Leica TCS SP5

User Manual

Leica
MICROSYSTEMS

Leica Microsystems CMS GmbH
Am Friedensplatz 3
68165 Mannheim
Germany

Telefon: **+49 621 7028 0**
Fax: **+49 621 7028 2980**
E-Mail: **CLSM.support@leica-microsystems.com**

1 Notes about this manual

1.1 Copyright

The contents of this manual are current as of the time of publication and are subject to change without notice.

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2 General

2.1 About these operating instructions

The main focus of these operating instructions is directed to the safety instructions that must be observed while working with the laser scanning microscope.

In addition, these operating instructions provide a rough overview of the operating principle of laser scanning microscopes. It presents you with the first steps for starting and startup of the system and provides a description of the **L**eica **A**pplication **S**uite **A**dvanced **F**luorescence (LAS AF).

The Leica TCS SP5 is supplied with the latest version of the licensed Leica Application Suite Advanced Fluorescence. To maintain information on the most current level, the description of software functions was intentionally omitted from these operating instructions. Instead, you are referred to the online help of the Leica Application Suite Advanced Fluorescence in which you can obtain the most up-to-date explanations and instructions about the corresponding software functions.

Please read the chapter ”Introduction to the help of the Leica Application Suite Advanced Fluorescence” in these operating instructions to familiarize yourself first with setup and operation. Additional information about specific functions can subsequently be obtained electronically at your TCS workstation.

3 Proper intended use

The system was designed for confocal recording (laser scanning images) of fluorescence-marked living and fixed specimens as well as for quantitative measurements in the area of life science.

This system is intended for use in a lab.

Applications of in-vitro diagnostics according to the medical products law are excluded from proper intended use.

The manufacturer assumes no liability for any improper intended use and for use outside the specifications of Leica Microsystems CMS GmbH as well as any risks resulting from it. In such cases, the declaration of conformity becomes invalid.

4 Legal Notes

Manufactured in the Federal Republic of Germany.

4.1 DISCLOSURE

This document contains Leica Microsystems CMS GmbH proprietary data and is provided solely to its customers for their express benefit of safe, efficient operation and maintenance of the product described herein.
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4.4 Patents

The product is protected by the following US patents:
5,886,784; 5,903,688; 6,137,627; 6,222,961;

6,285,019; 6,311,574; 6,355,919; 6,423,960; 6,433,814; 6,444,971; 6,466,381; 6,510,001; 6,614,526; 6,654,165; 6,657,187; 6,678,443; 6,687,035; 6,738,190; 6,754,003; 6,801,359; 6,831,780; 6,850,358; 6,867,899.
Further patents are pending.

4.5 TRADEMARKS

Throughout this manual, trademarked names may be used. Rather than including a trademark (™) symbol at every occurrence of a trademarked name, we state that we are using the names only in an editorial fashion, and to the benefit of the trademark owner, with no intention of infringement.

4.6 Software licenses

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.

