other color look-up tables to the detection channel. This setting can be changed both in the result image and while the image is being recorded. To do so, open the Select Look-up Tables dialog window. There are two ways of opening the dialog window:

▶	Click the Select Look-up Tables button.
▶	Hold the mouse pointer over any position in the Viewer window. Click the right mouse button. Click on the LUT item in the context menu that appears. The color bars for the active detection channels appear in the Viewer window. Double-click the corresponding color bar.

& see Selecting Color Look-Up Tables (LUT) (page 159)

ℹ

> *This method of assigning color look-up tables only influences the currently displayed image. As soon as you start a new scan, the color look-up tables configured in the Beam Path Setting dialog window are applied again.*

& see Setting the Beam Path (page 111)

Viewing Detection Channel 5

Ch 5

Function

Clicking the Channel 5 button displays the image data that has been recorded in detection channel 5 in the Viewer window. You can assign any of several other color look-up tables to the detection channel. This setting can be changed both in the result image and while the image is being recorded. To do so, open the Select Look-up Tables dialog window. There are two ways of opening the dialog window:

▶	Click the Select Look-up Tables button.
▶	Hold the mouse pointer over any position in the Viewer window. Click the right mouse button. Click on the LUT item in the context menu that appears. The color bars for the active detection channels appear in the Viewer window. Double-click the corresponding color bar.

& see Selecting Color Look-Up Tables (LUT) (page 159)

This method of assigning color look-up tables only influences the currently displayed image. As soon as you start a new scan, the color look-up tables configured in the Beam Path Setting dialog window are applied again.

& see Setting the Beam Path (page 111)

Viewing Detection Channel 6

Ch 6

Function

Clicking the Channel 6 button displays the image data that has been recorded in detection channel 6 in the Viewer window. You can assign any of several other color look-up tables to the detection channel. This setting can be changed both in the result image and while the image is being recorded. To do so, open the Select Look-up Tables dialog window. There are two ways of opening the dialog window:

▶	Click the Select Look-up Tables button.
▶	Hold the mouse pointer over any position in the Viewer window. Click the right mouse button. Click on the LUT item in the context menu that appears. The color bars for the active detection channels appear in the Viewer window. Double-click the corresponding color bar.

& see Selecting Color Look-Up Tables (LUT) (page 159)

This method of assigning color look-up tables only influences the currently displayed

> *image. As soon as you start a new scan, the color look-up tables configured in the Beam Path Setting dialog window are applied again.*

& see Setting the Beam Path (page 111)

Viewing Detection Channel 7

Ch 7

Function

Clicking the Channel 7 button displays the image data that has been recorded in detection channel 7 in the Viewer window. You can assign any of several other color look-up tables to the detection channel. This setting can be changed both in the result image and while the image is being recorded. To do so, open the Select Look-up Tables dialog window. There are two ways of opening the dialog window:

▶	Click the Select Look-up Tables button.
▶	Hold the mouse pointer over any position in the Viewer window. Click the right mouse button. Click on the LUT item in the context menu that appears. The color bars for the active detection channels appear in the Viewer window. Double-click the corresponding color bar.

& see Selecting Color Look-Up Tables (LUT) (page 159)

> *This method of assigning color look-up tables only influences the currently displayed image. As soon as you start a new scan, the color look-up tables configured in the Beam Path Setting dialog window are applied again.*

& see Setting the Beam Path (page 111)

Viewing Detection Channel 8

Ch 8

Function

Clicking the Channel 8 button displays the image data that has been recorded in detection channel 8 in the Viewer window. You can assign any of several other color look-up tables to the detection channel. This setting can be changed both in the result image and while the image is being recorded. To do so, open the Select Look-up Tables dialog window. There are two ways of opening the dialog window:

▶	Click the Select Look-up Tables button.
▶	Hold the mouse pointer over any position in the Viewer window. Click the right mouse button. Click on the LUT item in the context menu that appears. The color bars for the active detection channels appear in the Viewer window. Double-click the corresponding color bar.

& see Selecting Color Look-Up Tables (LUT) (page 159)

> *This method of assigning color look-up tables only influences the currently displayed image. As soon as you start a new scan, the color look-up tables configured in the Beam Path Setting dialog window are applied again.*

& see Setting the Beam Path (page 111)

Zooming Image(s) in the Viewer Window

Function

Clicking the Display button opens a drop-down list that you can use to set the graphical zoom. This enlarges or reduces the image displayed in the Viewer window:

n-1	The image is reduced by displaying n pixel as 1 pixel in the resulting image.
Automatic	The image is displayed in the original image format.
1-n	The image is enlarged by displaying 1 pixel as n pixel in the resulting image.

> *The Leica Confocal Software features three distinguishing zoom functions: graphic zoom, 3D zoom and electronic zoom.*

& see Zooming the 3D View (page 178)
& see Electronic Zoom (page 117)

Selecting Color Maps (LUT)

Function

Use the Look-up Tables button to open a dialog window, which you can use to assign color look-up tables to the detection channels. The color look-up tables can be configured both in the result image and while the image is being recorded:

▶	Click the detection channel to which you want to assign a new color look-up table in the Select Channel field.
▶	Select the desired color look-up table in the Select LUT field.
▶	Click Apply to check the results in the Viewer window.

It is also possible to open the Select LUT's dialog window from the Viewer window.

▶	Hold the mouse pointer over any position in the Viewer window. Then click the right mouse button. Click on the LUT item in the context menu that appears.
▶	The color bars for the active detection channels appear in the Viewer window to the right of the image window. Double-click one of the color bars.

Loading a second color look-up table

If you hold the mouse pointer over a color bar, grab points appear at the top and bottom of the color bar. You can use these grab points to limit the current color look-up table to a specific intensity range and to load a second color look-up table:

▶	Drag the top grab point down or the bottom grab point up.
▶	Double-click the area above or below the corresponding grab point.
▶	The Select LUT's dialog window opens so that you can select a second color look-up table.
▶	The upper and lower intensity ranges are represented in the colors of the second color look-up table.

> *This method of assigning color look-up tables only influences the currently displayed image. As soon as you start a new scan, the color look-up tables configured in the Beam Path Setting dialog window are applied again.*

& see Setting the Beam Path (page 111)

Typical Applications

Generally the selection of a suitable color look-up table for a specific use depends on the user's own judgment and needs. Experience shows however that certain color look-up tables are particularly useful for specific uses.

Color Map	**Application**
Green	This is commonly used for recording specimens that have been marked with FITC, Cy2, DTAF or other similar fluorochromes, which emit within the green spectral range.
Red	This is commonly used for recording specimens that have been marked with TRITC, Texas Red, Cy3, Rodamin or other similar fluorochromes, which emit within the red spectral range.
Blue	This is commonly used for recording specimens that have been marked with UV fluorochromes such as DAPI or Hoechst or other similar fluorochromes, which emit within the blue spectral range.
Gray	This is commonly used for displaying transmission recordings.
P. Color 1	This is especially suited for displaying 12 bit images.
P. Color 2, 3, 4, 5, 6	This is commonly used for recording specimens that have been marked with pH-sensitive or ion-sensitive fluorochromes and for displaying time series.
Geo (Land), Geo (Sea) Geo (Land & Sea) R & B	These are recommended for generating topographical images, i.e. for mapping surface structures. Geo Land & Sea is especially useful for visualizing depths and graduations.
Glow, Glow (Over), Glow (Under), Glow (Over	These are recommended for optimizing image contrast (the offset and gain of the detectors). Glow Over highlights in blue intensities at the upper end of the table while Glow Under represents in green intensities at the lower end of the table. Glow Over and Under is a combination of these two color tables.

& Under)	
Cyan, Magenta, Yellow	These are recommended for printing images. The CMY(K) colors are generally used for the printer system color output. RGB colors such as the other color look-up tables used here are applied generally for color representation on monitors. Because these two color systems vary greatly, the colors in the CMY(K) representation can differ strongly from those used in the monitor representation.

All color look-up tables are also available with inverse color flow, meaning high intensities are represented as dark and low intensities as light.

Viewing the Single Image

Single

Function

Click the Single button to view only one detection channel or several detection channels in only one image in the Viewer window. The following display modes are available depending on the other options you enable simultaneously.

Button Combination	**Display**
Single + Channel 1 / 2 / 3 / 4 / 5	Only the selected detection channel is displayed; for an image series, the first image of the selected detection channel is displayed.
Single + Gallery + Channel 1 / 2 / 3 / 4 / 5	All individual images in an image series are displayed for the selected detection channel only.
Single + Overlay + Channel 1 / 2 / 3 / 4 / 5	A single overlay image is generated from all selected detection channels; for an image series, the first image of each selected detection channel is used.
Single + Gallery + Overlay + Channel 1 / 2 / 3 / 4 / 5	Based on the number of individual images in an image series, overlay images are generated from all selected detection channels.

> *The Single and Tiled buttons can not be enabled simultaneously because they carry out opposing functions.*

Viewing a Multiple Image

Tiled

Function

If you click the Tiled button, the detection channels are displayed separately in the Viewer window. The following display modes are available depending on the other options you enable simultaneously.

Button Combination	**Display**
Tiled + Channel 1 / 2 / 3 / 4 / 5	All selected detection channels are displayed separately; in the case of image series, the first image in each is applied.
Tiled + Gallery + Channel 1 / 2 / 3 / 4 / 5	All individual images in an image series are displayed for all selected detection channels.
Tiled + Overlay + Channel 1 / 2 / 3 / 4 / 5	All selected detection channels are displayed both separately and, in an overlay image, together. In the case of image series, the first

	image in each is applied.
Tiled + Gallery + Overlay + Channel 1 / 2 / 3 / 4 / 5	All individual images of an image series are displayed both separately for all selected detection channels and, in overlay images, together.

The Tiled and Single buttons can not be enabled simultaneously because they carry out opposing functions.

Viewing an Overlay Image

Function
Clicking the Overlay button displays all selected detection channels together in an overlay image in the Viewer window. In contrast to the Overlay rgb button, the detection channels are always displayed with currently specified color look-up tables. For this purpose, the color values of the pixels are averaged. The following display modes are available depending on the other options you enable simultaneously.

Button Combination	**Display**
Single + Overlay + Channel 1 / 2 / 3 / 4 / 5	A single overlay image is generated from all selected detection channels; for an image series, the first image of each selected detection channel is used.
Gallery + Single + Overlay + Channel 1 / 2 / 3 / 4 / 5	Based on the number of individual images in an image series, overlay images are generated from all selected detection channels.
Tiled + Overlay + Channel 1 / 2 / 3 / 4 / 5	All selected detection channels are shown separately as well as together in an overlay image. In an image series, the first image is used.
Tiled + Gallery + Overlay + Channel 1 / 2 / 3 / 4 / 5	All individual images of an image series are displayed both separately for all selected detection channels and, in overlay images, together.

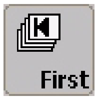
Use the Viewer Options dialog window to select from three different methods of color mixing for generating an overlay image:

▶	From the View menu, select Viewer Options.
▶	Click on the Overlay icon.

& see Viewer Options Dialog Window, Overlay Icon (page 151)

Viewing of Image Series

Viewing the First Image of a Series

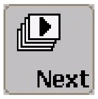


Function
You can view the individual images of an image series as a film sequence. Click First to jump to the first image in the series.

ℹ

> *If the Gallery button is enabled, the First and Last, Next and Previous and Play/Stop buttons are disabled and displayed in gray. The Gallery button is used to show all of the individual images of an image series and does not allow film sequence mode.*

Display the Next Image in a Series



Function
You can view the individual images of an image series as a film sequence. Click Next to jump to the next image in the series.

ℹ

> *If the Gallery button is enabled, the Next and Previous, First and Last and Play/Stop buttons are disabled and displayed in gray. The Gallery button is used to show all of the individual images of an image series and does not allow film sequence mode.*

Displaying the Previous Image in a Series

Function
You can view the individual images of an image series as a film sequence. Click Previous to jump to the previous image in the series.

ℹ

> *If the Gallery button is enabled, the Next and Previous, First and Last and Play/Stop buttons are disabled and displayed in gray. The Gallery button is used to show all of the individual images of an image series and does not allow film sequence mode.*

Viewing the Last Image of a Series

Last

Function
You can view the individual images of an image series as a film sequence. Click on the Last button to jump to the last image in the series.

If the Gallery button is enabled, the First and Last, Next and Previous and Play/Stop buttons are disabled and displayed in gray. The Gallery button is used to show all of the individual images of an image series and does not allow film sequence mode.

Displaying any Image From a Series

Sel

Function
When you click on the Selection button, a dialog window opens which lets you select any image of a series and display it in the Viewer window. The number of individual images for the series is displayed from 1 to n, as well as in an entry field the number of the currently displayed image.

▶	Use the mouse to move the slide of the scale. The corresponding image is displayed immediately.
▶	Enter the number of the desired image into the entry field, and click on Apply.

Starting and Ending a Film

Play

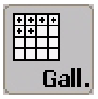
Function
You can view the individual, recorded images of an image series as a film sequence. Use the Play/Stop button to start and stop the film. The film speed, that is the number of single images per time unit, is variable and can be set in the Viewer Options dialog window:

▶	From the View menu, select Viewer Options.
▶	Click the Display icon and then on the Movie tab.

& see Viewer Options Dialog Window, Display Icon (page 147)

If the Gallery button is enabled, the Play/Stop, First and Last, Next and Previous buttons are disabled and displayed in gray. The Gallery button is used to show all of the individual images of an image series and does not allow film sequence mode.

Viewing the Series Image



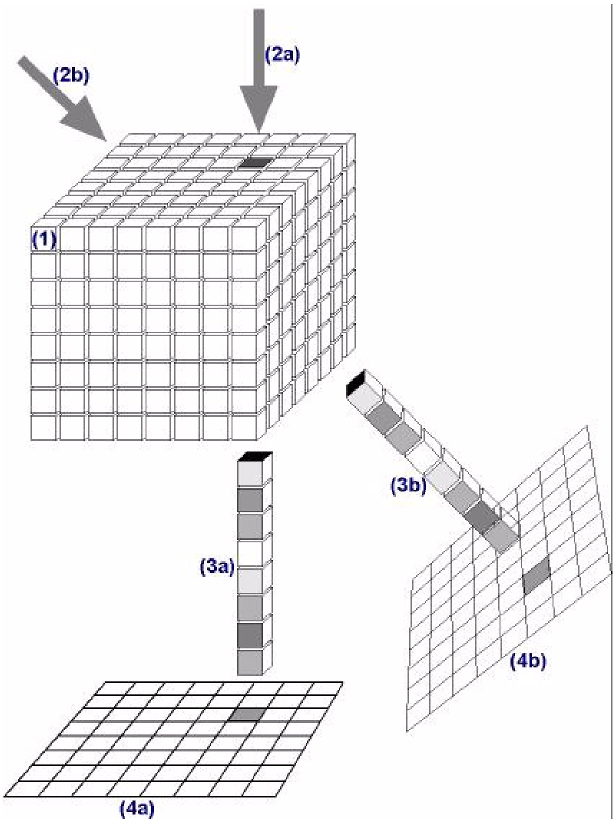
Function

If you click the Gallery button, all of the individual images in an image series are displayed in the Viewer window. The following display modes are available depending on the other options you enable simultaneously.

Button Combination	**Display**
Gallery + Single + Channel 1 / 2 / 3 / 4 / 5	All individual images in an image series are displayed for the selected detection channel only.
Gallery + Single + Overlay + Channel 1 / 2 / 3 / 4 / 5	Based on the number of individual images in an image series, overlay images are generated from all selected detection channels.
Gallery + Tiled + Channel 1 / 2 / 3 / 4 / 5	All individual images in an image series are displayed for all selected detection channels.
Gallery + Tiled + Overlay + Channel 1 / 2 / 3 / 4 / 5	All individual images of an image series are displayed both separately for all selected detection channels and, in overlay images, together.

Projections

Principles and Types of Projections



(2a) Projection with an invariable projection axis
(2b) Projection with a variable projection axis

Functional Principles of Projections
Biological specimens are recorded with a series of images. Each image is taken from a particular level in the specimen. The result is a three dimensional recording that contains the entire volume of a specimen. Each optical section (each individual image) depicts only the information from a single level in the specimen. Therefore, structures that extend throughout the entire specimen will be partially represented in several images. Projections are necessary to view these structures in their entirety. Using projection algorithms, it becomes possible to select the relevant information from many images and to depict this information in a single two dimensional image. This method can be used to reconstruct connected structures.

Accordingly, a series of images is the foundation of any projection. The series may consist of horizontal xy-sections or vertical xz-sections. In order to best understand the functionality of a projection, it is practical to think of an image series as a stack of individual images. In turn, each individual image consists of individual laser sampling points. Furthermore, the individual sampling points or Voxels (1), (the intensity values measured in each individual image) lie on top of each other. During creation of a projection, the sampling points of the individual images, which lie on top of each other along the projection axis (2a, 2b), are scanned through all of the optical sections. Then, either the intensity value that fulfills the selection criteria or another calculated value is depicted in a two-dimensional image (4a, 4b) as a representative of all intensity values within the column. This is repeated for each column of sampling points (3a, 3b).

Projections with invariable or variable projection axes
There are two main groups of projection types available in the Leica Confocal Software. These are distinguished by the type of projection axis. There are projections with invariable axes as well as projections with variable axes. The first type is orthogonal projections. The projection axis is always the optical axis that runs vertical to the stack of images (the z-axis with horizontal xy-cuts and the y-axis with vertical xz-cuts). In contrast, the projection axis for the second type may be freely selected. With the exception of the SFP projection, the same three projection types are available in both of the two main groups.

The Projection Types

Projection Type	Invariable Projection Axis	Variable Projection Axis
Maximum Projection	fix Max.	3D Max
Average Projection	fix Avg.	3D Avg.
Transparent Projection	fix Trans.	3D Trans.
SFP Projection	__________	3D SFP

Activate the projection type either with the corresponding button or in the Image Tool dialog window. If you activate the function using the button, the projection image will just be a temporary display on the monitor. When activated through the dialog window, the projection image will be stored as a new file in the current experiment:

▶	The projections with invariable projection axes are located in the Image Tool dialog window → 3D button → Orthogonal Projection icon.
▶	The projections with variable projection axes are located in the Image Tool dialog window → 3D button → Projections and Animations icon.
▶	The SFP projection is located in the Image Tool dialog window → 3D button → SFP icon.

Maximum Projection
During maximum projection, we assume that the maximum intensity values are the relevant information for the reconstruction of a structure. For this reason, the maximum value is found in each column of sampling points and is displayed in the two dimensional projection image as representative of the entire column:

$Ip = Max(Vn)$
whereby Ip is the pixel in the projection image and Vn is the investigated Voxel.

Average Projection
During average projection, every intensity value with equal weighting flows into the projection image. For this reason, during average projection the arithmetic average of all intensity values is calculated in each column of sampling points. This value is displayed in the two dimensional projection image as representative of the entire column:

$$Ip = \frac{\sum_{n=0}^{N-1} V_n}{N}$$

whereby IP is the pixel in the projection image, Vn
is the investigated Voxel, and N is the total number of Voxel.

Transparent Projection

During transparent projection, all intensity values should be included in the projection image as well. In contrast to average projection, the intensity values from the various individual images are weighted differently. The intensity values from the lower images in the image stack are weighted less than the intensity values from the upper images. For this reason, during transparent projection the weighted mean of all intensity values is calculated in each column of sampling points. This value is displayed in the two dimensional projection image as representative of the entire column:

$$I_0 = V_0$$

$$I_1 = (T_n \times \alpha) \times I_0 + V_1$$

$$I_2 = (T_n \times \alpha) \times I_1 + V_2$$

$$\ldots$$

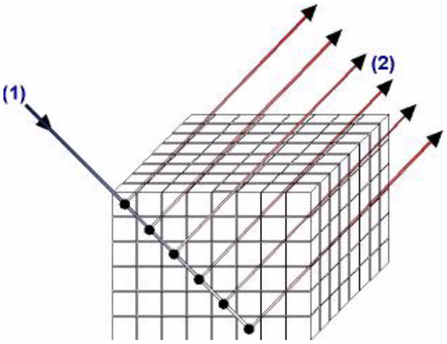
$$I_n = (T_n \times \alpha) \times I_{n-1} + V_n$$

whereby In is the pixel in the projection image, Tn = 1 - (Vn / Vmax) is the standardization, α is the variable transparent factor, In-1 is the value of the previously calculated Voxels, and Vn is the currently investigated Voxel

Calculation of the projection image begins with the lowest and ends with the highest individual image in the image stack. Weighting of the intensity values is based on two factors.
The first factor, Tn, is calculated from the relationship of the respective intensity value to the maximum possible intensity (standardization). This factor ensures that an image's gray value range from 0 to 255 at 8 bit, for example, will not be exceeded.
The second (user adjustable) transparent factor α ($0 < \alpha < 1$) determines the weighting of the previously measured intensity value. The higher the transparent factor is adjusted, the more the intensity values from the lower levels in the image stack will be included in the projection image.

SFP Projection (Simulated Fluorescence Process), mathematical simulation of a fluorescence process

This projection type uses arithmetic operations to simulate in a specimen the process of absorbing laser light and emitting fluorescent light. This procedure is common in fluorescence microscopy. It consists of two arithmetic steps.



The first step simulates the laser beam (1), which penetrates the sample and is thereby weakened. We assume a lateral light source striking the Voxel of the image stack. The weakening of the incoming beam while traversing the image stack is represented by the following expression:

$$I_{n+1} = I_n \left(1 - \alpha \cdot \frac{V_n}{V_{max}}\right)$$

whereby In+1 is the remaining intensity of the incoming light after absorption in the nth Voxel, In is the intensity of the incoming light in the nth Voxel, α is the absorption coefficient of the laser light in the sample, Vn is the intensity in the Voxel, and Vmax is the maximum Voxel intensity. The quotient Vn / Vmax corresponds to the standardization.

For each Voxel, the illumination strength VIn = In x Vn is calculated using the calculated intensities

(In). This information is temporarily saved in a table (In = intensity of the incoming light in Voxel n, Vn = intensity of the Voxel n). This calculation is completed in one step for the entire image stack (for all Voxel in the direction of the incoming beam). The result is a table of illumination strengths.