5 TCS SP5 specifications

5.1 System overview

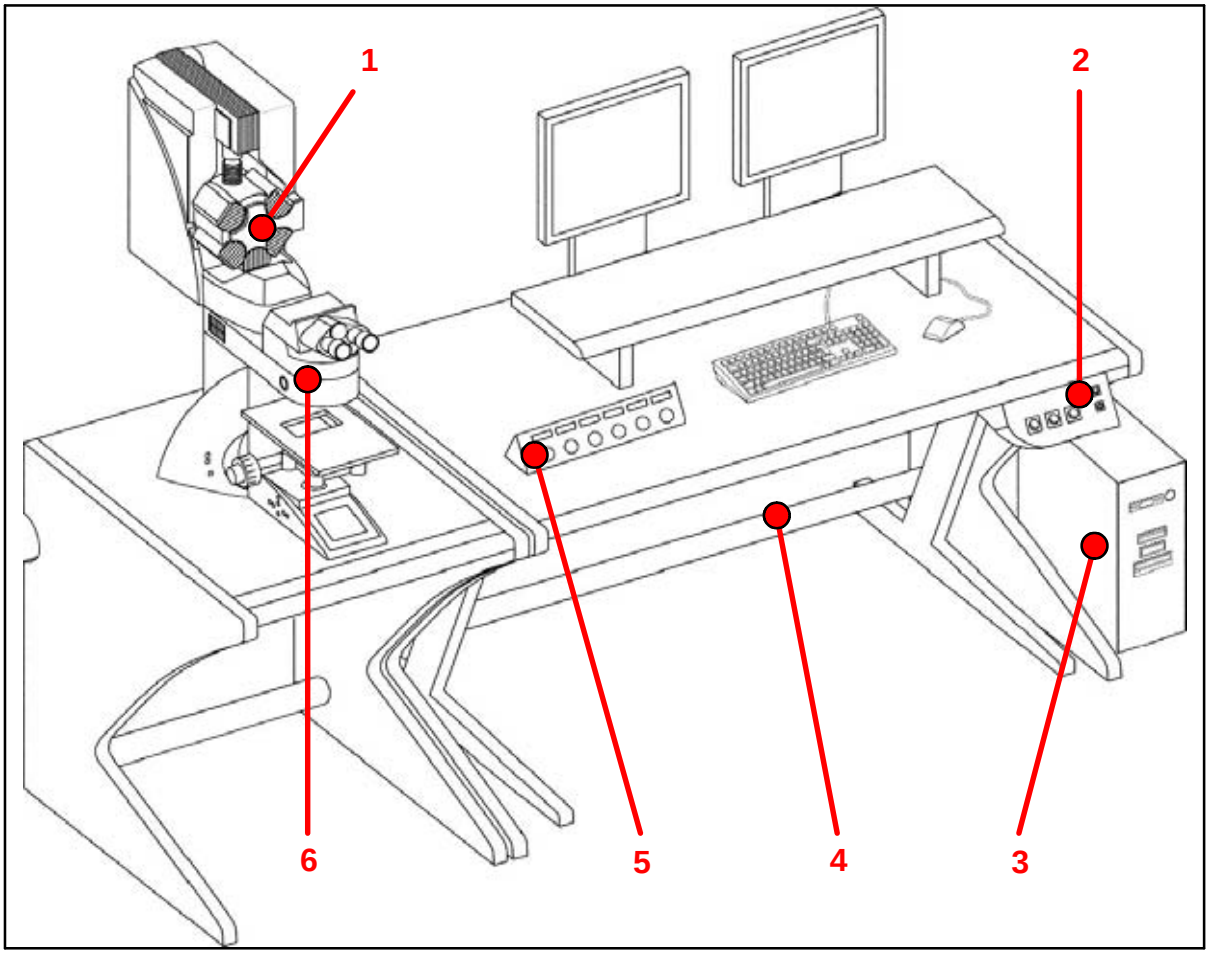

figure1. System components (overview)

1 TCS SP5 Scanner
2 Control panel
3 TCS workstation
4 Supply unit
5 Panel box
6 Stand with scanner

5.2 Dimensions

5.2.1 System with inverted stand:

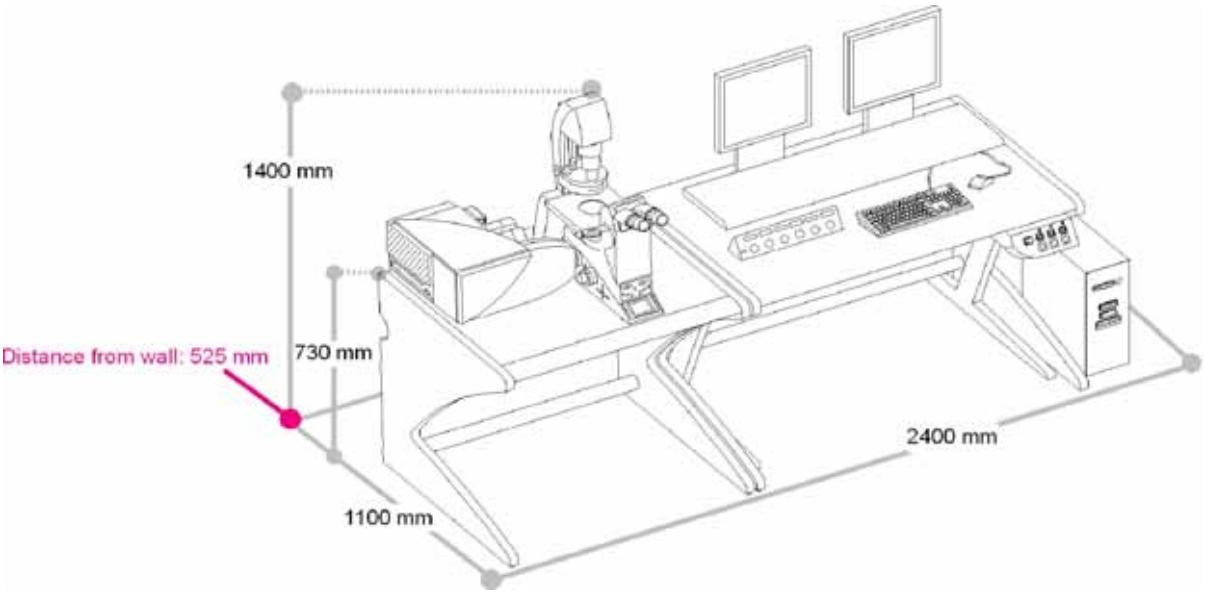

figure2. Dimensions of the TCS SP5 with inverted stand

5.2.2 System with upright stand

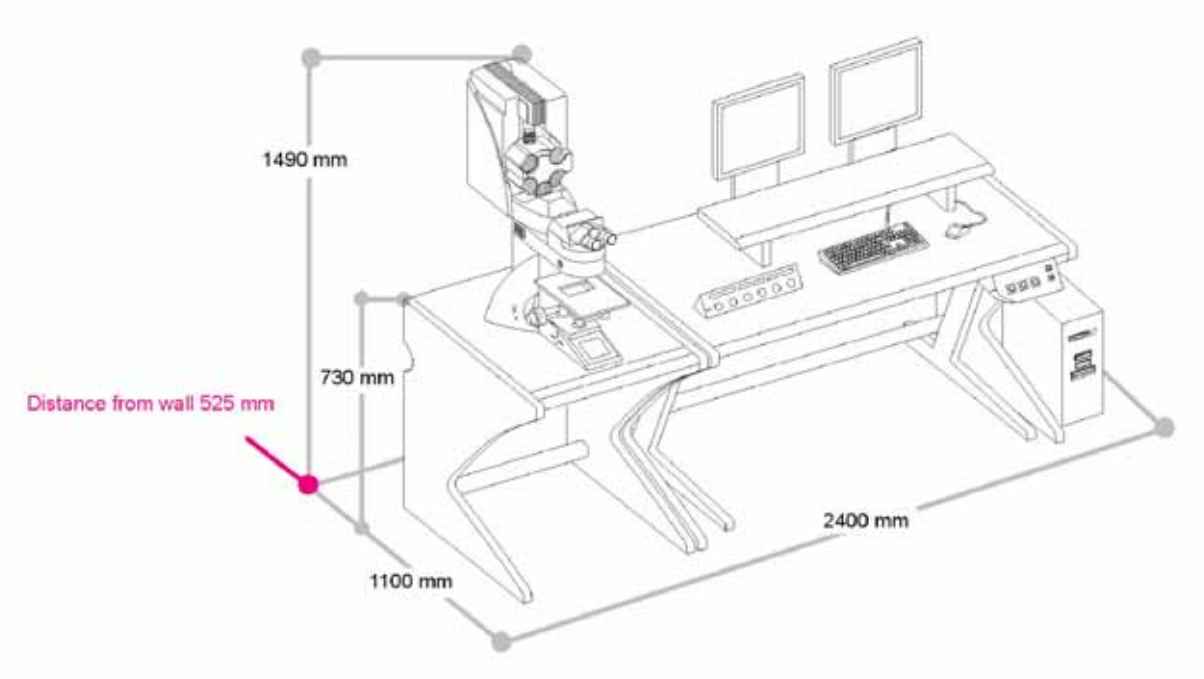

figure3. Dimensions of the TCS SP5 with upright stand

5.3 Installation site requirements

Do not expose the system to draft.

Ensure that the system is not installed next to elevators, air conditioners or ventilation systems. For this reason, the installation site should be carefully planned.

Ensure that the environment is as dust-free as possible.

Please also read the notes on protection against dust in the chapters on maintenance and cleaning. Installing the system in darkened rooms is also advisable.

For installation, maintenance and transport, the TCS SP5 system requires doors with inside spans of 1.00 m.

With regard to the load-bearing capacity of the floor, please note that the system will apply a static load of 200 kg/m@.

5.4 Permissible ambient conditions

Permissible temperature range for operation: 18 ... 25° C

Permissible relative humidity: 20-80% (non-condensing)

Permissible vibration speeds:

Frequency range [5 Hz–30 Hz]: less than 30 µm/s (effective value)
Frequency range [> 30 Hz]: less than 60 µm/s (effective value)

Pollution degree: Class 2

5.5 Electrical connections

Supply voltage (Input 1/Input 2): 100...240 V AC +/- 10%

Frequency: 50/60 Hz

Power consumption: 2 x 1.6kW

Overvoltage category: II

The building installation must feature two separate power connections with the following fuse protection:

D 100 V - 120 V AC power: 20 A

- 200 V - 240 V AC power: 12-16 A

For the specifications of external lasers such as UV and MP lasers, please refer to the manufacturer’s documentation.

5.6 Required cooling capacity

The TCS SP5 system has a maximum power consumption of 3.2
kW (VIS system).

For the specifications of external lasers such as UV and MP lasers, please refer to the manufacturer’s documentation.

5.7 Important additional notes

The optimal optical performance of the system can be achieved using standard objectives and standard immersions only at 22° Celsius + 1 Kelvin.

5.8 Required safety measures for VIS and UV systems

Please observe valid country-specific regulations pertaining to laser safety measures for laser class IIIb. The operator is responsible for observing the laser safety regulations.

5.9 Required laser safety measures for MP systems

Please observe valid country-specific regulations pertaining to laser safety measures for laser class 4/IV. The operator is responsible for observing the laser safety regulations.

5.10 Important additional notes

The optimal optical performance of the system can be achieved using standard objectives and standard immersions only at 22° Celsius + 1 °Celsius.

6 Safety Notes

**The instrument is Class 3B or 4 laser equipment
(depending on the laser used) in accordance with IEC/EN 60225-1.
This laser equipment may be operated only by persons who have been trained in the use of the systems and the potential dangers of laser radiation.**

As it is not possible to anticipate every potential hazard, please take care and apply common sense to the installation, operation and maintenance of this product. Observe all safety precautions relevant to Class IIIb lasers and Class IV MP systems.

Do not deviate from the operating and maintenance instructions provided herein.
The failure to observe these instructions shall be exclusively at the user's own risk and may void the warranty.

6.1 Which standards does this product meet?

This device was tested and meets the requirements of the following standards:

- IEC/EN 61010-1
  "Safety requirements for electrical equipment for measurement, control and laboratory use"
- IEC/EN 60825-1
  "Safety of laser products, Part 1: Equipment classification, requirements and user's guide"
- IEC/EN 61326
  "Electrical equipment for measurement, control and laboratory use - EMC requirements" (Class A).
  This is a Class A device. Operating this device on a public low-voltage grid may result in radio interference. The operator

must take suitable measures should this occur.