In the second step, the established illumination strengths are used to calculate the emitted fluorescence (2). The recursive formula is used to calculate the intensity of the pixel in the projection image as representative of the Voxel in the observer's viewing direction.

$$I_{n+1} = I_n (1 - \alpha \cdot \frac{V_n}{V_{max}}) + VI_n$$

In addition, α is the absorption coefficient of the fluorescent light in the sample and VIn is the illumination strength of the nth Voxel, calculated in the first step.

Maximum Projection of an Image Stack with Invariable Projection Axis

fix Max.

Function

Using the button fix Max. button starts a maximum projection. For this function, the projection axis is always the orthogonal axis (z-axis for horizontal xy-sections, and y-axis for vertical xz-sections).

The basis for a projection is an image stack, i.e. a series of horizontal xy-sections or vertical xz-sections. When a projection is generated, the sampling points of the individual images—superimposed along the projection axis—are examined throughout all optical sections. From each of these columns of sampling points, the maximum intensity value is displayed in the two-dimensional projection image as the representative of all intensity values within the column.

& see Principles and Types of Projections (page 166)

▶	Click the fix Max. button. A maximum projection of the current image stack is created and displayed in the Viewer window.
▶	You can return to the original image anytime by clicking on the Original button.

In the Viewer Options dialog window, you can also adjust a threshold and scale the intensity value range present in the image.

& see Viewer Options Dialog Window, Projections Icon (page 149)

> *A maximum projection can be activated using either the fix Max. or the Process operating step / Extended Focus folder. If you activate the function using the button, the projection image will just be a temporary display on the monitor.*

& see Process Operating Step / Materials Folder (page 195)
& see Creating an Experiment (page 230)

> *Note the difference between a maximum projection and a topographical image, which is generated from intensity maximums. In a maximum projection, the intensity maximums*

> *are assigned color values directly. In a topographical image based on intensity maximums, the intensity maximums are assigned real z-positions of the individual sampling points first and then are color-coded.*

& see Creating a Topographical Image (page 175)

Maximum Projection of an Image Stack with Variable Projection Axis (Optional)

Function

The 3D Max. button starts a maximum projection. The projection axis is freely variable.
The basis for a projection is an image stack, i.e. a series of horizontal xy-sections or vertical xz-sections. When a projection is generated, the sampling points of the individual images—superimposed along the projection axis—are examined throughout all optical sections. From each of these columns of sampling points, the maximum intensity value is displayed in the two-dimensional projection image as the representative of all intensity values within the column.

& see Principles and Types of Projections (page 166)

▶	Click on the 3D Max. button. A maximum projection of the current image stack is created and displayed in the Viewer window.
▶	You can now change the projection axis by clicking in the image in the Viewer window and holding the left mouse button pressed.
▶	Broken support lines are displayed that enclose the symbolically displayed image stack. Turn the image stack to the desired new position by moving the mouse pointer while holding down the left mouse button.
▶	If you release the left mouse button, a new maximum projection is calculated and displayed. The projection axis is the viewing direction of the user to the newly positioned image stack.
▶	Use the Zoom button to enlarge or reduce the projection. & see Zooming the 3D View (page 178)
▶	You can return to the original image anytime by clicking on the Original button.

In the Viewer Options dialog window, you can also adjust a threshold and scale the intensity value range present in the image.

& see Viewer Options Dialog Window, Projections Icon (page 149)

> *A maximum projection can be activated using either the 3D Max. button or the Process operating step / 3D Visualization and Animation folder. If you activate the function using the button, the projection image will just be a temporary display on the monitor.*

& see Process Operating Step / 3D Visualization and Animation Folder (page 179)
& see Creating an Experiment (page 230)

> *Note the difference between a maximum projection and a topographical image, which is generated from intensity maximums. In a maximum projection, the intensity maximums are assigned color values directly. In a topographical image based on intensity maximums, the intensity maximums are assigned real z-positions of the individual sampling points first and then are color-coded.*

& see Creating a Topographical Image (page 175)

Average Projection of an Image Stack with Invariable Projection Axis

Function

Using the fix Avg. button starts an average projection. For this function, the projection axis is always the orthogonal axis (z-axis for horizontal xy-sections, and y-axis for vertical xz-sections).

The basis for a projection is an image stack, i.e. a series of horizontal xy-sections or vertical xz-sections. When a projection is generated, the sampling points of the individual images—superimposed along the projection axis—are examined throughout all optical sections. From each of these columns of sampling points, the arithmetic average is calculated from all intensity values and displayed in the two-dimensional projection image as the representative of the complete column.

& see Principles and Types of Projections (page 166)

▶	Click the fix Avg. button. An average projection of the current image stack is created and displayed in the Viewer window.
▶	You can return to the original image anytime by clicking on the Original button.

In the Viewer Options dialog window, you can also adjust a threshold and scale the intensity value range present in the image.

& see Viewer Options Dialog Window, Projections Icon (page 149)

> *An average projection can be activated either by using the fix Avg. button or the Process operating step / Extended Focus folder. If you activate the function using the button, the projection image will just be a temporary display on the monitor.*

& see Process Operating Step / Materials Folder (page 195)
& see Creating an Experiment (page 230)

Average Projection of an Image Stack with Variable Projection Axis (Optional)

3D Avg.

Function

Use the 3D Avg. button to start an average projection. The projection axis is freely variable. The basis for a projection is an image stack, i.e. a series of horizontal xy-sections or vertical xz-sections. When a projection is generated, the sampling points of the individual images—superimposed along the projection axis—are examined throughout all optical sections. From each of these columns of sampling points, the arithmetic average is calculated from all intensity values and displayed in the two-dimensional projection image as the representative of the complete column.

& see Principles and Types of Projections (page 166)

▶	Click on the 3D Avg. button to create an average projection of the current stack of images. The projection is displayed in the Viewer window.
▶	You can now change the projection axis by clicking in the image in the Viewer window and holding the left mouse button pressed.
▶	Broken support lines are displayed that enclose the symbolically displayed image stack. Turn the image stack to the desired new position by moving the mouse pointer while holding down the left mouse button.
▶	After you release the left mouse button, the computer calculates and displays the new average projection. The projection axis is the viewing direction of the user to the newly positioned image stack.
▶	Use the Zoom button to enlarge or reduce the projection. & see Zooming the 3D View (page 178)
▶	You can return to the original image anytime by clicking on the Original button.

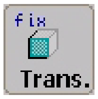
In the Viewer Options dialog window, you can also adjust a threshold and scale the intensity value range present in the image.

& see Viewer Options Dialog Window, Projections Icon (page 149)

An average projection can be activated using either the 3D Avg. button or the Process operating step / 3D Visualization and Animation folder. If you activate the function using the button, the projection image will just be a temporary display on the monitor.

& see Process Operating Step / 3D Visualization and Animation Folder (page 179)
& see Creating an Experiment (page 230)

Transparent Projection of an Image Stack with Invariable Projection Axis



Function
Use the fix Trans. button to start the transparent projection. For this function, the projection axis is always the orthogonal axis (z-axis for horizontal xy-sections, and y-axis for vertical xz-sections).

The basis for a projection is an image stack, i.e. a series of horizontal xy-sections or vertical xz-sections. When a projection is generated, the sampling points of the individual images—superimposed along the projection axis—are examined throughout all optical sections. From each of these sampling point columns, a weighted average is calculated from all intensity values, which is then displayed in the two-dimensional projection image as a representative of the entire column.

& see Principles and Types of Projections (page 166)

The user can vary the weighting of the sampling points to calculate the averages by setting the corresponding factors (transparent factor) in the Viewer Options dialog window. Furthermore, you can indicate a threshold and scale the intensity value range for the image.

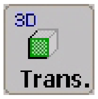
& see Viewer Options Dialog Window, Projections Icon (page 149)

▶	Click the fix Trans. button. A transparent projection of the current image stack is created and displayed in the Viewer window.
▶	You can return to the original image anytime by clicking on the Original button.

> *You can activate a transparent projection using the fix Trans. button or the Process operating step / Extended Focus folder. If you activate the function using the button, the projection image will just be a temporary display on the monitor.*

& see Process Operating Step / Materials Folder (page 195)
& see Creating an Experiment (page 230)

Transparent Projection of an Image Stack with Variable Projection Axis (Optional)



Function
You can activate a transparent projection using the 3D Trans. button. The projection axis is freely variable.
The basis for a projection is an image stack, i.e. a series of horizontal xy-sections or vertical xz-sections. When a projection is generated, the sampling points of the individual images—superimposed along the projection axis—are examined throughout all optical sections. From each of these sampling point columns, a weighted average is calculated from all intensity values, which is then displayed in the two-dimensional projection image as a representative of the entire column.

& see Principles and Types of Projections (page 166)

The user can vary the weighting of the sampling points to calculate the averages by setting the corresponding factors (transparent factor) in the Viewer Options dialog window. Furthermore, you can indicate a threshold and scale the intensity value range for the image.

& see Viewer Options Dialog Window, Projections Icon (page 149)

▶	Click on the 3D Trans. button. A transparent projection of the current image stack is created and displayed in the Viewer window.
▶	You can now change the projection axis by clicking in the image in the Viewer window and holding the left mouse button pressed.
▶	Broken support lines are displayed that enclose the symbolically displayed image stack. Turn the image stack to the desired new position by moving the mouse pointer while holding down the left mouse button.
▶	Upon releasing the left mouse button, the new transparent projection will be calculated and displayed. The projection axis is the viewing direction of the user to the newly positioned image stack.
▶	Use the Zoom button to enlarge or reduce the projection. & see Zooming the 3D View (page 178)
▶	You can return to the original image anytime by clicking on the Original button.

You can activate a transparent projection using the 3D Trans. button or the Process operating step / 3D Visualization and Animation folder. If you activate the function using the button, the projection image will just be a temporary display on the monitor.

& see Process Operating Step / 3D Visualization and Animation button (page 179)
& see Creating an Experiment (page 230)

Creating SFP Projection of an Image Stack (Optional)

Function

The SFP button starts an SFP projection, the mathematical simulation of a fluorescence process. The basis for a projection is an image stack, i.e. a series of horizontal xy-sections or vertical xz-sections. When a projection is generated, the sampling points of the individual images—superimposed along the projection axis—are examined throughout all optical sections. In the first step, the laser beam is simulated penetrating the sample and diminishing. In the second step, using the determined light density, the simulated fluorescence for each Voxel is calculated.

& see Principles and Types of Projections (page 166)

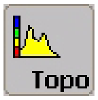
▶	Click the SFP button. A SFP projection of the current image stack is created and displayed in the Viewer window.
▶	You can now change the projection axis by clicking in the image in the Viewer window and holding the left mouse button pressed.
▶	Broken support lines are displayed that enclose the symbolically displayed image stack. Turn the image stack to the desired new position by moving the mouse pointer while holding down the left mouse button.

▶	Upon releasing the left mouse button, the new SFP projection will be calculated and displayed. The projection axis is the viewing direction of the user to the newly positioned image stack.
▶	You can return to the original image anytime by clicking on the Original button.

> *The SFP projection can be activated using the SFP button and the Process operating step / 3D Visualization and Animation folder. If you activate the function using the button, the projection image will just be a temporary display on the monitor.*

& see Process Operating Step / 3D Visualization and Animation Folder (page 179)
& see Creating an Experiment (page 230)

Creating a Topographical Image



Function

Use the Topography function to select specific intensity data from an image stack, i.e. a series of xy or xz-sections, and transfer them into a two-dimensional topographical image. This function examines the sampling points (voxels) that are superimposed along the z-axis throughout all optical sections. From each of these columns of sampling points, only the intensity value that is met by the selection criterion is displayed in the two-dimensional topographical image as the representative of all values within the column.

You can make your selection based on either the maximum intensity or the center of mass of the measured intensities. If you want to represent the intensity maximums in the topographical image, only the sampling point at which the maximum intensity was measured is selected. When determining the center of mass, a mean value is calculated from all superimposed sampling points (the center of mass of the area that is limited by the curve of the measured intensity values).

The intensity maximum or center of mass is then assigned the real z position of the corresponding sampling point and then color-coded. Based on this assignment, a topographical image maps **the real surface structure** of the specimen. Using the default settings, higher structures appear light and deeper structures dark.
Use the Viewer Options dialog window to set the selection criterion for the topographical image:

▶	From the View menu, select Viewer Options.
▶	Click on the Surface Calculation icon to view the Topography tab.

& see Viewer Options Dialog Window, Surface Calculation Icon
(page 153)

By applying a second step, you can display the topographical image as a three-dimensional graphical image using the 3D View function.

& see Creating the 3D View (page 176)

Typical Applications

Displaying the image data in a topographical image is especially informative in material scientific research. For the application of quantification functions, the topography function is indispensable.

Viewing the Original Image

Function
Click the Original button to undo a projection image or topographical image. The Viewer window then displays the recorded raw data of the image again.

Creating 3D Views

Creating the 3D View

Function
Use the 3D View button to display a two-dimensional data record three-dimensionally. When viewing an image series, the data record of the series that is currently being viewed in the Viewer window is always the one applied. You can display a single xy-section or xz-section from the raw data or a result image, such as a topographical image or projection image, in the 3D view. Depending on the dimension represented or calculated in the output image, either intensity values or height values are mapped to the z-axis of the 3D view when the 3D view is generated.

The spatial representation of intensity in a 3D view leads easily to the assumption that the topography of the specimen is being represented. Note however that you can only display ***the real surface structure*** *of a specimen if you have generated a topographical image first.*

& see Creating a Topographical Image (page 175)

The 3D view can be generated in three different modes: surface, wireframe or isolines. Set the display mode in the Viewer Options dialog window:

▶	From the View menu, select Viewer Options.
▶	Click the Surface View icon and then click the Visualization tab.

& see Viewer Options Dialog Window, Surface View Icon (page 152)

A separate 3D view is created for each detection channel. Therefore it is not possible to generate a single 3D view from one overlay image (Overlay button).

Rotating the 3D View

Rot

Function
Use the Rotate button to rotate a 3D view in all three spatial directions. Note that the 3D view is rotated around a fixed point, which is located in the center of the image.

▶	Hold the mouse pointer over any position in the 3D view.
▶	Press and hold the left mouse button.
▶	Move the mouse pointer in the direction in which you want to rotate the 3D view.

While the 3D view is being turned, the resolution of the image is decreased in order to speed up processing. When the left mouse button is released, the image is displayed with its original resolution.

As an alternative to rotating a 3D view manually, the Viewer Options dialog window also provides the option of specifying the angles of rotation for the three spatial axes:

▶	From the View menu, select Viewer Options.
▶	Click the 3D icon and then click the Navigation tab.
▶	You can then specify the angles of rotation for all three axes in the Rotation field.

& see Viewer Options Dialog Window, 3D Icon (page 146)

Moving the 3D View

Move

Function
Use the Move button to shift a 3D view:

▶	Hold the mouse pointer over any position in the 3D view.
▶	Press and hold the left mouse button.
▶	Move the mouse pointer in the direction in which you want to move the 3D view.

While the 3D view is being moved, the resolution of the image is decreased in order to speed up processing. When the left mouse button is released, the image is displayed with its original resolution.

As an alternative to moving a 3D view manually, the Viewer Options dialog window also provides the option of specifying the coordinate values for the position of the image:

▶	From the View menu, select Viewer Options.

▶	Click the 3D icon and then click the Navigation tab.
▶	Modify the coordinate values for the x-axis and z-axis in the Translation field.

& see Viewer Options Dialog Window, 3D Icon (page 146)

Zooming the 3D View

Function

Use the Zoom button to magnify and shrink a 3D view steplessly while maintaining the aspect ratio. This function only adjusts the scale of the generated image. It can not be used to improve its resolution.

To zoom in on the 3D view:	Click anywhere inside the image window of the Viewer window and, while pressing the left mouse button, drag the mouse pointer **toward the lower edge**.
To zoom out of the 3D view:	Click anywhere inside the image window of the Viewer window and, while pressing the left mouse button, drag the mouse pointer **toward the upper edge**.

> *While the 3D view is being zoomed, the resolution of the image is decreased in order to speed up processing. When the left mouse button is released, the image is displayed with its original resolution.*

As an alternative to zooming a 3D view manually, the Viewer Options dialog window also allows you to change the zoom scale by specifying numerical values:

▶	From the View menu, select Viewer Options.
▶	Click the 3D icon.
▶	You can then vary the zoom factor in the Navigation tab by changing the y value in the Translation field.

& see Viewer Options Dialog Window, 3D Icon (page 146)

> *The Leica Confocal Software features three distinguishing zoom functions: 3D zoom, electronic zoom and graphic zoom.*

& see Electronic Zoom (page 117)
& see Viewer Options Dialog Window, Display Icon (page 147)

Data Processing

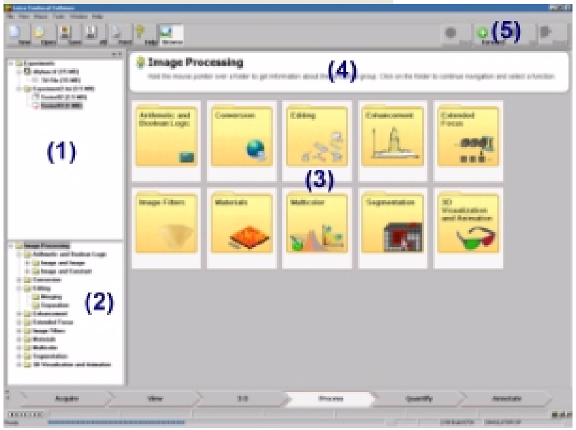
3D Projections and Animations of Image Series (Optional)

3D
Visualization
and Animation

This chapter contains the following topics

- Setup and operation of the Process operating step
- Navigation: 3D Visualization and Animation\ Projections
    - Navigation: 3D Visualization and Animation\ Projections with Animation
- Navigation: 3D Visualization and Animation\ Stereo
- Navigation: 3D Visualization and Animation\ Stereo with Animation

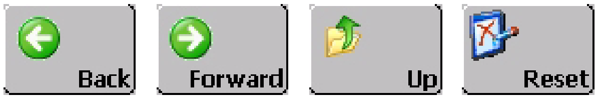
Setup and operation of the Process operating step



After clicking the Process arrow symbol, the usual setup of the user interface is completely changed. All function buttons of the other button areas/operating steps are hidden. The Experiment Overview **(1)** display window is shown on the left with the opened experiments. The navigation window **(2)** with the directory tree of all functions available in this operating step is located underneath it. The middle of the user interface displays 10 large tiles **(3)**, which comprise the 10 function groups. A help field **(4)** is located above these tiles, which can display a brief explanation of a function group by holding the mouse pointer over the corresponding tile.

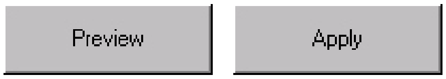
▶	Click the tile with the desired function group. On every navigational level, you can display an explanation of the function groups in the top help field by holding the mouse pointer over the corresponding tile.
▶	Select one tile each on the following navigational levels until you have reached the desired function with the corresponding dialog on the lowest level.
▶	You can directly select a function in the navigation window by clicking the corresponding folder.

The menu bar at the top right offers the following buttons **(5)**:



▶	Click Back to return to the previously displayed navigational level.
▶	Click Forward to display the level which you selected prior to clicking the Back button.
▶	Click Up to enter the next higher level in the hierarchy.
▶	Click Reset to return modified variables to their last setting.
▶	To exit the Process operating step, click the arrow symbol of another operating step (e.g. Acquire).

The bottom of every dialog displays the following two buttons:



▶	Click Preview to execute the selected function and to display the resulting image on the right in the preview window.
▶	Click Apply to apply the selected function to the current image data set, to display the resulting image in the Viewer display window and to create a new file in the current experiment.

Navigation: 3D Visualization and Animation\ Projections
Creating Projection Images Along a Definable Projection Axis

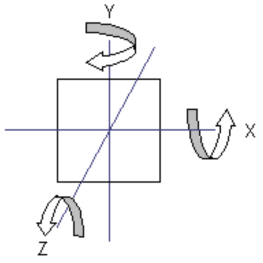
& see Principles and Types of Projections (page 166)

▶	In the Experiment Overview display window, select the image data set to be processed by double-clicking the file.
▶	Select one of the four projection types: Average Projection, Maximum Projection, Transparent Projection, SFP Projection (SFP Shadow Projection).

Options tab

▶	Click on Rescale in the Options field to standardize the intensity values of the image to the maximum possible intensity values when creating the projection image. This ensures that the calculated gray values of the projection image remain within the value range of, for example, 0 to 255 at 8 bits. This allows for brightening dark images.
▶	If you click on Invert in the Options tab, the projection axis is mirrored. The calculation of the projection image is thus in the opposite direction. This function makes sense above all in the case of inverse microscopes to match the calculation of the projection image to the inverse beam path of laser light.
▶	Move the slider in the Threshold field to define a threshold value. The intensity values below this value are not taken into account for the creation of the projection image.

Rotation tab

▶	In the Viewing Direction field, set the desired projection axis by entering a value for Z, Y and X. With these three values, you rotate the image stack into a certain position and thus define the angle at which the image stack is projected.
	The projection axis here is the viewing angle of the person viewing the rotated image stack. The scheme below illustrates the rotation direction of each image level; in the case of positive values for Z, Y and X. In the case of negative values, the rotation direction is exactly the other way around: 

▶	In the Depth Factor field, enter a value to scale the z-axis of the projection images.

Factor tab (only for transparent projection)

& see Principles and Types of Projections (page 166)

▶	The Factor tab is only displayed if the transparent projection was selected. Use the slider to vary the transparent factor α. The higher it is set, the stronger the intensity values flow from the lower levels in the image stack into the projection image.
▶	Click the Transparency Gallery tile to view a gallery of preview images of the selected image data set. Each of these preview images was calculated using a different transparency factor. Click a tile to display a dialog window with the corresponding settings.

Light tab (only for SFP projection)

& see Principles and Types of Projections (page 166)

▶	The Light tab is only displayed if the SFP projection was selected. In the Light Direction field, you define the projection angle for the first computing step of the SFP projection. This angle symbolizes the angle of incidence of the laser beam onto the specimen. Enter one coordinate each for x and y.
▶	Move the slider in the Absorption field to vary the absorption coefficient α. The higher it is set, the smaller are the values calculated in the projection image.If the coefficient is set low, many of the pixels in the projection image reach the maximum value, making it increasingly difficult to distinguish between the structures.
▶	If you click on Calc. Shadow in the Options field on the Options tab, a cast shadow is calculated for the SFP projection image and displayed.
▶	Click the SFP Light Gallery tile to view a gallery of preview images of the selected image data set. Each of these preview images was calculated using a different projection angle. Click a tile to display a dialog window with the corresponding settings.
▶	Click the SFP Absorption Gallery tile to view a gallery of preview images of the selected image data set. Each of these preview images was calculated using a different absorption coefficient. Click a tile to display a dialog window with the corresponding settings.

Navigation: 3D Visualization and Animation\ Projections and Animation
Running projection images as three-dimensional rotation animation

& see Principles and Types of Projections (page 166)

Animation tab

▶	To make settings for the rotation animation, click on the Animation check box. The corresponding fields are enabled.
▶	In the Rotation field, select the animation type: Full turn or 360°, half turn or 180°, quarter turn or 90°, or any other angle.
▶	In the Frames field, enter the number of projection images to be created for the animation. The number of projection images and the animation type specify the intervals at which the projection images are created. With a half turn (angle of 180°) and a number of images of, for example, 10, an image is created in steps of 20° (not 18°), as there are only 9 steps for 10 recorded images.
	ℹ *There is another calculation in the case of the full turn (angle of 360°). To prevent the first and last projection image from being recorded under the same angle (360° = 0°), the function calculates one recorded image more, but without creating the corresponding projection image. In the case of 10 images, for example, the interval between the projection images is 36° (not 40°),*

	as 11 recorded images (i.e. 10 steps) are used in the calculation.However, only 10 projection images are created.
▶	In the Rotation Axis field, vary the inclination of the rotation axis.
▶	To start the rotation animation, click on the Movie button. & see Starting and Ending a Film (page 164)

ℹ

> *The three projection types can also be enabled directly using the corresponding buttons (3D Proj / 3D Max / 3D Avg / 3D Trans). If you activate the function using the button, the projection image will just be a temporary display on the monitor. You can undo this using the Original button.*

& see Maximum Projection of an Image Stack with Variable Projection Axis (page 170)
& see Average Projection of an Image Stack with Variable Projection Axis (page 172)
& see Transparent Projection of an Image Stack with Variable Projection Axis (page 173)

Navigation: 3D Visualization and Animation\ Stereo
Navigation: 3D Visualization and Animation\ Stereo with Animation

Creating Red-Green Stereo Images (Anaglyphic Images)

Stereo View Options Field

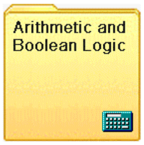
▶	In the Eye Angle list box determine the angle under which the two stereo images are recorded. For example, if you enter the value 30, a stereo image is recorded at an angle of 15° each left and right from the projection axis that was set on the Rotation tab.
▶	Stereo images can only be created from one detection channel. If you have an image series that was recorded in several detection channels, select the desired image data set in the Detection Channel list box.
▶	To display both stereo images in one image in the Viewer window, the buttons Channel 1, Channel 2, Single and Overlay must be pressed. & see Viewing Detection Channel 1 (page 154) & see Viewing Detection Channel 2 (page 155) & see a Viewing a Single Image (page 161) & see Viewing an Overlay Image (page 162)

Additional Information

Anaglyphic images create a stereoscopic sense of depth if they are viewed with corresponding 3D glasses (red-green stereo glasses). The stereoscopic effect of the anaglyphic image is possible since human beings use both eyes for viewing. Since the eyes are approximately 6.5 cm (2.56 inch) apart, the brain receives two images from different perspectives. Objects located at different distances are observed at different angles. This angular difference is a measure of distance for the human brain. The difference between the similar images generated by the two eyes is processed by the brain into one image with spatial depth impression. These image differences can also be simulated by holding a slightly different image in front of each eye.

In anaglyphic images, the complete stereoscopic image information is located on one image. The information of the left and right image is coded through colors. For this reason, glasses with a different color filter for each eye are needed in viewing these images. This filter separates the image information again for each eye. The red filter only passes red light, while completely absorbing the green light. On the other hand, the green filter absorbs the red light. Each eye perceives one image; both are merged in the brain into an overall spatial image.

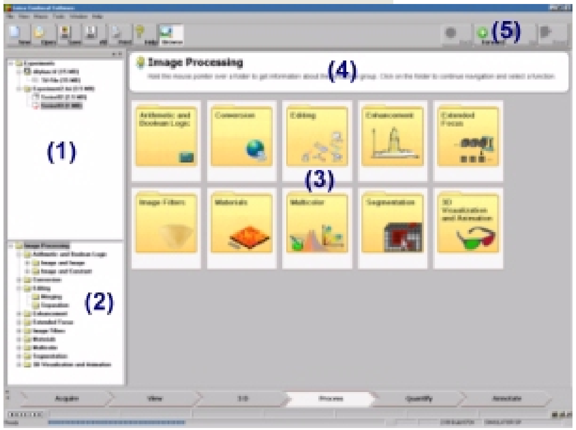
Arithmetic and Boolean Operations



This chapter contains the following topics

- Setup and operation of the Process operating step
- Navigation: Arithmetic and Boolean Logic\ Image and Image
- Navigation: Arithmetic and Boolean Logic\ Image and Constant

Setup and operation of the Process operating step



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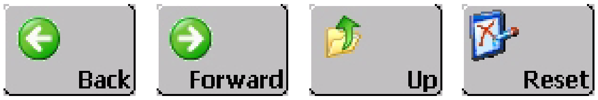
▶	Select one tile each on the following navigational levels until you have reached the desired function with the corresponding dialog on the lowest level.
▶	You can directly select a function in the navigation window by clicking the corresponding folder.

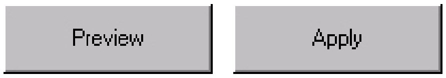
The menu bar at the top right offers the following buttons **(5)**:



▶	Click Back to return to the previously displayed navigational level.
▶	Click Forward to display the level which you selected prior to clicking the Back button.
▶	Click Up to enter the next higher level in the hierarchy.
▶	Click Reset to return modified variables to their last setting.

▶	To exit the Process operating step, click the arrow symbol of another operating step (e.g. Acquire).

The bottom of every dialog displays the following two buttons:



▶	Click Preview to execute the selected function and to display the resulting image on the right in the preview window.
▶	Click Apply to apply the selected function to the current image data set, to display the resulting image in the Viewer display window and to create a new file in the current experiment.

Navigation: Arithmetic and Boolean Logic\ Image and Image

Arithmetic and logical operations with two image data sets

> *A requirement for running arithmetical operations is equal dimensions or a 1/n relationship of the two data sets. Equal dimensions means that two images must have the same format in x and y, and that two image stacks must have the same number of single images (z). The 1/n relationship exists when, for example, 1 single image is offset against each (nth) single image of an image stack.*

Boolean Logic tile

& see Boolean Operations (page 236)

▶	In the Experiment Overview display window, select the first image data set by double-clicking the file.
▶	In the Second Source Image field, select the second image data set for the calculation process.
▶	Select the AND operator to combine the binary representations of the pixels of the two selected images with the logical AND operator. That means that pixels in the result image are given the value 1 when the value 1 is present in both of the pixels; otherwise 0 is entered.
	You achieve meaningful results using this Boolean operator when you link an image with a binary mask. The pixel is displayed in the result image at the point where the pixels of the image meet the pixels of the mask with the value 1. The areas defined by the mask are cut out of the image.
▶	Select the OR operator to combine the binary representations of the pixels of the two selected images with the logical OR operator. That means that pixels in the resulting image are given the value 1 when the value 1 is present in at least one of the two pixels; otherwise 0 is entered.
	You achieve meaningful results using this Boolean operator: 1. When you link two binary masks. The result image is a combination of the two masks. 2. When you link an image with a binary mask. The result image is an image in which the mask appears. 3. When you link two images. In contrast to the overlay image, pure intensity values are overlaid here. An RGB color addition does NOT take place here. & see Viewing an Overlay Image (page 162)
▶	Select the XOR operator to combine the binary representations of the pixels of the two selected images with the logical XOR operator. That means that pixels in the resulting image are given the value 1 when the two pixels have different values; otherwise 0 is entered.
	You achieve meaningful results using this Boolean operator when you create two binary masks of images and link them. The pixel is displayed in the result image at the point where the two

	images differ.

Arithmetic with World Coordinates tile
Arithmetic with Pixel Values tile

If you select Arithmetic with World Coordinates, world coordinates such as the z-position, the time or the wavelength are used for the arithmetic operations. If you selected Arithmetic with Pixel Values, the gray values standardized to the interval 0 – 255 for 8 bits or 0 – 4095 for 12 bits are used.

▶	In the Experiment Overview display window, select the first image data set by double-clicking the file.
▶	In the Second Source Image field, select the second image data set for the calculation process.
▶	Select Addition to add the intensity values of the two selected images according to the formula: pixel (resulting image) = pixel (first source image) + pixel (second source image).
▶	Select Subtraction to subtract the intensity values of the two selected images according to the formula: pixel (resulting image) = pixel (first source image) – pixel (second source image).
▶	Select Multiplication to multiply the intensity values of the two selected images according to the formula: pixel (resulting image) = pixel (first source image) x pixel (second source image).
▶	Select Division to divide the intensity values of the two selected images according to the formula: pixel (resulting image) = pixel (first source image) / pixel (second source image).
▶	Select Min to use the minimum value of the two selected images for each pixel in the resulting image according to the formula: pixel (resulting image) = MIN (pixel (first source image); pixel (second source image)).
▶	Select Max to use the maximum value of the two selected images for each pixel in the resulting image according to the formula: pixel (resulting image) = MAX (pixel (first source image); pixel (second source image)).
▶	Select Average to use the average value of the two selected images for each pixel in the resulting image according to the formula: pixel (resulting image) = 1/2 x (pixel (first source image) + pixel (second source image)).
▶	If you select 12 Bit Output in the Output Option field, each pixel in the result image is coded with 12 bits, i.e. the result values are saved in a value range from [0..4095].
▶	Click the Absolute Value check box if the amounts of the result data are to be used. This turns negative results into positive results.

Only for Arithmetic with Pixel Values

▶	Click the Cut at Maximum check box to cut off values that fall outside the value range following the calculation. Values less than 0 are set to 0. All values greater than 255 for 8-bit images or 4095 for 12-bit images are set to the value 255 or 4095.
▶	Click the Rescale check box to standardize the intensity values of the image to the maximum possible intensity values. The extreme values are determined. The minimum value is assigned to the 0. The maximum value for 8-bit images is assigned to the value 255, for 12-bit images to the value 4095. This ensures that the calculated pixel values of the resulting image remain within the respective value range.

Navigation: Arithmetic and Boolean Logic\ Image and Constant
Arithmetic and logical operations with one image data set and one constant

> *A requirement for running arithmetical operations is equal dimensions or a 1/n relationship of the two data sets. Equal dimensions means that two images must have the same format in x and y, and that two image stacks must have the same number of single images (z). The 1/n relationship exists when, for example, 1 single image is offset against each (nth) single image of an image stack.*

Boolean Logic tile

& see Boolean Operations (page 236)

▶	Enter a value for the constant in the Constant field. In the Experiment Overview display window, select the image data set to be processed by double-clicking the file.
▶	Select the AND operator to combine the binary representations of the pixels of the constant and the image with the logical AND operator. That means that pixels in the result image are given the value 1 when the value 1 is present in both of the pixels; otherwise 0 is entered.
▶	Select the OR operator to combine the binary representations of the pixels of the constant and the image with the logical OR operator. That means that pixels in the resulting image are given the value 1 when the value 1 is present in at least one of the two pixels; otherwise 0 is entered.
▶	Select the XOR operator to combine the binary representations of the pixels of the constant and the image with the logical XOR operator. That means that pixels in the resulting image are given the value 1 when the two pixels have different values; otherwise 0 is entered.

Arithmetic with World Coordinates tile
Arithmetic with Pixel Values tile

If you select Arithmetic with World Coordinates, world coordinates such as the z-position, the time or the wavelength are used for the arithmetic operations. If you selected Arithmetic with Pixel Values, the gray values standardized to the interval 0 – 255 for 8 bits or 0 – 4095 for 12 bits are used.

▶	Enter a value for the constant in the Constant field. In the Experiment Overview display window, select the image data set to be processed by double-clicking the file.
▶	Enter a value for the constant in the Constant field. The image data set is automatically the active image data record in the Viewer window.
▶	Select Addition to add the constant pixel by pixel to the intensity values of the selected image according to the formula: pixel (resulting image) = pixel (source image) + constant.
▶	Select Subtraction to subtract the constant pixel by pixel from the intensity values of the selected image according to the formula: pixel (resulting image) = pixel (source image) – constant.
▶	Select Multiplication to multiply the constant pixel by pixel with the intensity values of the selected image according to the formula: pixel (resulting image) = pixel (source image) x constant.
▶	Select Division to divide the intensity values of the selected image by the constant according to the formula: pixel (resulting image) = pixel (source image) / constant.
▶	Select Min to use the minimum value each of the selected image and constant for each pixel in the resulting image according to the formula: pixel (resulting image) = MIN (pixel (source image); constant).
▶	Select Max to use the maximum value each of the selected image and constant for each pixel in the resulting image according to the formula: pixel (resulting image) = MAX (pixel (source image); constant).
▶	Select Average to use the average value each of the selected image and constant for each pixel

	in the resulting image according to the formula: pixel (resulting image) = 1/2 x (pixel (source image) + constant).
▶	If you select 12 Bit Output in the Output Option field, each pixel in the result image is coded with 12 bits, i.e. the result values are saved in a value range from [0..4095].
▶	Click the Absolute Value check box if the amounts of the result data are to be used. This turns negative results into positive results.

Only for Arithmetic with Pixel Values

▶	Click the Cut at Maximum check box to cut off values that fall outside the value range following the calculation. Values less than 0 are set to 0. All values greater than 255 for 8-bit images or 4095 for 12-bit images are set to the value 255 or 4095.
▶	Click the Rescale check box to standardize the intensity values of the image to the maximum possible intensity values. The extreme values are determined. The minimum value is assigned to the 0. The maximum value for 8-bit images is assigned to the value 255, for 12-bit images to the value 4095. This ensures that the calculated pixel values of the resulting image remain within the respective value range.

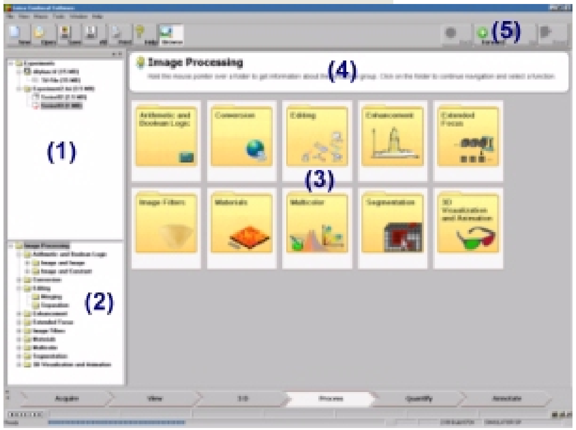
Conversion of the Bit Resolution of Images

Conversion

This chapter contains the following topics

- Setup and operation of the Process operating step
- Navigation: Conversion\ Bit Depth Conversion

Setup and operation of the Process operating step

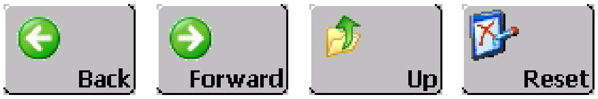


After clicking the Process arrow symbol, the usual setup of the user interface is completely changed. All function buttons of the other button areas/operating steps are hidden. The Experiment Overview **(1)** display window is shown on the left with the opened experiments. The navigation window **(2)** with the directory tree of all functions available in this operating step is located underneath it. The middle of the user interface displays 10 large tiles **(3)**, which comprise the 10 function groups. A help field **(4)** is located above these tiles, which can display a brief explanation of a function group by holding the mouse pointer over the corresponding tile.

▶	Click the tile with the desired function group. On every navigational level, you can display an explanation of the function groups in the top help field by holding the mouse pointer over the

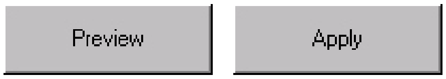
	corresponding tile.
▶	Select one tile each on the following navigational levels until you have reached the desired function with the corresponding dialog on the lowest level.
▶	You can directly select a function in the navigation window by clicking the corresponding folder.

The menu bar at the top right offers the following buttons **(5)**:



▶	Click Back to return to the previously displayed navigational level.
▶	Click Forward to display the level which you selected prior to clicking the Back button.
▶	Click Up to enter the next higher level in the hierarchy.
▶	Click Reset to return modified variables to their last setting.
▶	To exit the Process operating step, click the arrow symbol of another operating step (e.g. Acquire).

The bottom of every dialog displays the following two buttons:



▶	Click Preview to execute the selected function and to display the resulting image on the right in the preview window.
▶	Click Apply to apply the selected function to the current image data set, to display the resulting image in the Viewer display window and to create a new file in the current experiment.

Navigation: Bit Depth Conversion
Converting images of 8-bit depth to 12-bit depth and vice versa

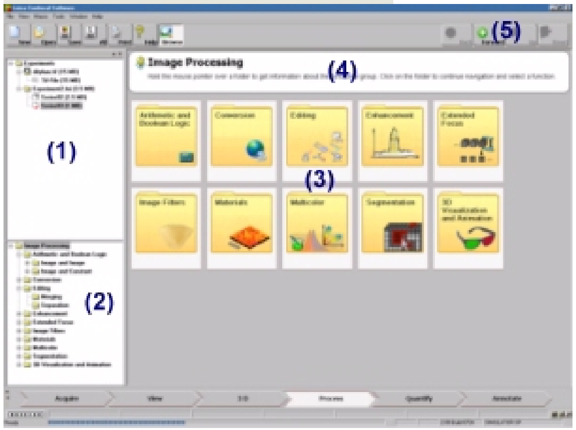
▶	In the Experiment Overview display window, select the image data set to be processed by double-clicking the file.
▶	The Source field displays the bit depth of the selected image.
▶	Select the bit depth in the New Resolution field to which the image data set is to be converted.

Merging or Separating of Image Segments, Images and Image Series

This chapter contains the following topics

- Setup and operation of the Process operating step
- Navigation: Editing\ Merging
- Navigation: Editing\ Separation

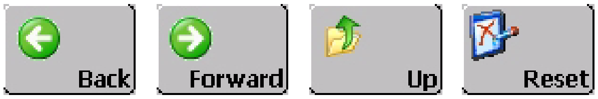
Setup and operation of the Process operating step



After clicking the Process arrow symbol, the usual setup of the user interface is completely changed. All function buttons of the other button areas/operating steps are hidden. The Experiment Overview **(1)** display window is shown on the left with the opened experiments. The navigation window **(2)** with the directory tree of all functions available in this operating step is located underneath it. The middle of the user interface displays 10 large tiles **(3)**, which comprise the 10 function groups. A help field **(4)** is located above these tiles, which can display a brief explanation of a function group by holding the mouse pointer over the corresponding tile.

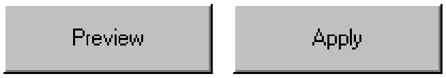
▶	Click the tile with the desired function group. On every navigational level, you can display an explanation of the function groups in the top help field by holding the mouse pointer over the corresponding tile.
▶	Select one tile each on the following navigational levels until you have reached the desired function with the corresponding dialog on the lowest level.
▶	You can directly select a function in the navigation window by clicking the corresponding folder.

The menu bar at the top right offers the following buttons **(5)**:



▶	Click Back to return to the previously displayed navigational level.
▶	Click Forward to display the level which you selected prior to clicking the Back button.
▶	Click Up to enter the next higher level in the hierarchy.
▶	Click Reset to return modified variables to their last setting.
▶	To exit the Process operating step, click the arrow symbol of another operating step (e.g. Acquire).

The bottom of every dialog displays the following two buttons:



▶	Click Preview to execute the selected function and to display the resulting image on the right in the preview window.
▶	Click Apply to apply the selected function to the current image data set, to display the resulting image in the Viewer display window and to create a new file in the current experiment.

Navigation: Editing\ Merging
Combining several images or data sets to a new image or data set

Amplitude tab
Adaptation of different bit resolutions and additional settings for calculating the resulting image

▶	In the Experiment Overview display window, select the first image data set by double-clicking the file.
▶	In the Second Source Image field, select the second image data set for the calculation process.
▶	In the Amplitude Image 1/2 Min. field, set the minimum intensity value. This value is «0» for data sets with gray values. For data sets with world coordinates, this field shows the actual minimum. The minimum is adjustable so that differently scaled data sets can be adapted to each other.
	If a data set with a small interval of intensity values is combined with a data set featuring a large intensity value range, the first one is shown darker than the second one. Adapting the minimum values of both data sets scales them in such a way that equal parts of the intensity values of both data sets are included in the merged image.
▶	In the Amplitude Image 1/2 Max. field, set the maximum intensity value. For data sets with gray values, this value is «255» with 8-bit data or «4095» with 12-bit data. For data sets with world coordinates, this field shows the actual maximum. The maximum is adjustable so that differently scaled data sets can be adapted to each other.
	If a data set with a small interval of intensity values is combined with a data set featuring a large intensity value range, the first one is shown darker than the second one. Adapting the maximum values of both data sets scales them in such a way that equal parts of the intensity values of both data sets are included in the merged image.
▶	In the Result field, you define the image resolution of the merged image. An 8-bit resolution can display 256 different intensity levels. A 12-bit resolution can display 4096 different intensity levels.
▶	If the Maximum Value option is selected in the Result field, the brightest pixel from the two images to be merged is used to calculate the corresponding pixel of the merged image.
▶	If the Average Value option is selected in the Result field, the arithmetical mean from the two images to be merged is used to calculate the corresponding pixel of the merged image.
	ℹ

If two strongly different images are calculated to a merged image by using the average method, the black image background causes a reduction of the image intensity!

x, y, z, t, la tab

Definition of the stereoscopic arrangement of the output images in the resulting image before combining

▶ In the Image 1 and Image 2 field, enter the position (x / y / z / t / λ) of the output image in the merged image in the Shift entry field. This value is typically «0». A value of > 0 is useful for example, if a reduction of one output image is to be shown at a certain position of the other output image. This function is also useful for lateral image series, such as those found in biomapping.
& see Example of Moving an Image (page 235)

▶ In the Image 1 and Image 2 field, enter the size of the dimensions (x / y / z / t / λ) of the output image in the merged image in the Size entry field.