- IEC/EN 61000-3-2 (EMC) ”Electromagnetic Compatibility” Part 3-2: Limits - Limits for harmonic currents
- IEC/EN 61000-3-3 (EMC) ”Electromagnetic Compatibility” Part 3-3: Limits - Limits - Section 3: Limits for voltage fluctuations and flikker in low-voltage networks.
- For use in the USA: CDRH 21 CFR 1040.10: Laser Products U.S. Food and Drug Administration (FDA) (”Complies with FDA performance standards for laser products except for deviations pursuant to laser notice No. 50, dated 26 July, 2001.”) **For the scope of the CDRH/FDA (USA), the designation Laser Class 3B in the text must be replaced by IIIb and Class 4 by IV.**

6.2 Which laser class does the product have?

Laser type	Wavelength range	Configuration	Laser class
VIS	400 - 700 nm, (visible laser radiation)	Combination of lasers from Chapter 6.11 (without lasers having wavelengths of 350 - 400 nm)	3B / IIIb
UV	350 - 700 nm, (visible and invisible laser radiation)	Combination of lasers from Chapter 6.11 (VIS and UV lasers)	3B / IIIb
MP	350 - 1050 nm, (visible and invisible laser radiation)	Combination of lasers from Chapter 6.11 (VIS/UV lasers) and Chapter 6.12 (IR lasers)	4 / IV

6.3 Laser class for VIS and UV systems

According to IEC/EN 60825-1, this laser scanning microscope is a laser device of **Class IIIb**.

Avoid exposing eyes or skin to direct radiation.

6.4 Laser class for MP systems

According to IEC/EN 60825-1, this laser scanning microscope is a laser device of **Class IV**.

Avoid exposing eyes or skin to direct and indirect radiation.

6.5 Warnings, Safety Cautions, and Notes

Notes either contain additional information on a specific topic or special instructions on the handling of the product.

A **safety note** points out an operation, a process, a condition or an instruction that must be observed strictly to prevent severe damage to the system or loss of data.

A **laser warning** points out an operation, a process, a condition or an instruction that must be observed strictly to prevent serious eye injuries to the persons using the system.

A **high-voltage warning** points out an operation, a process, a condition or an instruction that must be observed strictly to prevent possible injury or death of the persons using the system.

6.6 What should the user of the laser scanning microscope observe?

The user of this product is responsible for proper and safe operation and safe maintenance of the system as well as for following all applicable safety regulations. The user is fully liable for all consequences resulting from the use of the system for any other purposes than those listed in the Operating Instructions or the online help.

The user is obligated to perform and monitor suitable safety measures according to IEC / EN 60825-1 and the corresponding national regulations. Users must have received instructions concerning the risk potential associated with the operation of laser devices.

To assure classification as a 3B/IIIb or 4/IV laser product according to IEC/EN60825-1 and electrical safety compliance, all safety devices, interlocks, and safety systems of the laser device must be in operational condition.
Deactivating or damaging these safety devices or any intervention in any of these safety devices may lead to serious eye injuries, physical injuries or property damages. In these cases, Leica Microsystems CMS GmbH does not assume any liability.

According to IEC/EN 60825-1: ”Safety of laser products, Part 1: Equipment classification, requirements and user’s guide,” the user is required to

designate a Laser Safety Officer or a Laser Protection Advisor.

Repairs and servicing may only be performed by authorized Leica Microsystems CMS GmbH service personnel.

The user is fully liable for all consequences resulting from the use of the system if it is opened, improperly serviced or repaired by other persons than authorized Leica customer service representatives.

If repairs or service measures are performed that require opening parts of the housing, only trained Leica service technicians may occupy the room in which the laser scanning microscope is located.

Leica Microsystems CMS GmbH will not be liable for damages resulting from nonobservance 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 CMS GmbH.

6.7 Safety Notes for the User

Read and observe the safety notes in the Operating Instructions and the safety labels located on the system. Failure to observe the safety notes may lead to serious injuries and to significant damages to the system and loss of data.

Observe the instructions for operating the system located in the Operating Instructions.

Before performing operating steps for the first time with the system, read the corresponding description of the function in the online help first.
You can get an overview of the single functions in the contents file of the online help.

Do not connect any external equipment.
Connect only those electrical devices to the product that are listed in the Operating Instructions. Otherwise, please contact your local Leica service agency or Leica Microsystems CMS GmbH.