▶ If you select the Append option field, the second data set is placed next to the first data set. The direction of the placement depends on whether the check box on the x, y or z tab was selected. Selecting this function on all the different tabs for x, y and z shifts the second output image either in only one dimension or in several dimensions.

▶ In the Result field, you can specify the pixel after which the merged image ends.

ℹ

Definition background: Absolute lengths are used to determine the dimensions of the merged image. If the two data sets to be merged feature the same linear extension but a different number of pixels (due to a different scan format), it must be decided whether the number of pixels or the resulting total length is to be decisive for the merged image.

▶ If you select the Best Fit option in the Result field, the software uses the following procedure for calculating the resulting image:
The width of a single pixel is calculated for both images: µm/pixel. If the difference of this size between the two images is smaller than a factor of 2, this procedure uses the larger image resolution (i.e., the smaller µm/pixel value) for the resulting image. If the deviation of the image resolution between the two output images is larger than a factor of 2, the smaller image resolution (i.e., the larger µm/pixel value) is used for calculating the resulting image. The number of pixels of the resulting image (Element Number) divided by the total length results in the resolution of the resulting image.

ℹ

Definition background: Absolute lengths are used to determine the dimensions of the merged image. If the two data sets to be merged feature the same linear extension but a different number of pixels (due to a different scan format), it must be decided whether the number of pixels or the resulting total length is to be decisive for the merged image.

& see Selecting a Scan Format (page 120)

ch tab

Definition of the combination of images with different numbers of detection channels

▶	If two images or data sets with different numbers of detection channels are to be merged, you define in the Image 1 field the channel number in the merged image at which the channels of the output image 1 should be inserted.
▶	If two images or data sets with different numbers of detection channels are to be merged, you define in the Image 2 field the channel number in the merged image at which the channels of the output image 2 should be inserted.
▶	Selecting the option box distributes the two output images to different channels of the merged image.

Navigation: Editing\ Separation

Separating single images or image segments from a data set

▶	In the Experiment Overview display window, select the image data set to be processed by double-clicking the file.

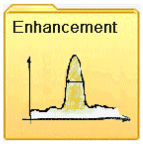
Dimension Selection field

▶	The ch entry field displays the detection channels of the image or the image series. Select the detection channels from which the image segment or the individual images are to be separated.
▶	The number of pixels in y that is dependent upon the scan format is indicated in the y entry field. Enter the number of pixels to be separated in the Start y and End y entry fields. Use the R (Reset) button to undo the entries.
▶	The number of pixels in x that is dependent upon the scan format is indicated in the x entry field. Enter the number of pixels to be separated in the Start x and End x entry fields. Use the R (Reset) button to undo the entries.
▶	The number of single images of an image series is displayed in the z entry field. Enter the number of images to be separated in the Start z and End z entry fields. Use the R (Reset) button to undo the entries.
▶	An interval within a dimension can be entered in the Step z / y / x entry field. For example, if you enter 2 in the Step z entry field, only every 2nd image is separated.

Examples

▶	If you want to separate all images of a detection channel from an image series, click in the ch entry field on the channel to be separated without changing the values in the entry fields of the other dimensions.
▶	If you want to separate complete single images from an image series, enter the corresponding numbers in the Start z and End z entry fields without changing the values in the entry fields of the other dimensions.
▶	If you want to separate a segment in all images of an image series, define the size of the segment in the Start x, End x and Start y, End y entry fields without changing the values in the entry fields for z.

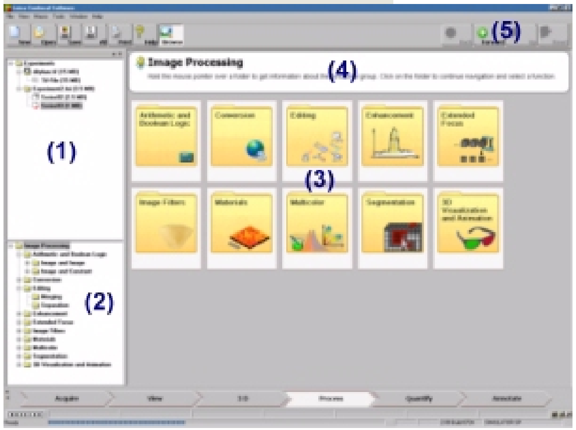
Adjusting Brightness, Contrast and Gamma Correction


Enhancement

This chapter contains the following topics

- Setup and operation of the Process operating step
- Navigation: Enhancement\ Contrast and Brightness
- Navigation: Enhancement\ Gamma Correction

Setup and operation of the Process operating step


(5)
Image Processing
(4)
(1)
Arithmetic and Boolean Logic
Conversion
Editing
Enhancement
Extended Focus
(3)
Image Filters
Materials
Multicolor
Segmentation
3D Visualization and Animation
(2)
Acquire
View
3-D
Process
Quantify
Annotate

After clicking the Process arrow symbol, the usual setup of the user interface is completely changed. All function buttons of the other button areas/operating steps are hidden. The Experiment Overview **(1)** display window is shown on the left with the opened experiments. The navigation window **(2)** with the directory tree of all functions available in this operating step is located underneath it. The middle of the user interface displays 10 large tiles **(3)**, which comprise the 10 function groups. A help field **(4)** is located above these tiles, which can display a brief explanation of a function group by holding the mouse pointer over the corresponding tile.

▶	Click the tile with the desired function group. On every navigational level, you can display an explanation of the function groups in the top help field by holding the mouse pointer over the corresponding tile.
▶	Select one tile each on the following navigational levels until you have reached the desired function with the corresponding dialog on the lowest level.
▶	You can directly select a function in the navigation window by clicking the corresponding folder.

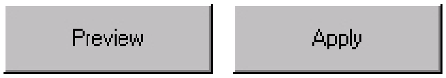
The menu bar at the top right offers the following buttons **(5)**:

Back
Forward
Up
Reset

▶	Click Back to return to the previously displayed navigational level.
▶	Click Forward to display the level which you selected prior to clicking the Back button.
▶	Click Up to enter the next higher level in the hierarchy.
▶	Click Reset to return modified variables to their last setting.

▶	To exit the Process operating step, click the arrow symbol of another operating step (e.g. Acquire).

The bottom of every dialog displays the following two buttons:



▶	Click Preview to execute the selected function and to display the resulting image on the right in the preview window.
▶	Click Apply to apply the selected function to the current image data set, to display the resulting image in the Viewer display window and to create a new file in the current experiment.

Navigation: Enhancement\ Brightness and Contrast
Adjusting brightness and contrast
If you change the brightness of an image, the high, medium and low brightness values of the image will change to the same degree. Contrast is used to vary the ratio of bright and dark values in the image.

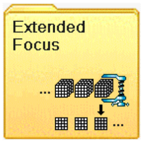
▶	In the Experiment Overview display window, select the image data set to be processed by double-clicking the file.
▶	For multichannel images, select the Lock Channels check box to make all changes for all channels at one time and to the same extent.
▶	For images that do not use the entire dynamic range, select the Linear Model check box. If the check box has been clicked, there is a linear relationship between the brightness in the output image and the resulting image. Whether an image uses the entire dynamic range available can be seen by creating a histogram of the image. If the histogram does not cover the entire intensity range (see minimum and maximum values), the dynamic range is not being used in full. & see Calculating a Histogram (page 212) If you have images in which you wish to highlight the medium brightness values more strongly, select the nonlinear model (Linear Model check box is not enabled).
▶	In the Channel Adjustment field, change the brightness of the resulting image using the Brightness slider. If the slider is exactly in the center, the output image and resulting image have exactly the same level of brightness. If the slider is moved from the center to the right, the result image becomes brighter than the output image. If the slider is moved from the center to the left, the resulting image becomes darker than the output image.
▶	In the Channel Adjustment field, change the contrast of the resulting image using the Contrast slider. If the slider is exactly in the center, the output image and result image have exactly the same level of contrast. If the slider is moved from the center to the right, the result image has greater contrast than the output image. If the slider is moved from the center to the left, the result image has less contrast than the output image.

Navigation: Enhancement\ Gamma Correction
Adjusting the medium brightness values (gamma correction)
The gamma correction can be used to influence the conversion of the output image contrast to the resulting image contrast in a nonlinear manner. The gamma correction only affects the medium brightness values of an image, whereas the very bright and very dark image sections remain unchanged.

▶	In the Experiment Overview display window, select the image data set to be processed by double-clicking the file.
▶	For multichannel images, select the Lock Channels check box to make all changes for all channels at one time and to the same extent.
▶	In the Channel Adjustment field, change the medium brightness values of the resulting image using the slider. If the slider is exactly in the center, the output image and resulting image have exactly the same level of brightness. If the slider is moved from the center to the right, the medium brightness values become brighter. If the slider is moved from the center to the left, the

	medium brightness values become darker.

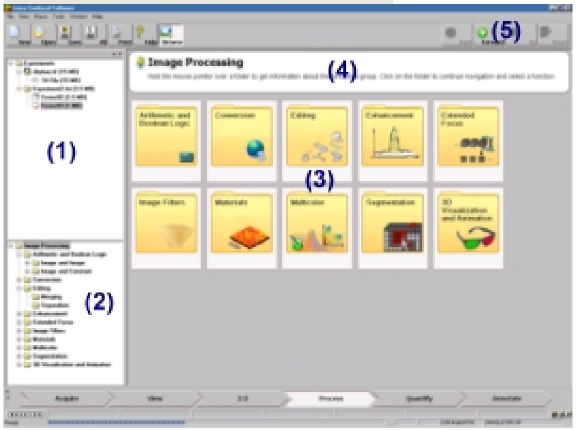
Expanded Focus by Projecting Image Stacks onto a Single Image



This chapter contains the following topics

- Setup and operation of the Process operating step
- Navigation: Extended Focus\ Average Projection, Maximum Projection, Transparent Projection

Setup and operation of the Process operating step



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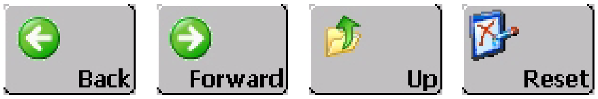
▶	Click the tile with the desired function group. On every navigational level, you can display an explanation of the function groups in the top help field by holding the mouse pointer over the corresponding tile.
▶	Select one tile each on the following navigational levels until you have reached the desired function with the corresponding dialog on the lowest level.
▶	You can directly select a function in the navigation window by clicking the corresponding folder.

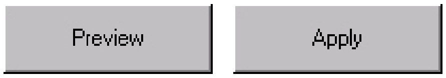
The menu bar at the top right offers the following buttons **(5)**:



▶	Click Back to return to the previously displayed navigational level.
▶	Click Forward to display the level which you selected prior to clicking the Back button.
▶	Click Up to enter the next higher level in the hierarchy.
▶	Click Reset to return modified variables to their last setting.

▶	To exit the Process operating step, click the arrow symbol of another operating step (e.g. Acquire).

The bottom of every dialog displays the following two buttons:



▶	Click Preview to execute the selected function and to display the resulting image on the right in the preview window.
▶	Click Apply to apply the selected function to the current image data set, to display the resulting image in the Viewer display window and to create a new file in the current experiment.

Navigation: Extended Focus\ Average Projection, Maximum Projection, Transparent Projection Creating a Projection Image Along the Orthogonal Axis (z-axis with horizontal xy-sections and y-axis with vertical xz-sections)

& see Principles and Types of Projections (page 166)

▶	In the Experiment Overview display window, select the image data set to be processed by double-clicking the file.
▶	Select one of the three projection types: Average Projection, Maximum Projection, Transparent Projection.

Options tab

▶	The Projection Direction field is used to specify the dimension to be used for the projection. Select [z] for stereoscopic series, [t] for time series or [λ] for wavelength series to create the projection from all images of a detection channel, such as from image 1 to image 10 in detection channel 1. Select [ch] to create the projection from a certain image in all detection channels, for example from image 1 in detection channel 1 and image 1 in detection channel 2.
▶	Click on Rescale in the Options field to standardize the intensity values of the image to the maximum possible intensity values when creating the projection image. This ensures that the calculated gray values of the projection image remain within the value range of, for example, 0 to 255 at 8 bits. This allows for brightening dark images.
▶	If you click on Invert in the Options field, the projection axis is mirrored. The calculation of the projection image is thus in the opposite direction. This function makes sense above all in the case of inverse microscopes to match the calculation of the projection image to the inverse beam path of laser light.
▶	Move the slider in the Threshold field to define a threshold value. The intensity values below this value are not taken into account for the creation of the projection image.

Factor tab (only for transparent projection)

& see Principles and Types of Projections (page 166)

▶	The Factor tab is only displayed if the transparent projection was selected. Use the slider to vary the transparent factor α. The higher it is set, the stronger the intensity values flow from the lower levels in the image stack into the projection image.

The three projection types can also be enabled directly using the corresponding buttons (Fix Max / Fix Avg / Fix Trans). If you activate the function using the button, the projection image will just be a temporary display on the monitor. You can undo this *using the Original button.*

& see Maximum Projection of an Image Stack with Invariable Projection Axis (page 169)
& see Average Projection of an Image Stack with Invariable Projection Axis (page 171)
& see Transparent Projection of an Image Stack with Invariable Projection Axis (page 173)

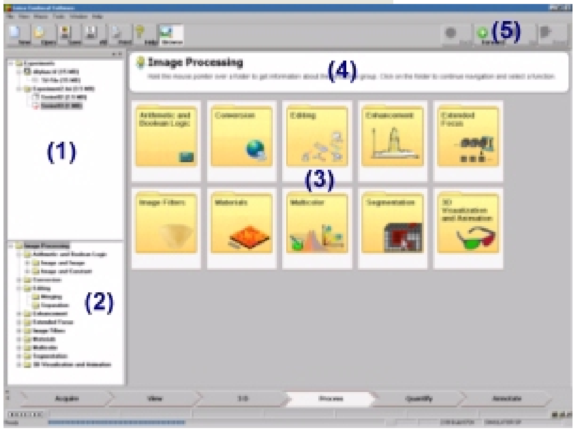
Linear Filters and Morphological Filters (Optional)

Image Filters

This chapter contains the following topics

- Setup and operation of the Process operating step
- What are linear filters?
- Navigation: Image Filters\ Linear Filters
- What are morphological filters?
- Navigation: Image Filters\ Morphological Filters

Setup and operation of the Process operating step



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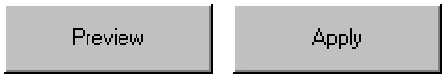
▶	Click the tile with the desired function group. On every navigational level, you can display an explanation of the function groups in the top help field by holding the mouse pointer over the corresponding tile.
▶	Select one tile each on the following navigational levels until you have reached the desired function with the corresponding dialog on the lowest level.
▶	You can directly select a function in the navigation window by clicking the corresponding folder.

The menu bar at the top right offers the following buttons **(5)**:

Back
Forward
Up
Reset

▶	Click Back to return to the previously displayed navigational level.
▶	Click Forward to display the level which you selected prior to clicking the Back button.
▶	Click Up to enter the next higher level in the hierarchy.
▶	Click Reset to return modified variables to their last setting.
▶	To exit the Process operating step, click the arrow symbol of another operating step (e.g. Acquire).

The bottom of every dialog displays the following two buttons:



▶	Click Preview to execute the selected function and to display the resulting image on the right in the preview window.
▶	Click Apply to apply the selected function to the current image data set, to display the resulting image in the Viewer display window and to create a new file in the current experiment.

What are linear filters?
Filters are used to enhance the quality of an image. As a rule, this means removing undesirable pixels from the image. The filter function available here—a low-pass filter—is used to suppress static noise in an image that could be generated by the detector.

The principle of a filter consists of taking the value of each pixel in an image and setting it off against the values of the adjacent pixels. The filter kernel describes the number and weighting of the adjacent pixels that come to bear in the calculation of the new pixel. The kernel used here can be described using Pascal's triangle:

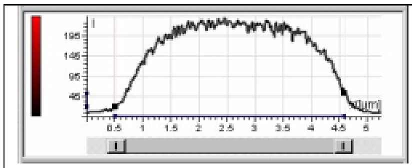
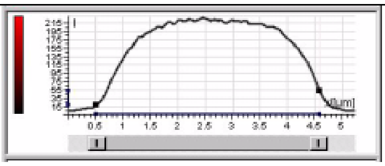
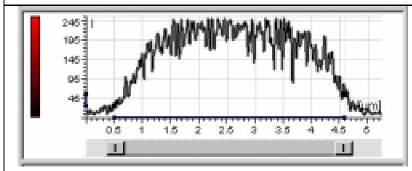
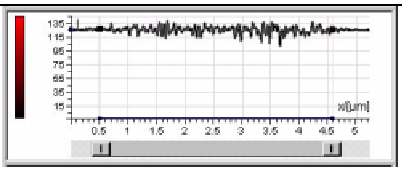
Filter kernel size	Pascal triangle	Scaling
	1 1	1/2
Kernel Size 3	1 2 1	1/4
	1 3 3 1	1/8
Kernel Size 5	1 4 6 4 1	1/16
	1 5 10 10 5 1	1/32
Kernel Size 7	1 6 15 20 15 6 1	1/64
	1 7 21 35 35 21 7 1	1/128
Kernel Size 9	1 8 28 56 70 56 28 8 1	1/256
...	...	...

If, for example, kernel size 5 is selected, 5 pixels of the original image are used in calculating 1 pixel in the filtered image. It is always the middle pixel in the filter kernel that is the pixel to be calculated. In this case, the gray values of the 5 pixels corresponding to the weighting scale 1-4-6-4-1, are multiplied by the factors 1/16, 4/16, 6/16, 4/16, 1/16 and together added to the final value. With respect to the calculation of the pixel C in the following schematic representation this means that the gray values of pixels A, B and D, E are included in the calculation of the filtered pixel C'. C' is calculated as follows: 16x1/16 + 4x4/16 + 8x6/16 + 32x4/16 + 8x1/16 = 1 + 1 + 3 + 8 + 0.5 = 13.5

	A	B	C	D	E
Gray values of pixels A-E in the original image	16	4	8	32	8
Factors of kernel size 5	1/16	4/16	6/16	4/16	1/16
Gray value of C' in the filtered image			13.5		

A calculation of this kind is performed for each pixel in the original image. At the edge of the image, the filter mask protrudes beyond the image. For these pixels, the value 0 is set.

Function of linear filters

 Intensity profile of an unfiltered image	 Intensity profile of the image after application of the Blur filter
 Intensity profile of the image after application of the Sharpen filter	 Intensity profile of the image after application of the High-Pass filter

▶	If you select the Blur filter, the low-pass filter described above is applied to the image. This filter type filters the high frequencies out of the image. That is, the very distinctive transitions from low to high intensity values are reduced. This filter can be used to remove noise from an image.
▶	If you select the Sharpen filter, the low-pass filter is applied to the image, and the values of the high-pass-filtered image are added to the values of the original image. This filter type filters the low frequencies out of the image. That is, the very distinctive transitions from low to high intensity values are intensified.
▶	If you select the High-Pass filter, the low-pass filter is applied to the image, and the values of the original image are subtracted from the values of the low-pass-filtered image. The result corresponds to an image that was processed with a high-pass filter. This filter type displays only the very distinctive transitions from low to high intensity values in the image.

Navigation: Image Filters\ Linear Filters
Applying linear filters (low-pass and high-pass filters) to images

▶	In the Experiment Overview display window, select the image data set to be processed by double-clicking the file.
▶	Select one of the three filter types: Blur, Sharpen, High-pass.
▶	In the Kernel Size field, select a value for the kernel size. The value specified in the Kernel Size field as Cut-off Wavelength is the size of the filter measured in pixels. This value is calculated on the basis of the scan parameters (enlargement of the objective, wavelength of the excitation light, scan format, electronic zoom factor...) used for each image.
▶	In the Filter Directions field, set the dimensions of the filter. Select the spatial axes to which the filter is to be applied sequentially. One-dimensional filters are applied, that is, the filter is calculated only via one axis at any given time. The value specified in the Filter Directions field as Cut-off Wavelength is the size of the filter measured in nanometer. For example, with a value of 160 nm all wavelengths lesser than or equal to 160 nm are filtered.

Sharp Factor (only with Sharpen filter)

	Use the slider in the Sharp Factor field to vary the effect of the Sharpen filter. If this factor is set to 0, the filter is not active.

Blur (Gallery), Sharpen (Gallery), High-Pass (Gallery) tiles

▶	Click the Blur (Gallery), Sharpen (Gallery) or High-Pass (Gallery) tile to view a gallery of preview images of the selected image data set. Each of the preview images was calculated with a different kernel size (and a different sharpness factor). Click a tile to display a dialog window with the corresponding settings.

What are morphological filters?

Using mathematical operators, morphological filters are used to change the form of connected structures in an image and to separate them from the background. Morphological operators are typically applied to binary images. Binary images feature pixels, which only have the value 1 or 0. The segmentation functions in the Leica Confocal Software can be applied to create binary images. However, these operators can also be used to process halftone images, that is images with a value range of, for example, 256 (8 bits) different gray values/intensity values.

& see Process Operating Step / Segmentation Folder (page 208)

The principle consists of moving a mask—also referred to as structural element—pixel by pixel over the image. The pixel to be examined is always the central pixel of the mask. The mask in the Leica Confocal Software can have the shape of a cube, cross or sphere. Shape and size of the mask determine which and how many adjacent pixels are included in the filtering. A certain criterion is used to distinguish which pixels are defined as structure and as background in the resulting image. For binary images, this criterion is the logical combination AND or OR; for halftone images, it is the size of the gray value/intensity value.

& see Boolean Operations (page 236)

The shape of the mask is used to influence the structures to be highlighted in the resulting image. For example, if the sphere is selected as mask form, round shapes are accentuated in the resulting image. However, the morphological operator determines in which proximity relation the corresponding pixel is coded as structure or as background in the resulting image.

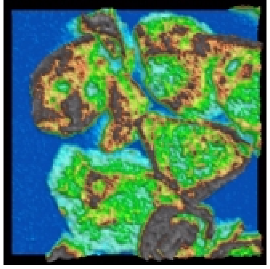
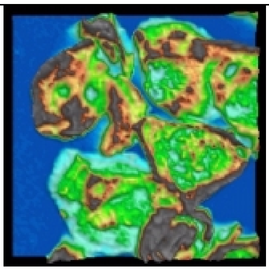
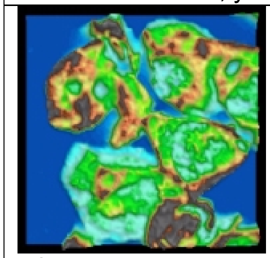
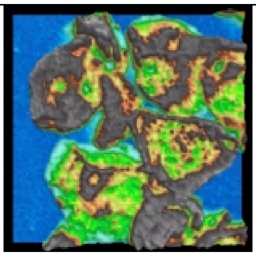
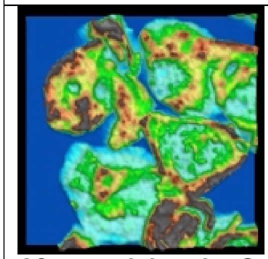
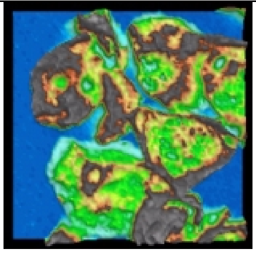
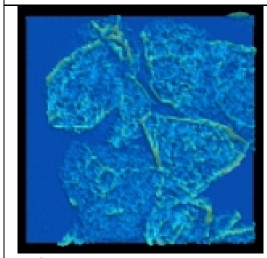
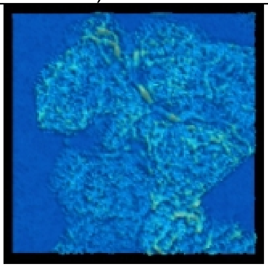
The following two elementary basic types of morphological operators, namely erosion and dilation, as well as combinations of these operators exist:

Operator	**Description**
Erosion	With binary images, the examined pixel is set to the binary value 1 in the resulting image if all adjacent pixels (determined by the shape and size of the mask) in the binary original image are coded with a 1. With halftone images, the minimum gray value of the adjacent pixels is entered in the resulting image instead of the value of the examined pixel.
Dilation	With binary images, the examined pixel is set to the binary value 1 in the resulting image if at least one adjacent pixel (determined by the shape and size of the mask) in the binary threshold image is coded with a 1. With halftone images, the maximum intensity value of the adjacent pixels is entered in the resulting image instead of the value of the examined pixel.

Combination of operators	**Description**
Open	Open is a combination of operators. First, an erosion is executed, followed by a dilation.
Close	Close is a combination of operators. First, a dilation is executed, followed by an erosion.
Top Hat	Top Hat processes the image with the Open operator combination and subtracts the resulting image from the original image.
Bottom Hat	Bottom Hat processes the image with the Close operator combination and subtracts the original image from the resulting image.

Filters	**Description**
Median	The Median filter belongs to the group of nonlinear hierarchical filters. In Median filtering, the intensity values of the adjacent pixels (determined by the shape and size of the mask) are sorted based on their size. The intensity value occupying the middle of this hierarchy is then entered in the resulting image of the median filtering instead of the examined pixel.

Effect of the morphological operators

 **Original image**	 **After applying the Median filter**
Structures (with very high intensity values) are color-coded in gray, the background (intensity value 0) in dark blue. Between these two extreme values, the range from higher to lower intensity values is color-coded from brown, yellow, green to turquoise.	The Median filter smoothes the overall image, while simultaneously accentuating and contouring structures in the image.
 **After applying the Erosion operator**	 **After applying the Dilation operator**
Connected structures shrink and small structures and connections between structures disappear (the number of high intensity values decreases, while the number of low intensity values increases).	Connected structures grow and spaces within a structure and between structures are filled (the number of high intensity values increases, while the number of low intensity values decreases).
 **After applying the Open operator (Erosion + Dilation)**	 **After applying the Close operator (Dilation + Erosion)**
Connected structures shrink and spaces become larger (the number of high intensity values decreases, while the number of low intensity values increases).	Connected structures grow and spaces remain almost unchanged (the number of high intensity values increases, while the number of low intensity remains almost identical).
 **After applying the Top Hat operator**	 **After applying the Bottom Hat operator**
The high intensity values are extracted	The low intensity values are extracted

Navigation: Image Filters\ Morphological Filters
Applying morphological filters to images

▶	In the Experiment Overview display window, select the image data set to be processed by double-clicking the file.
▶	Select one the seven filter types: Median, Erosion, Dilation, Open, Close as well as Top Hat and Bottom Hat.
▶	In the Mask field, select the basic form of the filter mask: Cube, Cross or Sphere. The selection of the shape of the structure element influences the image structures processed by the filter. For example, spheres accentuate round structures in the resulting image.
▶	Enter the size of the mask in pixels in the fields Size X, Size Y and Size Z. The size of the mask determines how many adjacent pixels are included in the filter mask.
▶	In the Repetition field, you specify the frequency with which the filter is to be applied.

Tiles with designation (Gallery)

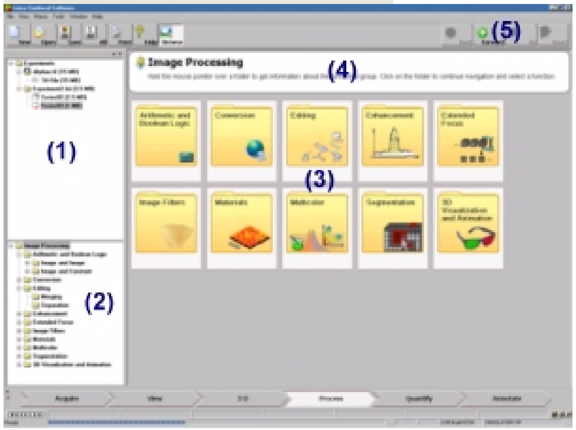
▶	Click one of the tiles labeled with (Gallery) to view a gallery of preview images of the selected image data set. Each of the preview images was calculated with a different structure element and/or mask size. Click a tile to display a dialog window with the corresponding settings.

Leveling and Filtering of Surface Images (Optional)

This chapter contains the following topics

- Setup and operation of the Process operating step
- Navigation: Materials\ Leveling
- What are linear filters?
- Navigation: Materials\ Roughness Filter
- Navigation: Materials\ Waviness Filter

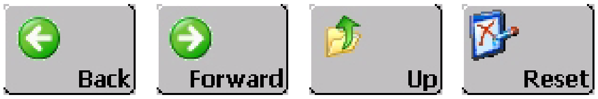
Setup and operation of the Process operating step



After clicking the Process arrow symbol, the usual setup of the user interface is completely changed. All function buttons of the other button areas/operating steps are hidden. The Experiment Overview **(1)** display window is shown on the left with the opened experiments. The navigation window **(2)** with the directory tree of all functions available in this operating step is located underneath it. The middle of the user interface displays 10 large tiles **(3)**, which comprise the 10 function groups. A help field **(4)** is located above these tiles, which can display a brief explanation of a function group by holding the mouse pointer over the corresponding tile.

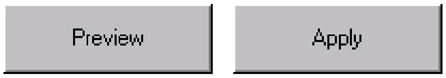
▶	Click the tile with the desired function group. On every navigational level, you can display an explanation of the function groups in the top help field by holding the mouse pointer over the corresponding tile.
▶	Select one tile each on the following navigational levels until you have reached the desired function with the corresponding dialog on the lowest level.
▶	You can directly select a function in the navigation window by clicking the corresponding folder.

The menu bar at the top right offers the following buttons **(5)**:



▶	Click Back to return to the previously displayed navigational level.
▶	Click Forward to display the level which you selected prior to clicking the Back button.
▶	Click Up to enter the next higher level in the hierarchy.
▶	Click Reset to return modified variables to their last setting.
▶	To exit the Process operating step, click the arrow symbol of another operating step (e.g. Acquire).

The bottom of every dialog displays the following two buttons:



▶	Click Preview to execute the selected function and to display the resulting image on the right in the preview window.
▶	Click Apply to apply the selected function to the current image data set, to display the resulting image in the Viewer display window and to create a new file in the current experiment.

Navigation: Materials\ Leveling

Adapting an interpolation function to a data set to correct trend curves (e.g. obliquely recorded data set)

This procedure aims at bringing an intensity curve that features a trend curve into a horizontal position. For this purpose, a suitable interpolation function (polynomial) is first adapted to the intensity curve. Next, the distance of the optimal interpolation function to a horizontal intensity plateau is calculated. The occurring intensity differences are added pixel by pixel to the original data set. This compensates the trend curve.

& see Adaptation Function for Correcting Trend Curves (page 234)

The target function to be optimized in determining the coefficients of the adaptation function is:

$$\int \| I - f(\Omega) \| d\Omega = \min$$

▶	Select one of the four types of the adaptation functions: Leveling (Linear), Leveling (Bilinear), Leveling (Quadratic), Leveling (Cubic).

Fitting Interpolation Function	**Formula**
Linear	$f(x) = a + bx + cy$
Bilinear	$f(x) = a + bx + cx + dxy$
Quadratic	$f(x) = a + bx + cx + dxy + fx^2$
Cubic	$f(x) = a + bx + cx + dxy + fx^2 + gxy^2 + hx^3 + iy^3$

▶	In the Experiment Overview display window, select the image data set to be processed by double-clicking the file.
▶	Select the step size in the Sample Step Width field. The step size sets the number of support positions for the polynomial. The smaller the step size, the more time-consuming and precise the calculation of the adaptation function.
▶	If the data set should show noise, it is recommended to hide the low intensity noise signals in the Threshold field by setting a threshold. All intensity values whose values are below the threshold are not used in the calculation. This prevents noise signals from being erroneously used as support positions for the adaptation function.

What are linear filters?

Filters are used to enhance the quality of an image. As a rule, this means removing undesirable pixels from the image. The filter function available here—a low-pass filter—is used to suppress static noise in an image that could be generated by the detector.

The principle of a filter consists of taking the value of each pixel in an image and setting it off against the values of the adjacent pixels. The filter kernel describes the number and weighting of the adjacent pixels that come to bear in the calculation of the new pixel. The kernel used here can be described using Pascal's triangle:

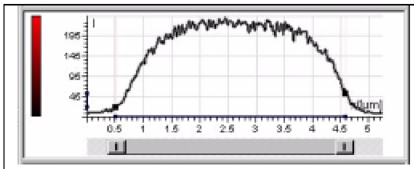
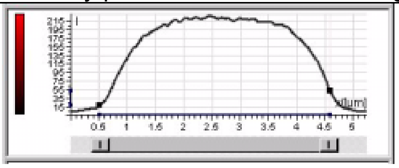
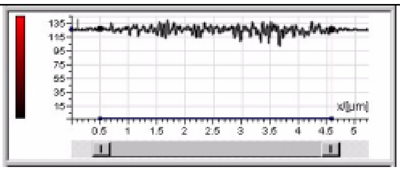
Filter kernel size	**Pascal triangle**	**Scaling**
	1 1	1/2
Kernel Size 3	1 2 1	1/4
	1 3 3 1	1/8
Kernel Size 5	1 4 6 4 1	1/16
	1 5 10 10 5 1	1/32
Kernel Size 7	1 6 15 20 15 6 1	1/64
	1 7 21 35 35 21 7 1	1/128
Kernel Size 9	1 8 28 56 70 56 28 8 1	1/256
...	...	...

If, for example, kernel size 5 is selected, 5 pixels of the original image are used in calculating 1 pixel in the filtered image. It is always the middle pixel in the filter kernel that is the pixel to be calculated. In this case, the gray values of the 5 pixels corresponding to the weighting scale 1-4-6-4-1, are multiplied by the factors 1/16, 4/16, 6/16, 4/16, 1/16 and together added to the final value. With respect to the calculation of the pixel C in the following schematic representation this means that the gray values of pixels A, B and D, E are included in the calculation of the filtered pixel C'. C' is calculated as follows: 16x1/16 + 4x4/16 + 8x6/16 + 32x4/16 + 8x1/16 = 1 + 1 + 3 + 8 + 0.5 = 13.5

	A	B	C	D	E
Gray values of pixels A-E in the original image	16	4	8	32	8
Factors of kernel size 5	1/16	4/16	6/16	4/16	1/16
Gray value of C' in the filtered image			13.5		

A calculation of this kind is performed for each pixel in the original image. At the edge of the image, the filter mask protrudes beyond the image. For these pixels, the value 0 is set.

Function of linear filters

 195 145 95 45 x[µm] 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 Intensity profile of an unfiltered image	
 Intensity profile of the image after applying the Waviness filter	 135 115 95 75 55 35 15 x[µm] 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 Intensity profile of the image after applying the Roughness filter

▶	If you select the Waviness filter, the low-pass filter described above is applied to the image. This filter type filters the high frequencies out of the image. That is, the very distinctive transitions from low to high intensity values are reduced. This filter can be used to remove noise from an image.
▶	If you select the Roughness filter, the low-pass filter is applied to the image, and the values of the original image are subtracted from the values of the low-pass-filtered image. The result corresponds to an image that was processed with a high-pass filter. This filter type displays only the very distinctive transitions from low to high intensity values in the image.

Navigation: Materials\ Roughness Filter
Applying high-pass filters to images

▶	In the Experiment Overview display window, select the image data set to be processed by double-clicking the file.
▶	In the Kernel Size field, select a value for the kernel size. The value specified in the Kernel Size field as Cut-off Wavelength is the size of the filter measured in pixels. This value is calculated on the basis of the scan parameters (enlargement of the objective, wavelength of the excitation light, scan format, electronic zoom factor...) used for each image.
▶	In the Filter Directions field, set the dimensions of the filter. Select the spatial axes to which the filter is to be applied sequentially. One-dimensional filters are applied, that is, the filter is calculated only via one axis at any given time. The value specified in the Filter Directions field as Cut-off Wavelength is the size of the filter measured in nanometer. For example, with a value of 160 nm all wavelengths lesser than or equal to 160 nm are filtered.

Navigation: Materials\ Waviness Filter
Applying low-pass filters to images

▶	In the Experiment Overview display window, select the image data set to be processed by double-clicking the file.
▶	In the Kernel Size field, select a value for the kernel size. The value specified in the Kernel Size field as Cut-off Wavelength is the size of the filter measured in pixels. This value is calculated on the basis of the scan parameters (enlargement of the objective, wavelength of the excitation light, scan format, electronic zoom factor...) used for each image.
▶	In the Filter Directions field, set the dimensions of the filter. Select the spatial axes to which the filter is to be applied sequentially. One-dimensional filters are applied, that is, the filter is calculated only via one axis at any given time. The value specified in the Filter Directions field as Cut-off Wavelength is the size of the filter measured in nanometer. For example, with a value of 160 nm all wavelengths lesser than or equal to 160 nm are filtered.

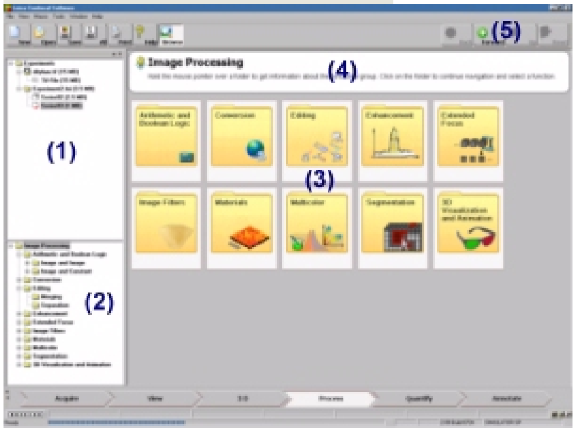
Reducing Crosstalk Between Detection Channels

Multicolor

This chapter contains the following topics

- Setup and operation of the Process operating step
- Navigation: Multicolor\ Crosstalk Reduction

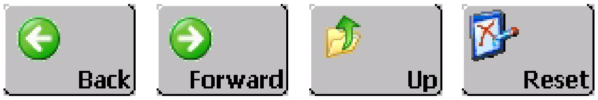
Setup and operation of the Process operating step


(5)
Image Processing
(4)
(1)
Arithmetic and Boolean Logic
Conversion
Editing
Enhancement
Extended Focus
(3)
Image Filters
Materials
Multicolor
Segmentation
3D Visualization and Animation
(2)
Acquire
View
Process
Quantify
Annotate

After clicking the Process arrow symbol, the usual setup of the user interface is completely changed. All function buttons of the other button areas/operating steps are hidden. The Experiment Overview **(1)** display window is shown on the left with the opened experiments. The navigation window **(2)** with the directory tree of all functions available in this operating step is located underneath it. The middle of the user interface displays 10 large tiles **(3)**, which comprise the 10 function groups. A help field **(4)** is located above these tiles, which can display a brief explanation of a function group by holding the mouse pointer over the corresponding tile.

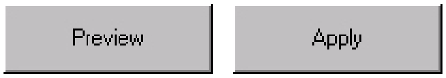
▶	Click the tile with the desired function group. On every navigational level, you can display an explanation of the function groups in the top help field by holding the mouse pointer over the corresponding tile.
▶	Select one tile each on the following navigational levels until you have reached the desired function with the corresponding dialog on the lowest level.
▶	You can directly select a function in the navigation window by clicking the corresponding folder.

The menu bar at the top right offers the following buttons **(5)**:


Back
Forward
Up
Reset

▶	Click Back to return to the previously displayed navigational level.
▶	Click Forward to display the level which you selected prior to clicking the Back button.
▶	Click Up to enter the next higher level in the hierarchy.
▶	Click Reset to return modified variables to their last setting.
▶	To exit the Process operating step, click the arrow symbol of another operating step (e.g. Acquire).

The bottom of every dialog displays the following two buttons:

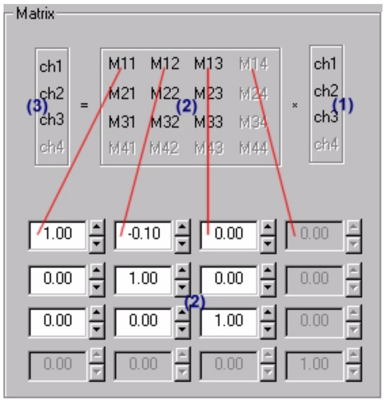


▶	Click Preview to execute the selected function and to display the resulting image on the right in the preview window.
▶	Click Apply to apply the selected function to the current image data set, to display the resulting image in the Viewer display window and to create a new file in the current experiment.

Navigation: Multicolor\ Crosstalk Reduction

Reducing crosstalk between detection channels

Crosstalk of fluorescence signals may occur during the recording of specimens marked with fluorescent dyes whose emission spectrums are close to each other or overlap. This leads to an unwanted transfer of signals from one detection channel to another. This function allows you to reduce this unwanted signal information of an image data set with up to four detection channels by means of a matrix multiplication.



1.00	-0.10	0.00	0.00
0.00	1.00	0.00	0.00
0.00	0.00	1.00	0.00
0.00	0.00	0.00	1.00

The dialog window displays the matrix multiplication. The pixel values of the 4 possible channels of the original image to be processed **(1)** are calculated with each other using the factors M11 through M44 **(2)**. The characteristic numbers of the M factors refer to the channels. For example, factor M12 represents the portion of signal information, which channel 2 of the original image **(1)** should have on channel 1 of the resulting image **(3)**. Since the factors M11, M22, M33 and M44 refer to the same channel in the original image and resulting image, the value of these factors is set to 1.

▶	In the Experiment Overview display window, select the image data set to be processed by double-clicking the file.
▶	By changing the M factor, you increase or decrease the signal information in the channel of the resulting image. For example, if the crosstalk of channel 2 in channel 1 is to be removed from the resulting image, enter a negative number between 0 and 1 in the M12 field. If you enter positive numbers between 0 and 1 in the M12 field, the signal information in channel 1 of the resulting image is increased.

Segmentation of Image Structures (Optional)

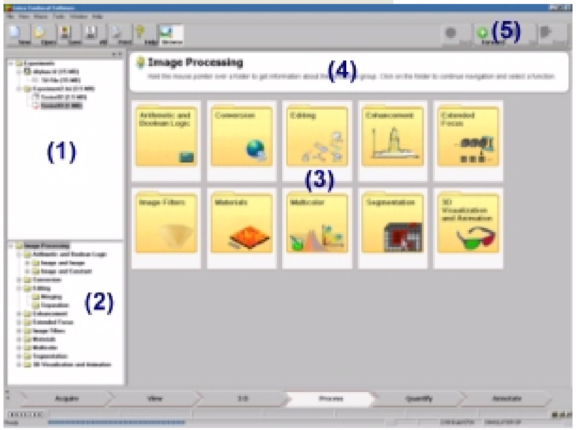
Segmentation

This chapter contains the following topics

- Setup and operation of the Process operating step
- What is segmentation in image processing?
- Navigation: Segmentation\ Thresholding
- Navigation: Segmentation\ Seeding (Threshold)
- Navigation: Segmentation\ Seeding (Single Channel)
- Navigation: Segmentation\ Seeding (Cross-Channel)

Setup and operation of the Process operating step

Process



After clicking the Process arrow symbol, the usual setup of the user interface is completely changed. All function buttons of the other button areas/operating steps are hidden. The Experiment Overview **(1)** display window is shown on the left with the opened experiments. The navigation window **(2)** with the directory tree of all functions available in this operating step is located underneath it. The middle of the user interface displays 10 large tiles **(3)**, which comprise the 10 function groups. A help field **(4)** is located above these tiles, which can display a brief explanation of a function group by holding the mouse pointer over the corresponding tile.