6.8 Safety Notes for Operation

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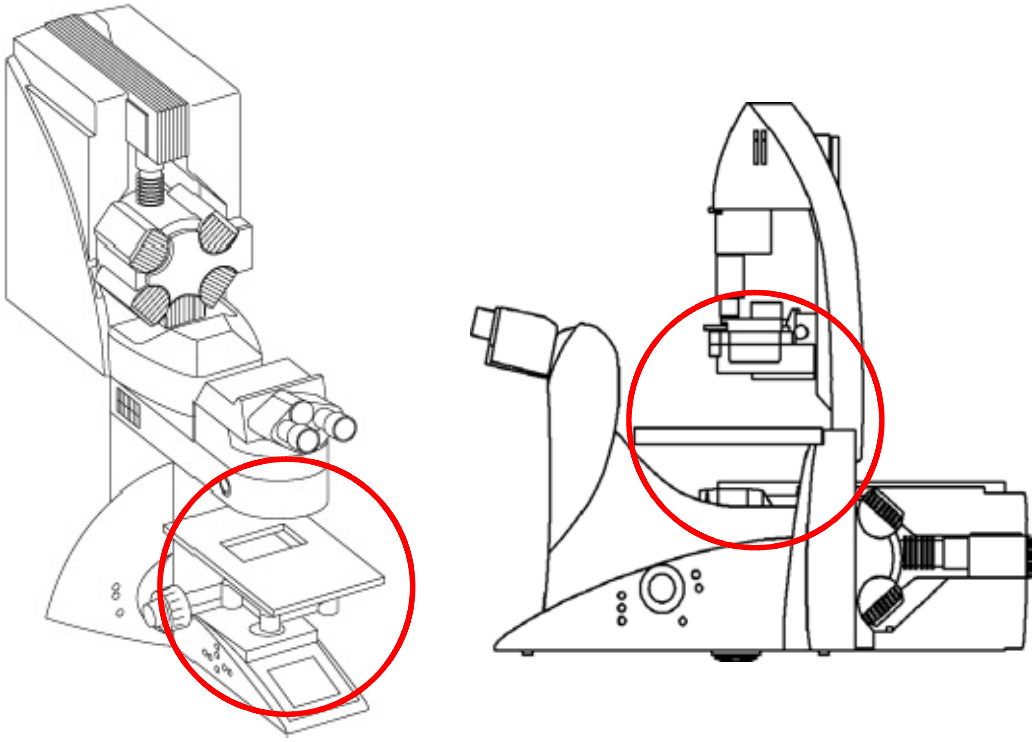


figure4. Specimen area of upright and inverted stand

During scanning, the laser radiation is freely accessible after exiting the objective in the specimen area of the laser scanning microscope.

This circumstance demands special attention and caution. If the laser radiation comes in contact with the eyes, it may cause serious eye injuries. For this reason, prudent handling is absolutely necessary as soon as one or several laser emission warning indicators are lit.
If the laser scanning microscope is used as prescribed and the safety notes are observed during operation, there are no dangers to the user. Maintain a safety distance of 20 cm between your eyes and the eyepiece opening.

Do not look into the eyepieces during the scan process.

Do not look into the eyepieces when switching the beam path in the stand..

Never look directly into a laser beam or a reflection of the laser beam. Avoid all contact with the laser beam.

Never deactivate the laser protection devices. Please read the chapter ”Laser protection devices” to familiarize yourself with the safety devices of the laser scanning microscope.

Do not introduce any reflective objects into the laser beam path.
If, for example, micromanipulators are used in the specimen area, you must ensure that no uncontrolled laser light leaves the safe beam path due to reflection or scattering during the scanning process, as it could pose a hazard to the surrounding area.

Do not change specimens during scanning.

Proceed as follows:

Upright microscope	Inverted microscope
Finish the scan process.	Finish the scan process.
Ensure that no laser radiation is present in the specimen area.	Ensure that no laser radiation is present in the specimen area. Tilt the transmitted-light arm back.
Exchange the specimen. Insert the specimen correctly into the specimen holder.	Exchange the specimen. Insert the specimen correctly into the specimen holder.
	Tilt the transmitted-light arm back into the working position.

Do not change objectives while scanning.

Should it become necessary nevertheless, please follow these procedures:

1 Finish the scan process.

2 Rotate the objective turret so that the objective to be changed is swiveled out of the beam path and points outward.

3 Exchange the objective.

- All unoccupied positions in the objective turret must be closed using the supplied caps.

Do not change any filter cubes or beam splitters during scanning.

Proceed as follows:

Upright microscope	Inverted microscope
Finish the scan process.	Finish the scan process.
Remove the cover of the fluorescence module (see Microscope Stand operating instructions).	Pull out the fluorescence module.
Remove the filter cube/beam splitter.	Remove the filter cube/beam splitter.
Insert the desired filter cube/beam splitter.	Insert the desired filter cube/beam splitter.
Reattach the cover to the front of the fluorescence module.	Reinsert the fluorescence module.

Never disconnect an optical waveguide.

Never remove the scanner from the microscope stand during operation. Before removing the scanner, the system must be completely switched off.

Do not use an S70 microscope condenser.The large working distance and the low numeric aperture of the S70 microscope condensers could result in a threat from laser radiation. Therefore, only S1 and S23 Leica microscope condensers should be used.

6.9 Specific Safety Notes

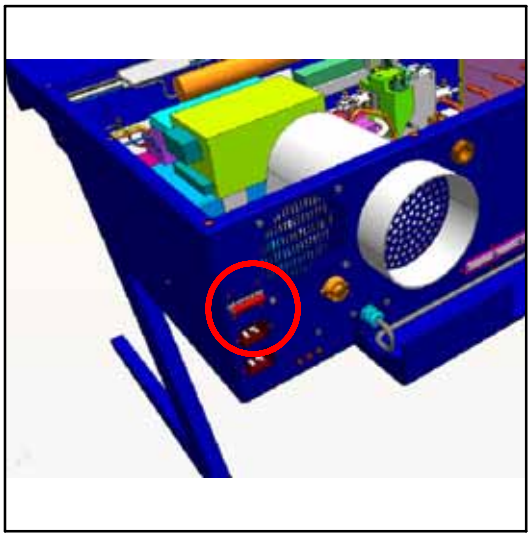
6.9.1 Operational reliability

This instrument must not be used together with life-support systems such as those found in intensive-care wards.

This instrument may only be used with a grounded AC power supply.

Contact with liquids or the entry of liquids into the housing must be avoided.