▶	Click the tile with the desired function group. On every navigational level, you can display an explanation of the function groups in the top help field by holding the mouse pointer over the corresponding tile.
▶	Select one tile each on the following navigational levels until you have reached the desired function with the corresponding dialog on the lowest level.
▶	You can directly select a function in the navigation window by clicking the corresponding folder.

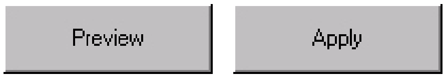
The menu bar at the top right offers the following buttons **(5)**:

Back Forward Up Reset

▶	Click Back to return to the previously displayed navigational level.
▶	Click Forward to display the level which you selected prior to clicking the Back button.

▶	Click Up to enter the next higher level in the hierarchy.
▶	Click Reset to return modified variables to their last setting.
▶	To exit the Process operating step, click the arrow symbol of another operating step (e.g. Acquire).

The bottom of every dialog displays the following two buttons:

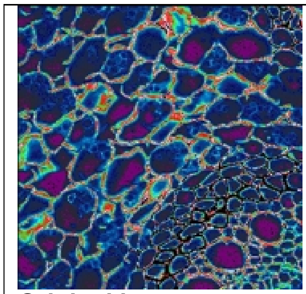
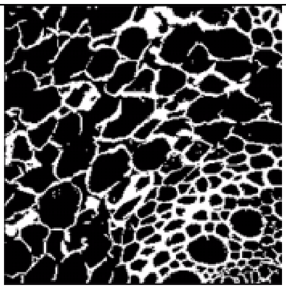
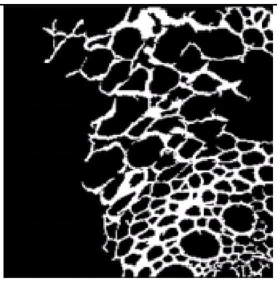


▶	Click Preview to execute the selected function and to display the resulting image on the right in the preview window.
▶	Click Apply to apply the selected function to the current image data set, to display the resulting image in the Viewer display window and to create a new file in the current experiment.

What is segmentation in image processing?
Many applications of confocal microscopy require extracting structures in an image and, for example, make them accessible as a whole for quantitative analyses. The segmentation process is applied to separate relevant from irrelevant image information. In image processing, segmentation is not only the separation of background and structure, but also the graduation of an image into coherent structures. There are pixel-oriented and region-oriented segmentation processes.

Pixel-oriented processes examine the intensity value of an isolated pixel. This process acquires the intensity value distribution of an image in a histogram and attempts to locate a threshold in it. This process is implemented in the Leica Confocal Software by means of the «Thresholding» function. It is possible to set an upper and a lower threshold. All pixels whose intensity values lies within the range defined by the threshold values receive the binary value 1 in the resulting image and all other pixels receive the binary value 0. The result is a binary image with the structure versus background information. The pixels coded as structure are all represented with the maximum gray value 255 (at 8-bit depth), those coded as background with gray value 0.

Compared to the pixel-oriented process, the region-oriented process of the segmentation takes into account that structures are coherent formations. For image processing, this means that every pixel must always be examined together with its adjacent pixels. In this case, the isolated examination of the intensity value of one pixel is no longer decisive for the identification of a pixel as structure or background, but the intensity value of a pixel within the context of the intensity values of its adjacent pixels. This process is implemented in the Leica Confocal Software by means of the «Seeding» functions (range growth functions). Starting with a start pixel (also referred to as «seed pixel»), the relation of the intensity values of start pixel and adjacent pixel is examined. A criterion is then used to decide which pixels in the binary resulting image are counted towards the structure (binary value 1) and which ones are counted towards the background (binary value 0). This criterion, for example, can be defined such that the intensity value of an adjacent pixel may not deviate more than 75% from the value of a seed pixel.

 **Original image**	 **Binary image** Pixel-oriented process, Thresholding	 **Binary image** Region-oriented process, Seeding

Navigation: Segmentation\ Thresholding

Pixel-oriented segmenting by means of a threshold

It is possible to define an upper and a lower threshold. The intensity value of each pixel in the image is then examined whether it falls inside or outside this threshold range. This method of segmentation is especially useful if the structures in the image do not touch and stand out clearly from the background.

▶	In the Experiment Overview display window, select the image data set to be processed by double-clicking the file.
▶	Specify the lower threshold separately for each detection channel in the Channels field on the Lower tab by entering a value between 0 and 255 (at 8-bit depth) or positioning the slider accordingly. Intensity values that fall below this threshold are set to 0 in the binary resulting image.
▶	Specify the upper threshold separately for each detection channel in the Channels field on the Upper tab by entering a value between 0 and 255 (at 8-bit depth) or positioning the slider accordingly. Intensity values that fall above this threshold are set to 0 in the binary resulting image.
▶	Click Lock Channels in the Options field if you want to specify the same threshold for all detection channels.
▶	Click the Suggest button if the software is to calculate a suitable lower threshold. The calculation of the threshold utilizes the algorithm that was selected on the Estimation tab (see below).

Estimation tab

Entropy Maximization: Calculating the maximum entropy

The foundation of this algorithm is the premise that a connection exists between the probability of an event and its information content. Therefore, more frequent events (often the background) have a lower information content and less frequent events (often the structure) have a higher one. The intensity values present in the image are divided into two sets and examined based on the intensity value at which the entropy—the probability of its occurrence—is the highest. The optimum threshold is the value at which the sum of the entropies of both sets is the highest or, in other words, at which the information content is the lowest.

Within Group Variance Minimization: Calculating the minimum value of group variance

The foundation of this algorithm is the premise that two normal distributions of low as well as high intensity values can be found in an image, which correspond to the structure and the background. The intensity values present in the image are examined for each possible threshold and two normal distribution curves are calculated. The threshold is then placed between these two curves.

Navigation: Segmentation\ Seeding (Threshold)

Region-oriented segmenting by means of a threshold

The user can define a lower and an upper threshold. Based on a selectable start pixel, the intensity values of all adjacent pixels are examined whether they fall inside or outside this threshold range. If no more adjacent pixels are found that fall inside the value range, the operation is finished.

▶	In the Experiment Overview display window, select the image data set to be processed by double-clicking the file.
▶	Specify the lower threshold in the Lower Threshold field and the upper threshold in the Upper Threshold field by entering a value between 0 and 255 (at 8-bit depth) or positioning the slider accordingly. Intensity values that fall below or above this threshold range are set to 0 in the binary resulting image.
▶	In the Position field of the Channels list field, select the detection channel whose image data is to be segmented. If the All option is selected, the segmentation is carried out separately for each detection channel.
▶	If the image data set is a time series or a wavelength series, select the fourth dimension in the list field on the right, i.e. a certain time or a certain wavelength.
▶	Position the graticule in the image for the definition of the start pixel by entering one value each for the x-, y- and z-coordinate in the Position field. Or move the graticule in the image to the desired position by pressing and holding the left mouse button.

Navigation: Segmentation\ Seeding (Single Channel)

Region-oriented segmenting in one channel

The user can define a permissible deviation (in per cent) from the intensity value of a selectable start pixel. Based on this start pixel, the intensity values of all adjacent pixels are examined, for example, whether they deviate by more than 75% from the value of the start pixel. If no more adjacent pixels are found that fall inside the permissible value range, the operation is finished. This operation is carried out separately for each selected detection channel.

▶	In the Experiment Overview display window, select the image data set to be processed by double-clicking the file.
▶	Specify the permissible deviation in per cent in the Threshold field by entering a value or positioning the slider accordingly. Intensity values that deviate more than the specified percentage range, are set to 0 in the binary resulting image.
▶	In the Position field of the Channels list field, select the detection channel whose image data is to be segmented. If the All option is selected, the segmentation is carried out separately for each detection channel.
▶	If the image data set is a time series or a wavelength series, select the fourth dimension in the list field on the right, i.e. a certain time or a certain wavelength.
▶	Position the graticule in the image for the definition of the start pixel by entering one value each for the x-, y- and z-coordinate in the Position field. Or move the graticule in the image to the desired position by pressing and holding the left mouse button.

Navigation: Segmentation\ Seeding (Cross-Channel)

Region-oriented segmenting across all channels

The user can define a permissible deviation (in per cent) from the intensity value of a selectable start pixel. Based on this start pixel, the intensity values of all adjacent pixels are examined, for example, whether they deviate by more than 75% from the value of the start pixel. If no more adjacent pixels are found that fall inside the permissible value range, the operation is finished. In contrast to the previous «Seeding (Single Channel)» function, this operation is carried out simultaneously across all detection channels.

▶	In the Experiment Overview display window, select the image data set to be processed by double-clicking the file.
▶	Specify the permissible deviation in per cent in the Threshold field by entering a value or positioning the slider accordingly. Intensity values that deviate more than the specified percentage range are set to 0 in the binary resulting image.
▶	The All option is fixed in the Position field of the Channels list field. If the image data set is a time series or a wavelength series, select the fourth dimension in the list field on the right, i.e. a certain time or a certain wavelength.
▶	Position the graticule in the image for the definition of the start pixel by entering one value each for the x-, y- and z-coordinate in the Position field. Or move the graticule in the image to the desired position by pressing and holding the left mouse button.

Measurement and Analysis Functions

Calculating a Histogram

Hist

Function

The Histogram function measures the frequency of a measurement variable, displays it in a curve and in addition, calculates various statistical values. The histogram is measured from the data set selected in the Viewer window. This data set can be a spatial series, a time series or a wavelength series. The statistical values are calculated dependent on the size of the third dimension—height (z) for spatial series, time (t) for time series, wavelength (λ) for wavelength series.

▶	Click on the Histogram button.
▶	A viewer window opens that displays the measurement curve for each detection channel and the statistical values:

Parameter	**Meaning**	**Formula**
# Pixel	The total number of pixels that are included in the calculation of the histogram. This corresponds to the configured scan format.	
Mean	Arithmetical mean value	$\mu(I) = \frac{1}{N_{Pixel}} \sum_i I_i$
Maximum	Maximum value	Max (I)
Minimum	Minimum value	Min (I)
Variance	Variance	$VAR(I) = \frac{1}{N-1} \sum_i (I_i - \mu(I))^2$
Average Deviation	Average deviation	$DISP(I) = \frac{1}{N-1} \sum_i \lvert I_i - \mu(I) \rvert$
Standard Deviation	Standard deviation	$s(I) = \sqrt{VAR(I)} = \sqrt{\frac{1}{N-1} \sum_i (I_i - \mu(I))^2}$
Mean Energy	Average image energy	$I^2 mean = \frac{1}{N_{Pixel}} \sum_i I_i^2$
Root Mean Square (RMS)	Root mean square value	$RMS = \sqrt{\frac{1}{N_{Pixel}} \sum_i I_i^2}$
Skewness	Skewness of the distribution	$Skew(I) = \frac{1}{N} \sum_i \left[\frac{I_i - \mu(I)}{\sqrt{VAR(I)}} \right]^3$

Some of the parameters are specified in scientific exponential notation, e.g. Pixel = 3.28e+005 = 327680 (corresponds to a scan format of 640 x 512).

Using the maximum and minimum values of intensity, the gain values and offset values can be optimally configured for the detectors.

Measuring a Profile Within a Region of Interest (ROI)

Function

The Profile (z) function measures a measurement variable within a region of interest, displays it in a curve and calculates various statistical values. The profile is measured from the data record selected in the Viewer window. This data set can be a spatial series, a time series or a wavelength series. The statistical values are calculated dependent on the size of the third dimension—height (z) for spatial series, time (t) for time series, wavelength (λ) for wavelength series.

▶	Click on the Profile (z) button and draw a region of interest (ROI) in the image. The buttons used to define a region of interest are activated together with the Profile (z) button. & see Defining the Region of Interest (ROI) as an Ellipse (page 218 ff.) & see Defining the Region of Interest (ROI) as a Polygon & see Defining the Region of Interest (ROI) as a Rectangle & see Automatically Defining the Region of Interest (ROI) & see Selecting and Moving the Region of Interest (ROI) & see Deleting Regions of Interest (ROI)
▶	A window opens automatically to display the measurement curve for every detection channel as well as the statistical values.

Parameter	Meaning	Formula
# pixel (ROI)	Number of pixels within the selected region of interest	
Area	Area of the region of interest	$A = \int_0^A d_A\, A$
Length	Height of image stack (spatial series) Recording time (time series) Bandwidth (wavelength series)	$L = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$
Mean Amplitude	Arithmetical mean	$\mu(I) = \frac{1}{N_{Pixel}} \sum_{Pixel} I_i$
Maximum Amplitude	Maximum value	Max (I)
Maximum amplitude position	Position of maximum value	Arg Max I (z), (t), (λ)
Minimum Amplitude	Minimum value	Min (I)
Minimum amplitude position	Position of minimum value	Arg Min I (z), (t), (λ)
Average Deviation	Average deviation	$DISP(I) = \frac{1}{N-1} \sum_i \lvert I_i - \mu(I) \rvert$
Standard Deviation	Standard deviation	$s(I) = \sqrt{VAR(I)} = \sqrt{\frac{1}{N-1} \sum_i (I_i - \mu(I))^2}$
Variance	Variance	$VAR(I) = \frac{1}{N-1} \sum_i (I_i - \mu(I))^2$

The following four measurement values appear only when the Two-Point Measurement function is selected in the Viewer Options dialog window.

▶	Select the View menu, the Experiment Overview option, and the Charts icon. & see Viewer Options Dialog Window, Charts Icon (page 148)
▶	In the Measurement field you can switch two-point measurement on or off by selecting 2 Point or Off.

I(µm); I(s); I(nm)	Intensity measured at measuring point 1 (z, t λ)	$I(z_1)$; $I(t_1)$; $I(\lambda_1)$
I(z); I(t); I(λ)	Intensity measured at measuring point 2 (z, t λ)	$I(z_2)$; $I(t_2)$; $I(\lambda_2)$
dI, dz	Difference of values between measuring point 1 and 2	$\lvert I(z_1) - I(z_2)\rvert$; $\lvert I(t_1) - I(t_2)\rvert$, $\lvert I(\lambda_1) - I(\lambda_2)\rvert$
d(z), (t), (λ)	Height (z), recording time (t), bandwidth (λ) between the measuring points 1 and 2	$\lvert z_1 - z_2\rvert$; $\lvert t_1 - t_2\rvert$, $\lvert \lambda_1 - \lambda_2\rvert$

Typical Applications
This function can be used to determine the extreme values of the intensity within an image stack. They in turn allow for the optimized setting of the detectors. In addition, this function can be used to determine the emission maximum of a wavelength series. By adapting the width and the position of the screens in front of the detectors, more emission light from the specimen can be recorded than with conventional optical filters. In time series, this function can be used to determine the time of the highest fluorescence activity.

This quantification function is only available off-line, i.e. for previously recorded data sets.

Measuring a Profile Along a Line Segment

Function
The Profile function measures a measurement variable along a line segment, displays it in a curve and calculates various statistical values. The profile is measured from the data record selected in the Viewer window. This data set can be a spatial series, a time series or a wavelength series. The statistical values are calculated dependent on the size of the third dimension—height (z) for spatial series, time (t) for time series, wavelength (λ) for wavelength series.

▶	Click on the Profile button. The measurement section is displayed as a white line in the image in the Viewer window. You can change the length and position of the line segment by clicking the line and, while clicking and holding the grab point with the left mouse button, dragging it to the desired position.
▶	A window opens automatically to display the measurement curve for every detection channel as well as the statistical values.

Parameter	**Meaning**	**Formula**
Length	Length of the measured section	$L = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$

Mean Amplitude	Arithmetic mean of measurement variable	$\mu(I) = \frac{1}{N_{Pixel}} \sum_{Pixel} I_i$
Maximum Amplitude	Maximum value of measurement variable	Max (I)
Minimum Amplitude	Minimum value of measurement variable	Min (I)
Average Deviation	Mean standard deviation of the values from the average value of the measurement variable	$DISP(I) = \frac{1}{N-1} \sum_i \lvert I_i - \mu(I) \rvert$
Standard Deviation	Standard deviation of the values of the measurement variable	$s(I) = \sqrt{VAR(I)} = \sqrt{\frac{1}{N-1} \sum_i (I_i - \mu(I))^2}$
Variance	Variance of the measurement variable	$VAR(I) = \frac{1}{N-1} \sum_i (I_i - \mu(I))^2$

The following four measurement values appear only when the Two-Point Measurement function is selected in the Viewer Options dialog window.

▶	Select the View menu, the Experiment Overview option, and the Charts icon. & see Viewer Options Dialog Window, Charts Icon (page 148)
▶	In the Measurement field you can switch two-point measurement on or off by selecting 2 Point or Off.

Parameter	**Meaning**	**Formula**
I(µm); I(s); I(nm)	Value measured at measuring point 1 (z, t, λ)	$I(z_1)$; $I(t_1)$; $I(\lambda_1)$
I(µm); I(s); I(nm)	Value measured at measuring point 2 (z, t, λ)	$I(z_2)$; $I(t_2)$; $I(\lambda_2)$
dI, dz	Difference of values between measuring point 1 and 2	$\lvert I(z_1) - I(z_2)\rvert$; $\lvert I(t_1) - I(t_2)\rvert$, $\lvert I(\lambda_1) - I(\lambda_2)\rvert$
d(z), (t), (λ)	Height (z), recording time (t), bandwidth (λ) between the measuring points 1 and 2	$\lvert z_1 - z_2\rvert$; $\lvert t_1 - t_2\rvert$, $\lvert \lambda_1 - \lambda_2\rvert$

This quantification function is only available off-line, i.e. for previously recorded data sets.

Measuring Surfaces and Volumes

Function

The Materials function calculates surfaces and volumes of the three-dimensional, spatial data set. Click on the Materials button to automatically open a window in which a measurement curve and the size of the accumulated volume are displayed depending on the z position. Accumulated volume means that for each z position the value of the volume of the data set lying below it is calculated.

A requirement for calculating the surfaces and volumes is a typographical image. Thus, the Materials button is only active if the Topography button was used before to create a topographical image of the data set. Only one detection channel can be quantified at a time.

& see Creating a Topographical Image (page 175)

Parameter	**Meaning**
Scanned Area A	This value represents the horizontal projection of the surface of an xy section.
Surface Area A'	This value represents the actual surface of an xy section. In relation to the horizontal projection the actual surface is always at least as large, in general even larger.
Ratio A'/A	This value represents the relation of surface to base area. The larger it is, the more the two-dimensional surface is embedded in the three-dimensional space.

The following four measurement values appear only when the Two-Point Measurement function is selected in the Viewer Options dialog window.

▶	Select the View menu, the Experiment Overview option, and the Charts icon. & see Viewer Options Dialog Window, Charts Icon (page 148)
▶	In the Measurement field you can switch two-point measurement on or off by selecting 2 Point or Off.

V(z<=upper position value)	This value represents the volume located below the upper position value of the measurement slider (right corner point of the slider).
V(z<=lower position value)	This value represents the volume located below the lower position value of the measurement slider (right corner point of the slider).
dV	Volume between the position values of the slider
dz	Distance between the position values of the slider

This quantification function is only available off-line, i.e. for previously recorded data sets.

Copying Quantification Graphs to the Annotation Sheet

QSnap

Function

The Snap Quantification Data function copies the data calculated by the quantification functions to the annotation sheet. The calculated values for the following quantification functions, which are also displaying an evaluation graph, can be copied to an annotation sheet:

& see Creating an Annotation Sheet (page 223)

Prof.	& see Measuring a Profile Along a Line Segment (page 214)
Pr. (z)	& see Measuring a Profile Within a Region of Interest (ROI) (page 213)

Mat.	& see Measuring Surfaces and Volumes (page 215)
R-Prof.	& see Measuring Roughness Along a Line Segment (page 231)

Printing Quantification Graphs

QPrint.

Function

The Print Quantification Data function sends the data calculated by the quantification functions to the standard printer. The calculated values for the following quantification functions, which are also displaying an evaluation graph, can be printed:

Prof.	& see Measuring a Profile Along a Line Segment (page 214)
Pr. (z)	& see Measuring a Profile Within a Region of Interest (ROI) (page 213)
Mat.	& see Measuring Surfaces and Volumes (page 215)
R-Prof.	& see Measuring Roughness Along a Line Segment (page 231)

This function is also available in the context menu within the quantification function window:

▶	Move the mouse pointer into the view window opened by a quantification function.
▶	Click the right mouse button, and select the Print menu item.

Exporting Quantification Data

QExp.

Function

Using he Export Quantification Data function, you can export the data calculated by the quantification functions as ASCII standard data. This enables you to read and edit data in Excel. The calculated values for the following quantification functions, which are also displaying an evaluation graph, can be exported:

Prof.	& see Measuring a Profile Along a Line Segment (page 214)
Pr. (z)	& see Measuring a Profile Within a Region of Interest (ROI) (page 213)
Mat.	& see Measuring Surfaces and Volumes (page 215)
R-Prof.	& see Measuring Roughness Along a Line Segment (page 231)

This function is also available in the context menu within the quantification function window:

▶	Move the mouse pointer into the view window opened by a quantification function.
▶	Click the right mouse button, and select the Export menu item.

Defining the Region of Interest (ROI) as an Ellipse

Ellip

Function

Using the Ellipse button, you can define an elliptic Region of Interest (ROI) within the image:

▶	Click the Ellipse button.
▶	Click the left mouse button on the image position where you would like to place a ROI corner.
▶	Then, keeping the left mouse button pressed, drag the mouse pointer diametrically to the opposite corner to define the second corner of the ROI.

& see Selecting and Moving the Region of Interest (page 221)
& see Moving and Rotating a Region of Interest (ROI) (page 222)
& see Deleting Regions of Interest (ROIs) (page 223)

Other Requirements

The definition of a region of interest in the image is required for quantification functions. Therefore, the Ellipse button is deactivated until you activate a quantification function using one of the following buttons:

Pr. (z)	& see Measuring a Profile Within a Region of Interest (ROI) (page 213)
Hist	& see Calculating a Histogram (page 212)
R-Area	& see Measuring Roughness Within a Region of Interest (ROI) (page 232)

Defining the Region of Interest (ROI) as a Polygon

Poly

Function

Using the Polygon button, you can define any polygonal Region of Interest (ROI) within the image:

▶	Click the Polygon button.
▶	Click the left mouse button on the image position where you would like to place a ROI corner.
▶	In order to define the other polygon corners, release the mouse button, move the cursor to the next polygon corner, and click the left mouse button again. Repeat this step for each desired polygon corner.
▶	If you want to draw the polygon by hand, keep the left mouse button pressed and draw the ROI into the image.
▶	Close the polygon with a double click.

& see Selecting and Moving the Region of Interest (page 221)
& see Moving and Rotating a Region of Interest (ROI) (page 222)
& see Deleting Regions of Interest (ROIs) (page 223)

Other Requirements

The definition of a region of interest in the image is required for quantification functions. Therefore, the Polygon button is deactivated until you activate a quantification function using one of the following buttons:

Pr. (z)	& see Measuring a Profile Within a Region of Interest (ROI) (page 213)
Hist	& see Calculating a Histogram (page 212)
R-Area	& see Measuring Roughness Within a Region of Interest (ROI) (page 232)

Defining the Region of Interest (ROI) as a Rectangle

Rect

Function
Using the Rectangle button, you can define a rectangular Region of Interest (ROI) within the image:

▶	Click the Rectangle button.
▶	Click the left mouse button on the image position where you would like to place a ROI corner.
▶	Then, keeping the left mouse button pressed, drag the mouse pointer diametrically to the opposite corner to define the second corner of the ROI.

& see Selecting and Moving the Region of Interest (page 221)
& see Moving and Rotating a Region of Interest (ROI) (page 222)
& see Deleting Regions of Interest (ROIs) (page 223)

Other Requirements
The definition of a region of interest in the image is required for quantification functions. Therefore, the Rectangle is deactivated until you activate a quantification function using one of the following buttons:

Pr. (z)	& see Measuring a Profile Within a Region of Interest (ROI) (page 213)
Hist	& see Calculating a Histogram (page 212)
R-Area	& see Measuring Roughness Within a Region of Interest (ROI) (page 232)

Automatically Defining the Region of Interest (ROI)

Wiz.

Function
You can use the Wizard button to have the computer define any structure in the image as a Region of Interest (ROI) automatically in the image. Depending on which of the buttons you have clicked, an elliptical, rectangular or polygonal region of interest is placed around the structure.

▶	Click on the Ellipse, Rectangle or Polygon buttons then on the Wizard button.
▶	Double-click with the left mouse button on the structure in the image that you want to mark as region of interest.
▶	The structure is automatically marked as an elliptical, rectangular or polygonal region of interest.

& see Defining the Region of Interest (ROI) as an Ellipse (page 218 ff.)
& see Defining the Region of Interest (ROI) as a Polygon
& see Defining the Region of Interest (ROI) as a Rectangle
& see Deleting Regions of Interest (ROIs)

Other Requirements
The definition of a region of interest in the image is required for quantification functions. The Wizard

button is only enabled when you have previously enabled a quantification function using one of the following buttons:

Pr. (z)	& see Measuring a Profile Within a Region of Interest (ROI) (page 213)
Hist	& see Calculating a Histogram (page 212)
R-Area	& see Measuring Roughness Within a Region of Interest (ROI) (page 232)

Selecting and Moving the Region of Interest (ROI)

Sel.

Function
Using the Select ROI button, you can select and move a Region of Interest (ROI) within the image:

▶	Click first the Select ROI button and then the ROI you want to move.
▶	Move the mouse pointer into the ROI.
▶	As soon as the mouse pointer changes to a crossed double arrow, press and hold the left mouse button.
▶	Keeping the left mouse button pressed, move the ROI.

You can change the size of the region of interest you have drawn:

▶	Hold the mouse pointer over one of the ROI's edges until a double arrow appears. Press the left mouse button to shift the shape of the ROI in one direction.
▶	If you place the mouse pointer exactly over a corner of the ROI, you can change its shape in two directions at once.

& see Moving and Rotating a Region of Interest (ROI) (page 222)
& see Deleting a Region of Interest (ROI) (page 223)

Other Requirements
The definition of a region of interest in the image is required for quantification functions. Therefore, the Select ROI is deactivated until you activate a quantification function using one of the following buttons:

Pr. (z)	& see Measuring a Profile Within a Region of Interest (ROI) (page 213)
Hist	& see Calculating a Histogram (page 212)
R-Area	& see Measuring Roughness Within a Region of Interest (ROI) (page 232)

Moving and Rotating the Region of Interest (ROI)

Rot.

Function
Using the Rotate ROI button, you can move and rotate a Region of Interest (ROI) within the image:

▶	Click first the Rotate ROI button and then the region of interest you want to rotate.
▶	Place the mouse pointer exactly on the corner of the selected region of interest.
▶	As soon as the mouse pointer changes to a double arrow, press and hold the left mouse button.
▶	Keeping the left mouse button pressed, rotate the region of interest. The region of interest will be rotated around its center point.

You can change the size of the region of interest you have drawn:

▶	Hold the mouse pointer over one of the ROI's edges until a double arrow appears. Press the left mouse button to shift the shape of the ROI in one direction.

& see Deleting Regions of Interest (ROIs) (page 223)

Other Requirements
The definition of a region of interest in the image is required for quantification functions. Therefore, the Rotate ROI is deactivated until you activate a quantification function using one of the following buttons:

Pr. (z)	& see Measuring a Profile Within a Region of Interest (ROI) (page 213)
Hist	& see Calculating a Histogram (page 212)
R-Area	& see Measuring Roughness Within a Region of Interest (ROI) (page 232)

Deleting Regions of Interest (ROIs)

Clear

Function
The Clear button deletes all defined ROIs at once. To delete an individual ROI, click the Select ROI button, then the ROI in the image, and press the DELETE key on the keyboard.

Other Requirements
The definition of a region of interest in the image is required for quantification functions. Therefore, the Clear is deactivated until you activate a quantification function using one of the following buttons:

Pr. (z)	& see Measuring a Profile Within a Region of Interest (ROI) (page 213)
Hist	& see Calculating a Histogram (page 212)
R-Area	& see Measuring Roughness Within a Region of Interest (ROI) (page 232)

Data Documentation

Creating an Annotation Sheet

Annot.

Function
Use the Annotation button to open a window in which you can prepare recorded images for presentation purposes. If you have opened the annotation sheet, the Snap, Line, Rectangle and Text buttons are also enabled. Use these buttons to copy the image loaded in the Viewer window into the annotation sheet, to highlight specific areas of the copied image with lines and rectangles and to enter an image comment into a text field.

& see Drawing a Line (page 224)
& see Drawing a Rectangle (page 225)
& see Adding a Text Field (page 225)

If you hold the mouse pointer over the annotation sheet and press the right mouse button, a context menu containing the following commands appears:

Command	Function
Line	Use this to insert a line of default size onto the annotation sheet.
Rectangle	Use this to insert a rectangle of default size onto the annotation sheet.
Text	Use this to insert a text field with a default font onto the annotation sheet.
Zoom	Use this to select from four magnifications for viewing the annotation sheet.
Grid	This displays a grid on the annotation sheet, which can not be printed.

The Line, Rectangle and Text commands can be activated both from the context menu and by clicking their corresponding buttons.

Saved annotation sheets are given the *.ano file extension.

Copying an Image into the Annotation Sheet

Function
If you click the Snap button, the image loaded in the Viewer window is copied onto the annotation sheet. If you mark the copied image and then press the right mouse button, a context menu containing the following commands appears:

Command	**Function**
Original size	This displays the image in its original size.
Fit to page	This enlarges the image to the size of the annotation sheet.
Bring to front	This brings the image to the foreground.
Send to back	This sends the image to the background.
Delete	This deletes the image.

You can change the size of the image by dragging one of the image grab points. To move the image without changing its size, mark the image and move it while pressing the left mouse button.

The Snap button can be applied only if an annotation sheet has been opened using the Annotation button first and the Viewer window has been clicked.

Drawing a Line on the Annotation Sheet

Function
Use the Line button to draw a line on the annotation sheet. On the annotation sheet, click the position where you want the line to begin. Keeping the left mouse button depressed, drag the mouse pointer across the page to the position where you want the line to end. If you mark the line and then press the right mouse button, a context menu containing the following commands appears:

Command	**Function**
Style	This opens a dialog window where you can configure the style, thickness and length of the line.
Color	This opens a dialog window for selecting a color for the line.
Bring to front	This brings the line to the foreground.
Send to back	This sends the line to the background.
Delete	This deletes the line.

You can change the length of the line by dragging one of its grab points. To move the line without changing its size, click the center grab point and move the line while pressing and holding the left mouse button.

> *The Line button can be applied only if the annotation sheet has been opened using the Annotation button first.*

Drawing a Rectangle on the Annotation Sheet

Rect

Function

Use the Rectangle button to draw a rectangle on the annotation sheet. On the annotation sheet, click the position where you want the corner of the rectangle to be. Keeping the left mouse button depressed, drag the mouse pointer across the page to define the size of the rectangle. If you mark the rectangle and then press the right mouse button, a context menu containing the following commands appears:

Command	**Function**
Style	This opens a dialog window where you can configure, among other options, the style and thickness of the line.
Color	This opens a dialog window for selecting a color for the rectangle.
Bring to front	This brings the rectangle to the foreground.
Send to back	This sends the rectangle to the background.
Delete	This deletes the rectangle.

You can change the size of the rectangle by dragging one of the image grab points of the rectangle. To move the rectangle without changing its size, click in the middle of the rectangle and move it while pressing and holding the left mouse button.

> *The Rectangle button can be applied only if the annotation sheet has been opened using the Annotation button first.*

Adding a Text Field to an Annotation Sheet

Text

Function

Use the Text button to insert a text field onto the annotation sheet. On the annotation sheet, click the position where you want the text field to be placed. Keeping the left mouse button depressed, drag the mouse pointer across the page to define the size of the text field. If you mark the text field and then press the right mouse button, a context menu containing the following commands appears:

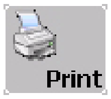
Command	**Function**
Font	This opens a dialog window used to select from various fonts and other formatting

	options.
Transparent	This makes the background visible through the text field.
Bring to front	This brings the text to the foreground.
Send to back	This sends the text to the background.
Delete	This deletes the text.

You can change the size of the text field by dragging one of the image grab points of the text field. To move the text field without changing its size, click in the middle of the text field and move it while pressing and holding the left mouse button.

> *The Text button can be applied only if the annotation sheet has been opened using the Annotation button first.*

Printing



Function

You can trigger the print command either by clicking the Print button or from the Printer Selection dialog window. To open this dialog window, select the Print option in the File menu. The image loaded in the Viewer window or an annotation sheet can be printed.

When you initiate the Print command, the image currently loaded in the Viewer window or the currently open annotation sheet is printed using the default printer and default page layout. You can change these default settings in the Printer Selection dialog window to suit your needs. Use the following buttons provided in the dialog window to do so:

Button	**Function**
Printer setup	This opens the dialog window for selecting a printer and changing the printer settings.
Print	This prints the image or the annotation sheet.
Background color	Use this to open a dialog window for selecting a background color for the page.
Center on page	This centers the image on the page.
Fit to page	This option changes the size of the image to fit the size of the printable area.
Aspect ratio	The height-to-width ratio remains the same when the size of the image changes.

Use the Image field to change the height (Size Y) and width (Size X) of the image and to define a top margin (Offset Y) and a left margin (Offset X). The image size and margins can not be modified if the Fit to page option is enabled. If you have enabled Center on Page, you can change the size but not the margins.

Check the results of your settings in the Print Preview field. Use the Page field to specify the height and width of the printable area (which is not identical to the paper size). The two bottom lines of the dialog window display the currently installed printer and configured paper size.

> *Close the Printer Selection dialog window by clicking OK to save the new settings for the active Viewer window. This allows you to store different printer settings and page layouts for different images. However, these settings are not permanent and are lost when you exit the Leica Confocal Software.*

Data Handling

Opening a File

Function
With the Leica Confocal Software, you can open various file types using the Open button.

File Type	Description
Experiment (*.lei)	A Leica-specific, binary data format. When the experiment is loaded, both the image data and the experiment settings are loaded.
Annotation sheet (*.ano)	A Leica-specific, binary data format. The elements on the annotation sheet, such as images, texts and graphic images, are each available as individual objects.
Tiff files (*.tif)	These are Leica image files in single and multi Tiff format. External files in RGB-Tiff format can be loaded as well.

& see Creating an Experiment (page 230)
& see Creating an Annotation Sheet (page 223)

Saving a File

Function
Click the Save button to save the data of the current experiment (*.lei) or the current annotation sheet (*.ano).

If you use the Save button to save an experiment or annotation sheet for the first time, the Save As dialog window opens.

> *It is easy to inadvertently overwrite your original data using the Save function. To avoid doing so, use the Save As function to save an experiment that has already been saved once or more under a new name.*

& see Saving a File As... (page 228)

Saving a File As...

Save..

Function
Use the Save As button to save an experiment (*.lei) or annotation sheet (*.ano) while giving it a specific name and file type.

When saving an experiment, a folder is created at the file level with the name of the experiment. This folder then contains the description file (*.lei) for the experiment as well as the individual image files. The format of the description file is Leica-specific and binary. This file contains the parameter settings and the color information (in the form of color look-up tables) for each image belonging to the experiment.

The image files of an experiment can be saved in *tif* or *raw* format. The standard format, *tif*, also contains the experiment settings and the color information for the images. In the *raw* format, just the image data is saved.

Typical Applications
The advantage of the *raw* format is the smaller file size, but this is only significant for image recordings with relatively small amounts of data. For example, if you record an image series with a large number of individual images but at a low scan format, you can save and open it faster in *raw* format than in *tif*.

Storing All Data

Σ All

Function
Use the Save All button to save experiments (*.lei) or annotation sheets (*.ano) while giving them a specific name and file type.

When saving an experiment, a folder is created at the file level with the name of the experiment. This folder then contains the description file (*.lei) for the experiment as well as the individual image files. The format of the description file is Leica-specific and binary. This file contains the parameter settings and the color information (in the form of color look-up tables) for each image belonging to the experiment.

The image files of an experiment can be saved in *tif* or *raw* format. The standard format, *tif*, also contains the experiment settings and the color information for the images. In the *raw* format, just the image data is saved.

Typical Applications
The advantage of the *raw* format is the smaller file size, but this is only significant for image recordings with relatively small amounts of data. For example, if you record an image series with a large number of individual images but at a low scan format, you can save and open it faster in *raw* format than in *tif*.

User-Specific Adjustments

Controlling Functions Using the Control Panel

Function
At frequent points during an image recording, various parameters have to be reset. For this reason, the control panel can be used to control functions quickly and directly. You can freely allocate the radio buttons of the control panel to a selection of functions:

▶	In the View menu, select the Status Bars option and then Control Panel Status Bar.

The status bar for the control panel is displayed at the bottom of the user interface of the Leica Confocal Software. Viewed from left to right, the status bar consists of the icon for the control panel, the seven fields that represent the radio buttons of the control panel, and three buttons.

▶	Click on one of the fields arranged in the same way as the radio buttons of the control panel.
▶	A list is opened where you can select the desired function. The name of the allocated function is shown in the field.

The sensitivity of the radio buttons can be adjusted:

▶	Click with the right mouse button on a recording parameter in the status bar of the control panel.
▶	The recording parameters Phase, Pinhole, Scan Field Rotation and Zoom allow selecting one of three sensitivity stages: Smooth, Moderate and Rough.
▶	For the recording parameters Gain, Offset, Smart Gain and Smart Offset, the number of volts can be adjusted by rotating the radio button.
▶	For the recording parameters Z Position and Z Wide Position, the number of micrometers can be adjusted by rotating the radio button.

Additional information about this topic can be found in the separate online help:

Tutorials (Exercises for Using Leica Confocal Software)

If you have assigned a function to all the radio buttons, you can save this configuration as a template.

▶	Click on the left of the three buttons located on the right side of the status bar.
▶	A dialog window is opened in which you can give the configuration a name and save it.
▶	Click on the middle button to select and load a configuration.

In addition to these user-defined configurations, you can also access other configurations predefined at the factory:

▶	Click on the right of the three buttons located on the right edge of the status bar.
▶	This opens a dialog window listing all the configurations for the control panel.

The configurations set as default, indicated by a **L** (Leica), can only be loaded but not modified. The user-defined configurations are created in the drop-down list under User and marked with a **U** (User). Click one of the configurations and then press the right mouse button to open a context menu which contains the following commands:

Command	**Function**
Set as default template	The configuration is loaded as the default configuration when the software is started.
Remove default setting	The setting of this configuration as default setting is undone.
Load	Loads the configuration.
Rename (only for **U**)	Use this to change the name of the configuration.
Delete (only for **U**)	The configuration is deleted.

Creating an Experiment

New

Function
Click the New Experiment button to open a new Viewer window, which creates a new experiment. An experiment is a file that consists of one ore more individual images or image series. This allows you to keep several images, each recorded using different scan parameters, or edited versions of images in one experiment. The format of the experiment file (*.lei) is Leica-specific.

Saving the Viewer Window as a Template

Templ

Function
Using the Template button, you can save a user-defined design of the Viewer window as a template. The elements of the Viewer window that can be shown or hidden, are the buttons the color bars of the color look-up tables and the Experiment legend.

& see Viewer Window (page 143)

Clicking the Template button opens a dialog window that you can use to enter a name for the template. This configuration of the Viewer window is saved and will be loaded each time you open a file or create a new experiment.

Additional information
In the Tools menu, click on Options and select the Viewer Template tab to access the user-defined templates. In the Leica Templates list box, you can choose from the following predefined templates:

Viewer Template	**Design**
Viewer (Pure)	The view window consists only of the image window.
Viewer (LUT)	The view window consists of the image window and the color bars of the color look-up tables.
Viewer (Standard)	The view window consists of the buttons, the image window, the color bars of the color look-up table, and the Experiment legend.

In the Personal Templates List box, the user-defined templates are entered. Using the Add Active Viewer and Remove Template buttons, you can save a new template or delete an existing one.

In the Tools menu, click on Options to indicate in the Workspace tab how many view windows, if any, Viewer should open when the Leica Confocal Software is started.

Optional Software Packages

Materials

Measuring Roughness Along a Line Segment

R-Prof.

Function
The Roughness Profile function measures height, distance, and angular values of a surface along a specific line and displays these values in a curve. The height profile is measured from the data record selected in the Viewer window. This data set should be a spatial series.

A topographical image is the prerequisite for the generation of a height profile. For this reason, the Roughness Profile button is active only when a topographic image of the data record has been created in advance with the Topography button. Only one detection channel can be quantified at a time.

& see Creating a Topographical Image (page 175)

▶	Click on the Roughness Profile button. The measurement section is displayed as a white line in the image in the Viewer window. You can change the length and position of the line segment by clicking the line and, while clicking and holding the grab point with the left mouse button, dragging it to the desired position.
▶	A window opens automatically to display the measurement curve for every detection channel as well as the statistical values.

Parameter	**Meaning**	**Formula**
Evaluation length: l(P)	Length of the plotted section, according to DIN EN ISO 4287	$L = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$
Max. Peak: Pp	Height of the highest profile peak (based on the average height), according to DIN EN ISO 4287	Max (Pp)
Min. Valley: Pv	Depth of the greatest profile valley (based on the average height), according to DIN EN ISO 4287	Min (Pv)
Deviation: Pa	Arithmetic average of the profile ordinates within the measured section (average height) according to DIN EN ISO 4287	$P_a = \frac{1}{l}\int_0^l \lvert Z(x)\rvert dx$ with $Z(x) = Z_i - \bar{Z}$
RMS: Pq	Root mean square value of the profile ordinates within the measured section, according to DIN EN ISO 4287	$P_q = \sqrt{\frac{1}{l}\int_0^l \lvert Z^2(x)\rvert dx}$ with $Z(x) = Z_i - \bar{Z}$

The following four measurement values appear only when the Two-Point Measurement function is selected in the Viewer Options dialog window.

▶	Select the View menu, the Experiment Overview option, and the Charts icon. & see Viewer Options Dialog Window, Charts Icon (page 148)
▶	In the Measurement field you can switch two-point measurement on or off by selecting 2 Point or Off.

Parameter	**Meaning**	**Formula**
h (position of the slide's left measurement point)	Profile height at the position of the slide's left measurement point	$h_1 (l_1)$
h (position of the slide's right measurement point)	Profile height at the position of the slide's right measurement point	$h_2 (l_2)$
Step Height dh	The profile's height difference between the slide's left and right measurement points	$\lvert h_2 - h_1 \rvert$
dx	Distance between the slide's left and right corners	$\lvert l_2 - l_1 \rvert$
alpha	The angle of the (left measurement point to right measurement) connection line from horizontal	$\arctan\left(\frac{dh}{dx}\right)$

Saving the measurement values
The software can save the values on the positions of both measurement points as well as the height difference between the measurement points.

►	Select the View menu, Experiment Overview option, Surface Measure icon. & see Viewer Options Dialog Window, Surface Measure Icon (page 153)
►	To save the values, click on the Remember button in the field Multipoint Measurement. The Clear button deletes them again.

This quantification function is only available off-line, i.e. for previously recorded data sets.

Measuring Roughness Within a Region of Interest (ROI)

Function
The Roughness Area function measures height, distance, and angular values of a surface within a region of interest. The profile is measured from the data record selected in the Viewer window. This data set should be a spatial series.