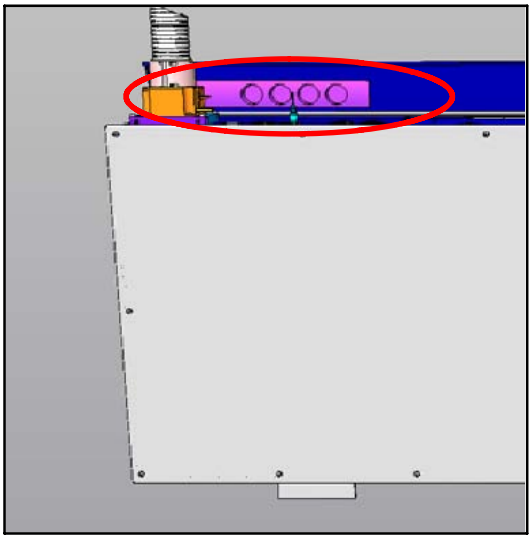
6.9.2 De-energizing the system



The main circuit breaker is located on the right rear side of the supply unit. It is used to de-energize the complete system using a single switch.
The main circuit breaker functions as a switch and as an overcurrent fuse.
The main circuit breaker is not to be used as the regular on/off switch for the system.
The supply unit must be set up so that the main circuit breaker is freely accessible at all times.

figure5. Supply unit with main circuit breaker

6.9.3 Maximum current load of the power outlet strip at the supply unit



The total power consumption of all loads connected to the power outlet strip must not exceed

800 VA.

The connectors are intended for:

- TCS control computer
- Monitor 1
- Monitor 2
- Microscope

figure6. Power outlet strip, rear side of supply unit

6.10 Laser safety devices

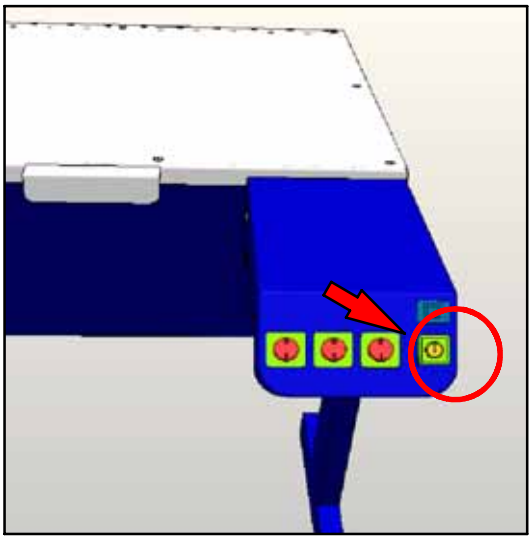
The light of all employed VIS lasers (wavelength range 400-800 nm, visible spectrum
) and UV lasers (
wavelength range < 400 nm, invisible) is fed through an optical waveguide and, therefore, completely shielded until it leaves the microscope objective and reaches the specimen. For MP systems, see the chapter “Shielding for MP systems”.

6.10.1 Eye protection for VIS or UV systems

It is not necessary to wear eye protection. If the device is used as prescribed and the safety notes are observed, the limit of the laser radiation is maintained so that eyes are not endangered.

For MP systems, see the chapter “Eye protection for MP systems”.

6.10.2 Detachable-key switch



The detachable-key switch for protection against unauthorized use of the laser devices is located on the control panel.

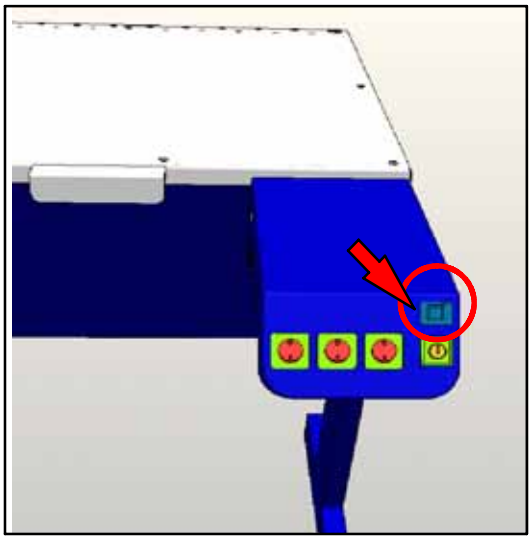
figure7. Detachable-key switch for the internal lasers

In the case of an optional external 405 nm advanced laser, the detachable-key switch for the laser is located on its power pack**.**

figure8. Detachable-key switch of the external 405-nm Advance laser

For lasers that are not connected as described above, please refer to the supplied manual of the laser manufacturer for the position of the detachable-key switches.

6.10.3 Emissions warning indicators



The operational readiness of lasers located in the supply unit is signaled by an emission warning indicator.
The emission warning indicator is located above the detachable-key switch and is yellow when lit.
As soon as the emission warning indicator of the lasers is lit, it is possible from a functional standpoint that laser radiation is present in the specimen area.

figure9. Emission warning indicators on the control panel

The optional external 405
nm advanced laser features the yellow emission warning indicator (1) above the detachable-key switch.

figure10. Emission warning indicator of the external 405-nm Advance laser

Immediately disconnect the system from the
power supply if any of the following occur:

- The emission warning indicator is not lit after being switched on using the keyswitch.