A topographical image is the prerequisite for the generation of a height profile. For this reason, the Roughness Area button is active only when a topographic image of the data set has been created in advance with the Topography button.
& see Creating a Topographical Image (page 175)

►	Click on the button Roughness Area and draw a region of interest (ROI) into the image. The buttons with which you define a ROI are activated together with the Roughness Area button. & see Defining the Region of Interest (ROI) as an Ellipse (page 218 ff.) & see Defining the Region of Interest (ROI) as a Polygon & see Defining the Region of Interest (ROI) as a Rectangle & see Automatically Defining the Region of Interest (ROI) & see Selecting and Moving the Region of Interest (ROI) & see Deleting Regions of Interest (ROI)
►	A window opens automatically to display the statistical values.

Parameter	**Meaning**	**Formula**
# Pixel	Number of pixels within the selected region of interest	

Area	Area	$A = \int_0^A d_A\ A$
Avg. Height	Average height of the profile ordinates	$H = \frac{1}{A}\int_0^A Z(x,y)\,dA$
Pa	Arithmetic average of the profile ordinates within the measured section (average height) according to DIN EN ISO 4287	$P_a = \frac{1}{A}\int_0^A \lvert Z(x,y)\rvert\,dA$ with $Z(x,y) = Z_i - \bar{Z}$
RMS (Pq)	Root mean square value of the profile ordinates within the measured section, according to DIN EN ISO 4287	$Pq = \sqrt{\frac{1}{A}\int_0^A \lvert Z^2(x,y)\rvert\,dA}$ with $Z(x,y) = Z_i - \bar{Z}$
Min Valley (Pv)	Minimal height of the profile ordinates (based on the average height)	Min (Pv)
Max Peak (Pp)	Maximal height of the profile ordinates (based on the average height)	Max (Pp)

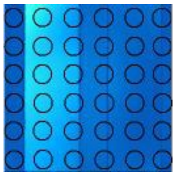
This quantification function is only available off-line, i.e. for previously recorded data sets.

Appendix

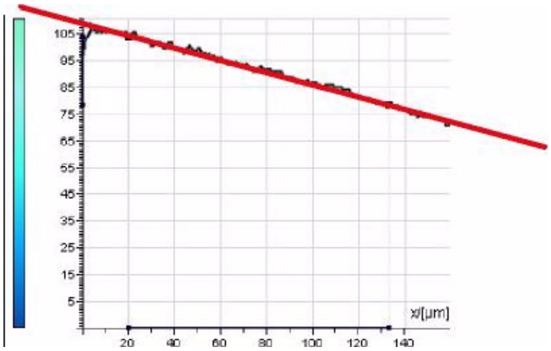
Adaptation Function for Correcting Trend Curves

& see Process Operating Step / Materials Folder (page 202)

1. Selecting the step width, that is, the distance of the support positions

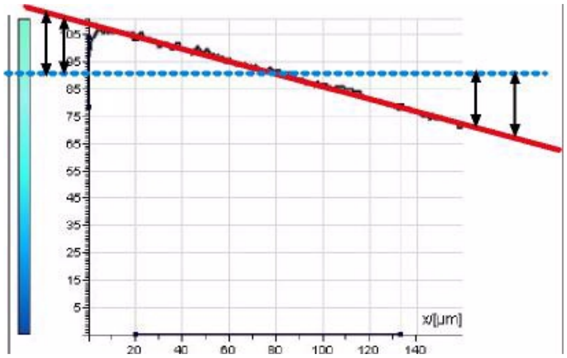


2. Selecting the character of the interpolation function to be adapted

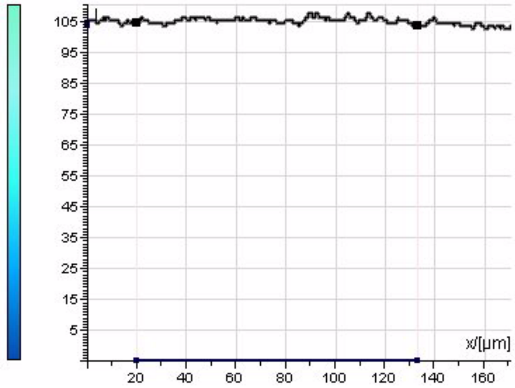


3. Calculating the interpolation function f(x) = a + bx + cy

4. Adding the intensity differences to the leveling of the trend curve

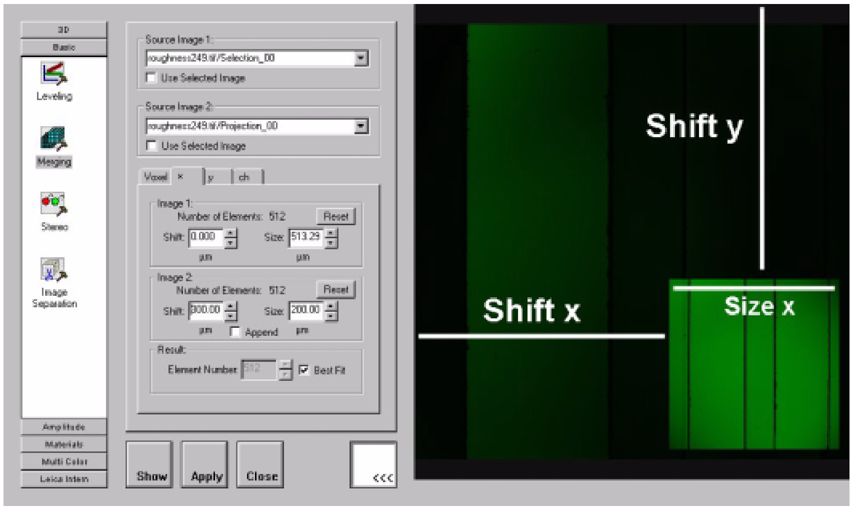


before leveling



after leveling

Example for Moving an Image in the Resulting Image


3D
Basic
Leveling
Merging
Stereo
Image Separation
Amplitude
Materials
Multi Color
Leica Intern
Voxel
x
y
ch
Reset
Reset
Show
Apply
Close
<<<
Shift y
Shift x
Size x

Source Image 1: roughness249.tif/Selection_00
☐ Use Selected Image

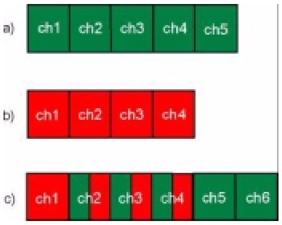
Source Image 2: roughness249.tif/Projection_00
☐ Use Selected Image

Image 1:
Number of Elements: 512
Shift: 0.000 µm
Size: 513.29 µm

Image 2:
Number of Elements: 512
Shift: 300.00 µm
Size: 200.00 µm
☐ Append

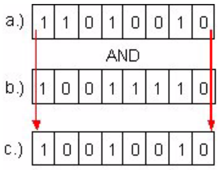
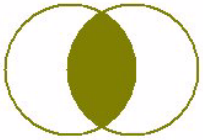
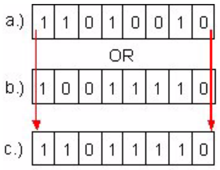
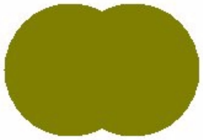
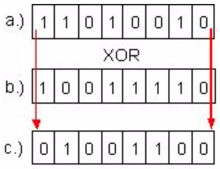
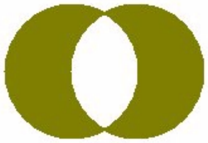
Result:
Element Number: 512
☑ Best Fit

Example for Merging Images of Different Channel Numbers (Merging)


a)
ch1
ch2
ch3
ch4
ch5
b)
ch1
ch2
ch3
ch4
c)
ch1
ch2
ch3
ch4
ch5
ch6

a.) First output image (setting: Start=2)
b.) Second output image (setting: Start=1)
c.) Merged image

Operating Principle of Boolean Operations

AND	Write a 1 if there is a 1 in pixel 1 AND in pixel 2; otherwise write 0	 a.) 1 1 0 1 0 0 1 0 AND b.) 1 0 0 1 1 1 1 0 c.) 1 0 0 1 0 0 1 0 a.) Binary representation of pixel 1 b.) Binary representation of pixel 2 c.) Binary representation of result pixel	 AND represents the intersection of two images
OR	Write a 1 if there is a 1 in pixel 1 OR in pixel 2; otherwise write 0	 a.) 1 1 0 1 0 0 1 0 OR b.) 1 0 0 1 1 1 1 0 c.) 1 1 0 1 1 1 1 0 a.) Binary representation of pixel 1 b.) Binary representation of pixel 2 c.) Binary representation of result pixel	 OR represents the union of sets of two images
XOR	Write a 1 if the values in pixel 1 and in pixel 2 differ; otherwise write 0	 a.) 1 1 0 1 0 0 1 0 XOR b.) 1 0 0 1 1 1 1 0 c.) 0 1 0 0 1 1 0 0 a.) Binary representation of pixel 1 b.) Binary representation of pixel 2 c.) Binary representation of result pixel	 XOR represents the union of sets without the intersection of two images.

Glossary

Aberration, chromatic	*An optical image distortion conditional on the varying refraction of light rays of different wavelengths on a lens. Thus light rays of shorter wavelengths have longer focal distances than light rays of longer wavelengths.*
Aberration, spherical	*An optical image distortion conditional on the varying distance of paraxial light rays of the same wavelength from the optic axis. Light rays that travel through outer lens zones have shorter focal distances than rays that travel through the lens center (optic axis).*
Achromatic	*Describes a correction class for objectives. The chromatic aberration for two wavelengths is corrected for objectives of this type. Usually an objective of this type is corrected to a wavelength below 500 nm and above 600 nm. Furthermore, the sine condition for one wavelength is met. The image curvature aberration is not corrected.*
Airy disc	*The Airy disc refers to the inner, light circle (surrounded by alternating dark and light diffraction rings) of the diffraction pattern of a point light source. The diffraction discs of two adjacent object points overlap some or completely, thus limiting the spatial resolution capacity.*
Aliasing	*An image distortion caused by a sampling frequency that is too low in relation to the signal frequency.*
AOTF **Acousto-Optical Tunable Filter**	*The acousto-optical tunable filter is an optic transparent crystal that can be used to infinitely vary the intensity and wavelength of radiated light. The crystal generates an internal ultrasonic wave field, the wavelength of which can be configured to any value. Radiated light is diffracted vertically to the ultrasonic wave field like through a grid.*
Aperture, numerical	*Aperture is the sine of the opening angle under which light enters into the front lens of a microscope objective; its symbol is NA. The aperture influences both the light intensity and the resolution capacity of an objective optical system. Since various media can be present between specimen and objective lens (such as the embedding medium for the specimen), the numerical aperture (NA = n * sinα) is usually applied as the unit of measurement for the light intensity and the resolution capacity.*
Apochromatic	*Describes a correction class for objectives. The chromatic aberration for three wavelengths is corrected for objectives of this type (usually 450 nm, 550 nm and 650 nm) and the sine condition for at least two colors is met. The image curvature aberration is not corrected.*
Bleaching, optical	*The destruction of fluorochromes, by intense lighting. In fluorescence microscopy, fluorochromes are excited with laser light to a high state of energy, the singlet state. When the excited molecules return to their normal energy state, a fluorescent signal is emitted. If the intensity of the excitation is too high however, the color molecules can change via intercrossing from a singlet state to a triplet state. Due to the significantly longer life of triplet states (phosphorescence), these excited molecules can react with triplet-oxide and be lost for further fluorescence excitation.*
Confocality	*While the optical design of conventional microscopes allows the uniform detection of focussed and unfocussed image components, the confocal principle suppresses the structures found outside of the focal plane of the microscope objective. Screens are implemented in optically conjugated locations to achieve this. They function as point light source (excitation screen) and point detector (detection screen). The diameter of the detection screen, along with the wavelength and numerical aperture of the objective being used, determines the axial extension of an optical section (optical resolution).*
Dichroic	*Dichroic filters are interference filters at an angle of incidence of light of 45°. The transmissivity or reflectivity of dichroites depends on a specific wavelength of light. For an* ***RSP*** *510 filter (****r****eflection* ***s****hort* ***p****ass) for example, the excitation light below 510 nm is reflected and the excitation light above this value is transmitted. The transmission values are generally between 80% and 90% and*

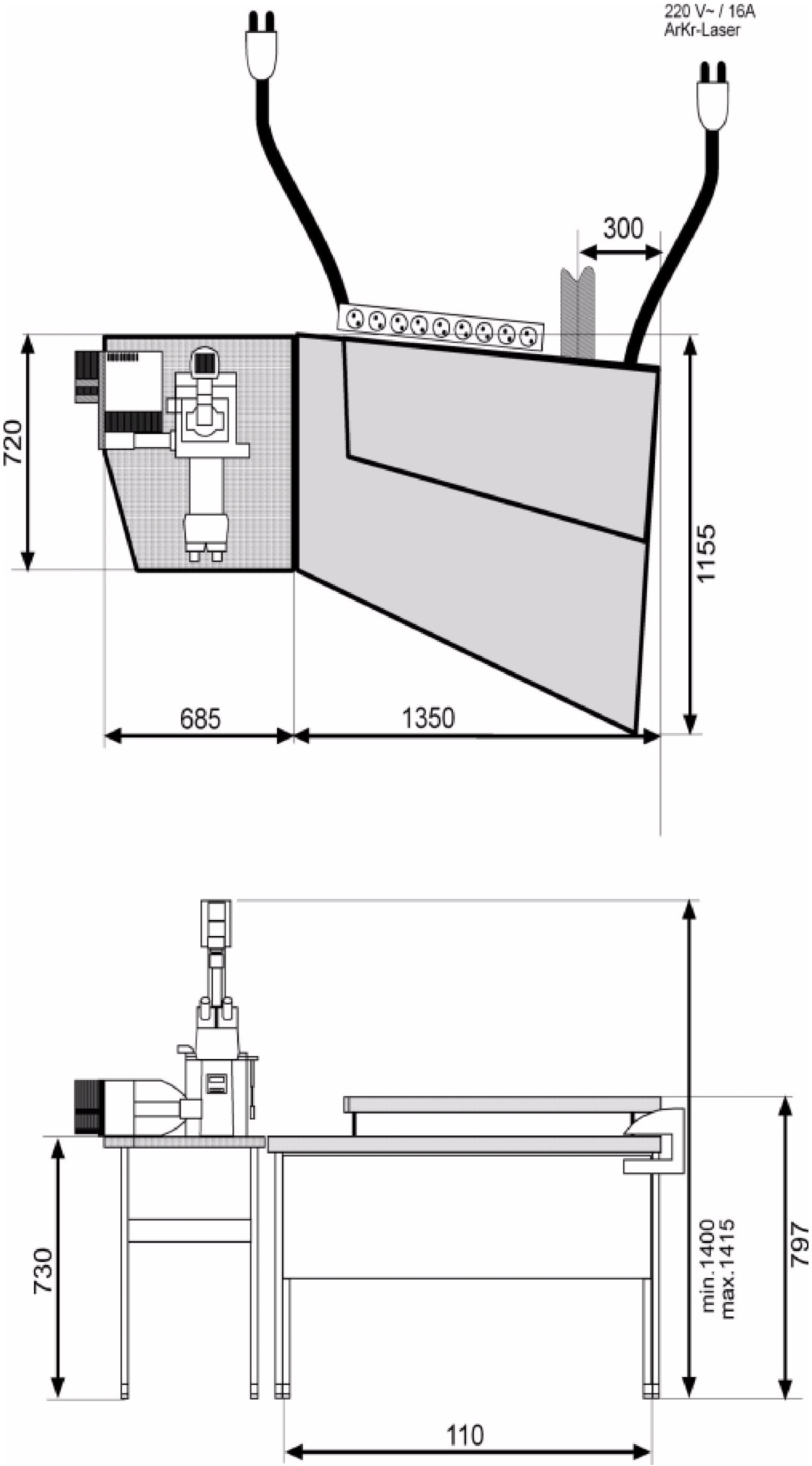
	the reflection values between 90% and 95%.
Double dichroite	*Double dichroic filters are interference filters at an angle of incidence of light of 45°. The transmissivity or reflectivity of double dichroites depends on two specific wavelengths of light. For a **d**ouble**d**ichroite DD 488/568, for example, the excitation light at 488 nm and 568 nm is reflected and the excitation light above these values is transmitted. The transmission values are generally at 80% and the reflection values are between 90% and 95%.*
Dry objective	*A microscopic objective used without immersion media. Between the objective lens and the specimen is air.*
Empty magnification	*A magnification without additional gain of information. Empty magnification is used as soon as distances are displayed that are smaller than the optical resolution. Magnifications with a larger scale than that of the empty magnification do not provide any additional information about the object but, instead, only diminish the focus and the contrast.*
Filter, digital, phase-true	*A digital filter consists of a computing rule used to modify image data. Filters are always applied to remove disturbing image components. A phase-true filter ensures that quantifiable image values do not change through filtering and remain a requirement for standardized measuring methods (e.g., characterization of surfaces in accordance with ISO).*
Fluorescence microscopy	*A light-optical contrast process for displaying fluorescent structures. Auto-fluorescent samples have a so-called primary fluorescence. They do not need to be enriched with additional, fluorescent substances. Secondary fluorescent substances, on the other hand, have to be treated with appropriate dyes or stains called fluorochromes. Specific dyeing methods additionally allow the precise localization of the stained structure elements of an object. Fluorescence microscopy allows both potential morphological examinations and the ability to carry out dynamic examinations on a molecular level.*
Fluorite objectives	*Describes a correction class for objectives. Fluorite objectives are semi-apochromatic, meaning their degree of correction lies between the achromatic and apochromatic.*
Frame	*A frame corresponds to the acquisition of a single optical section. For example, if a single optical section is acquired 4 times (to average the data and to eliminate noise), then 4 frames are created for this optical section.*
Image curvature aberration	*The curved surface to which a microscopic image is to be clearly and distinctly mapped is described as image curvature aberration. It is conditional on the convex shape of the lens and makes itself apparent as an error due to the short focal distances of microscope objectives. Here the object image is not in focus both in the center and at the periphery at the same time. Objectives that are corrected for image curvature aberration are called plane objectives (plane = flat image field).*
Immersion objective	*A microscopic objective, developed with the requirements for applying immersion media. The use of incorrect or no immersion medium with an immersion objective can lead to resolution loss and impairment of the correction.*
Neutral filter	*Neutral filters are semi-reflective glass plates. They are used to distribute the light path independent of wavelength. The incident light is partially reflected and partially transmitted. Neutral filters are usually placed at a 45° angle in the path of the beam. The ratings of a neutral filter are based on its reflectivity-to-transmissivity ratio. A neutral filter **RT** 30/70, for example, **r**eflects 30% of the excitation light and **t**ransmits 70%.*
Phase visualization	*The principle of phase visualization as used by Leica is an optimized alternative method to ratiometric displaying. The main area of application lies in measuring ion concentrations in physiology. In contrast with ratiometric procedures, phase visualization obtains more information on the specimen. In addition, this method allows for adapting the display of physiological data to the dynamics of the human eye. Detailed information on the principle of phase visualization can be obtained directly from Leica Microsystems Heidelberg GmbH.*
Pixel	*An acronym based on the words, picture and element. A pixel represents the smallest, indivisible image element in a two-dimensional system. In this*

	documentation, both the sampling points of the specimen as well as the image points are referred to as pixels.
Plane objectives	*Describes a correction class for objectives. The image curvature aberration is corrected for objectives of this type. Correcting this error requires lenses with stronger concave surfaces and thicker middles. Three types of plane objectives, plane achromate, plane apochromate and plane fluorite, are based on the type of additional correction for chromatic aberration.*
Reflection long pass filter	*Reflection long pass filters are interference filters that reflect short-wave light but are transparent for long-wave light. An optical reflection long pass filter is characterized by the reading of the wavelength edge at which the filter changes from reflection to transmission (50% threshold).*
Reflection short pass filter	*Reflection short pass filters are interference filters that transmit short-wave light while reflecting long-wave light. An optical reflection short pass filter is characterized by the reading of the wavelength edge at which the filter changes from transmission to reflection (50% threshold).*
Refraction index	*The factor by which the light velocity in an optical medium is less than in a vacuum.*
ROI	*Abbreviation for "Region of Interest". ROI encloses an area for which a measurement analysis is to be performed. On top of that, an ROI can also designate the area of a specimen to be scanned (ROI scan).*
Signal-to-noise ratio	*The ratio of signals detected in the specimen to the unwanted signals that are caused randomly by various optic and electronic components, which are also recorded by the detector.*
Stokes shift	*The Stokes shift is a central term in fluorescence microscopy. If fluorescent molecules are excited with light of a specific wavelength, they radiate light of another, larger wavelength. This difference between excitation light and fluorescent light is referred to as Stokes shift. Without Stokes shift, separating the high-intensity excitation light from the low-intensity fluorescence signals in a fluorescence microscope would not be possible.*
Triple dichroic	*Triple dichroic filters are interference filters at an angle of incidence of light of 45°. The transmissivity or reflectivity of triple dichroites depends on three specific wavelengths of light. For a* ***t****riple****d****ichroite TD 488/568/647, for example, the excitation light at 488 nm, 568 nm and 633 nm is reflected and the excitation light above these values is transmitted. The transmission values are generally at 80% and the reflection values are between 90% and 95%.*
Voxel	*An acronym based on the words, volume and pixel. A voxel represents the smallest, indivisible volume element in a three-dimensional system. In this documentation, both the volume elements of the specimen as well as the 3D image points are referred to as voxels.*
Working distance	*The distance from the front lens of an objective to the focal point. For a variable working distance, the gap between the front lens of the objective and the cover slip or uncovered sample is specified. Usually objectives with large working distances have low numerical apertures, while high-aperture objectives have small working distances. If a high-aperture objective with a large working distance is desired, the diameter of the objective lens has to be made correspondingly large. These, however, are usually low-correction optic systems, because maintaining extreme process accuracy through a large lens diameter can only be achieved with great effort.*

Specifications

Dimensions of the Leica TCS SP2

Inverse stand:


220 V~ / 16A
ArKr-Laser
300
720
1155
685
1350
730
min.1400
max.1415
797
110

Dimensions of the Leica TCS SP2 confocal system (all measurements in mm)

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