- The indicator continues to be lit after being switched off using the keyswitch
- Scanning of the specimen is not activated after being switched on properly (laser radiation in the specimen area).

Contact Leica Service immediately.

For lasers whose readiness is not indicated as described above, please refer to the supplied manual of the laser manufacturer for the location of the emission warning indicator.

6.10.4 Remote interlock connector on the supply unit

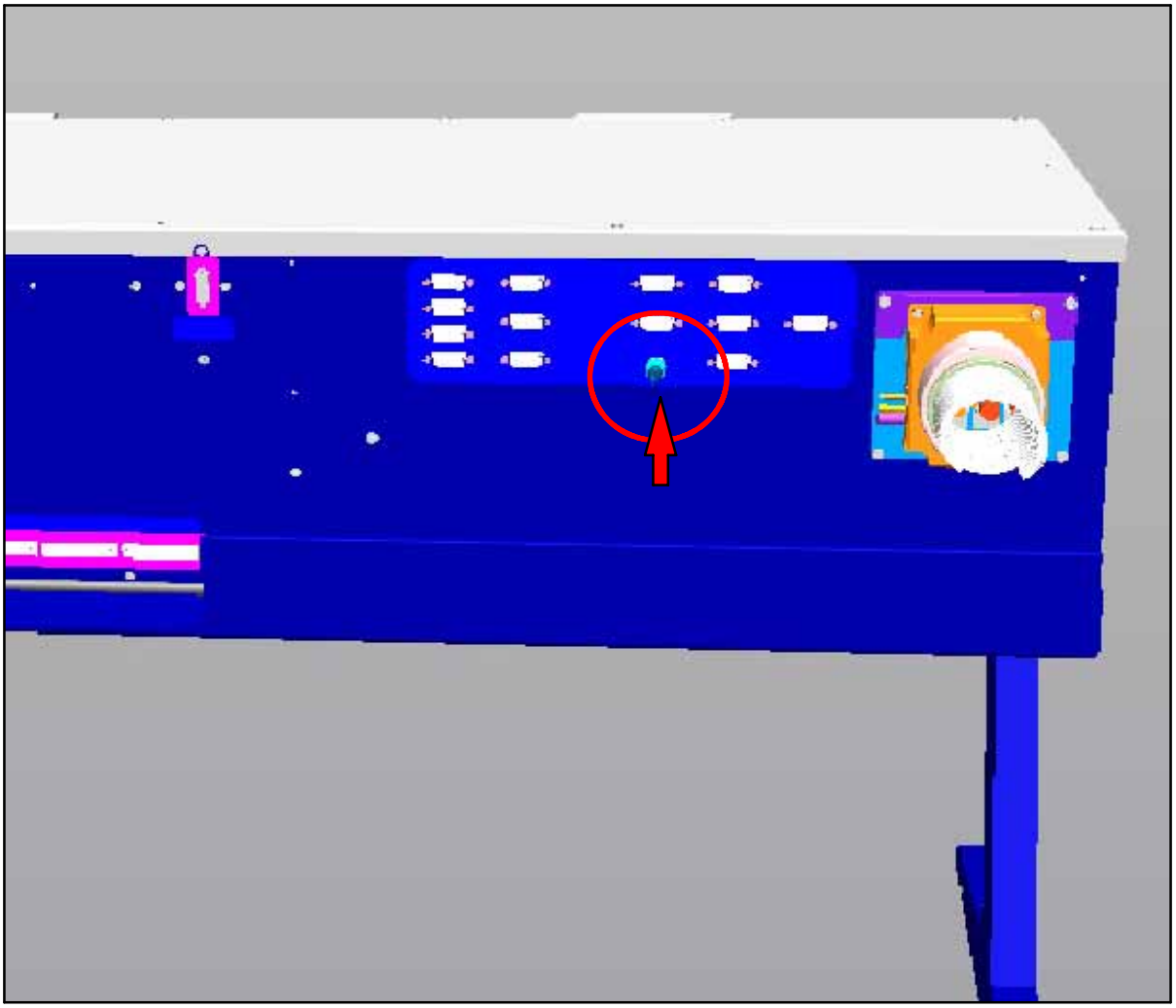
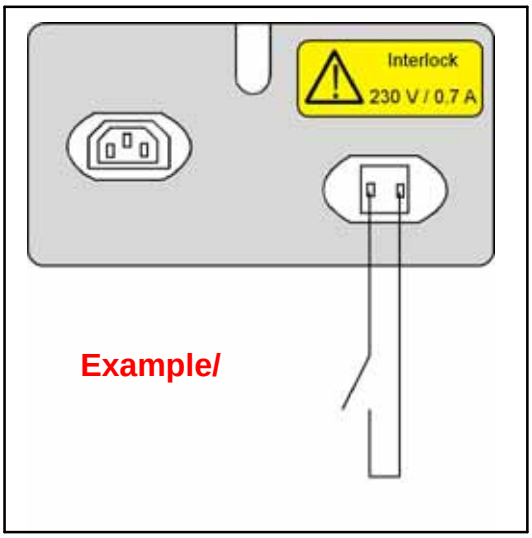


figure11. Position of the remote interlock connector

The remote interlock connection is located on the rear side of the supply unit (12V DC operating voltage). The remote interlock connector is plugged into this connection.
Remote interlocks that are connected to the room, the door or other locally fixed safety interlocks can be connected to the remote interlock connection. The laser beam path is interrupted if the contact is open.
The overall length of the cable between the two connecting pins of the remote interlock connector should not exceed 10 m.

6.10.5 Remote interlock connector on the external 405nm advanced laser



If the laser scanning microscope is equipped with the optional 405-nm laser (non-pulsed), the “Interlock“ remote interlock connection is located on the rear side of the laser power supply.
The remote interlock connector contains a shorting bridge.
Remote interlocks that are connected to the room, the door or other locally fixed safety interlocks can be connected to the remote interlock connection.
The laser beam path is interrupted if the contact is open.

figure12. Example of a remote interlock

The supply voltage of the remote interlock circuit of the 405 nm Advance laser is 100-240V AC. For this reason, the remote interlock circuit of the 405 nm Advance laser must never be connected to other remote interlock circuits but, instead, must be securely separated from them.

Due to the live voltage of 100-240 V, replacing the shorting plug by an external interrupt circuit (e.g. door interlock switch) may only be performed by a qualified electrician.

6.10.6 Remote interlock connectors on additional external lasers

For lasers whose remote interlock connector is not indicated in Chapter 6.10.6, please refer to the supplied manual of the laser manufacturer for the location of the remote interlock connector.

6.10.7 Interlock connection on scanner

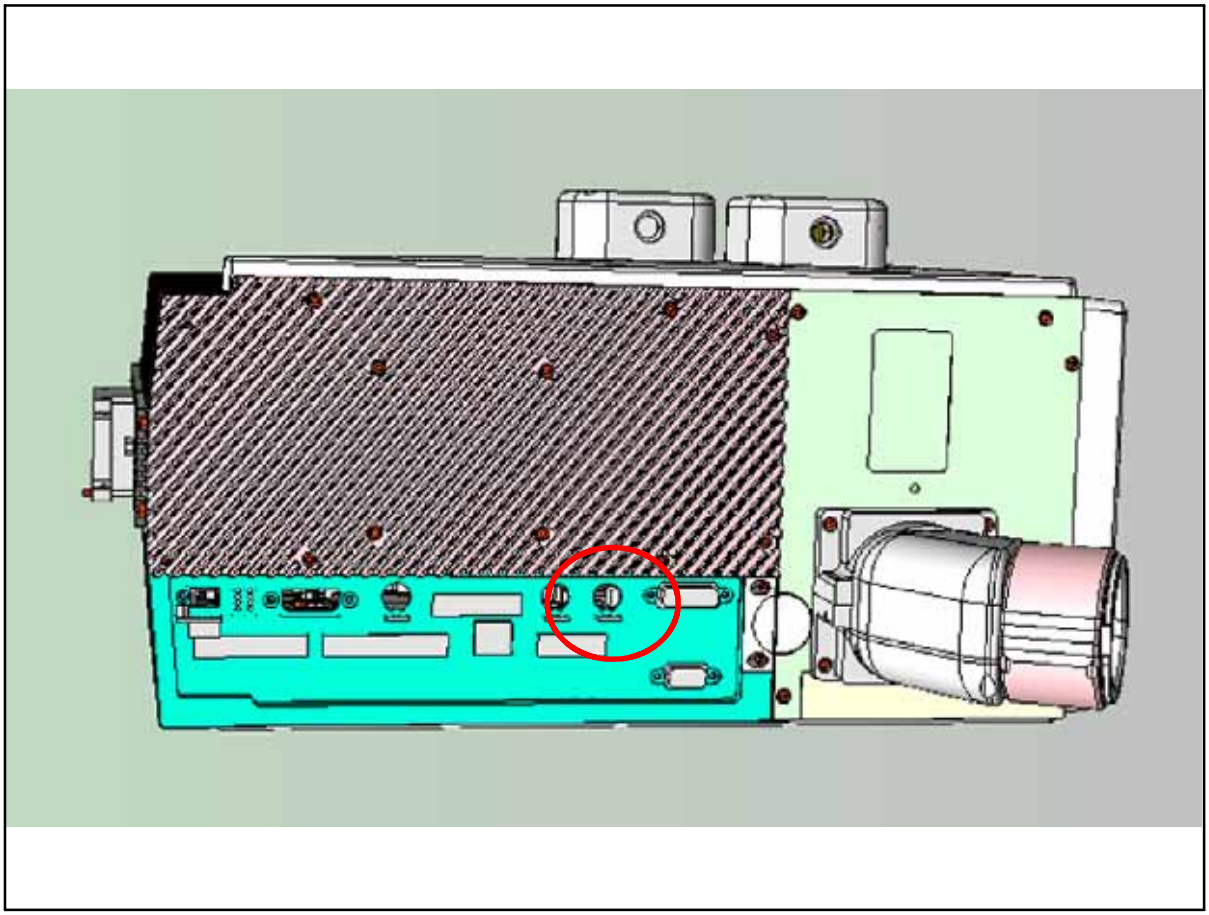


figure13. Position of the interlock connector

The interlock connector (operating voltage 12 V DC) is located on the rear side of the scanner.
The inverted microscope or, if an upright microscope is used, the mirror housing is connected to this connector. This ensures that the safety switch of the microscope is integrated in the interlock circuit.

6.10.8 Function and position of safety switches

When the safety switches are released, the light path of the laser beam is interrupted.

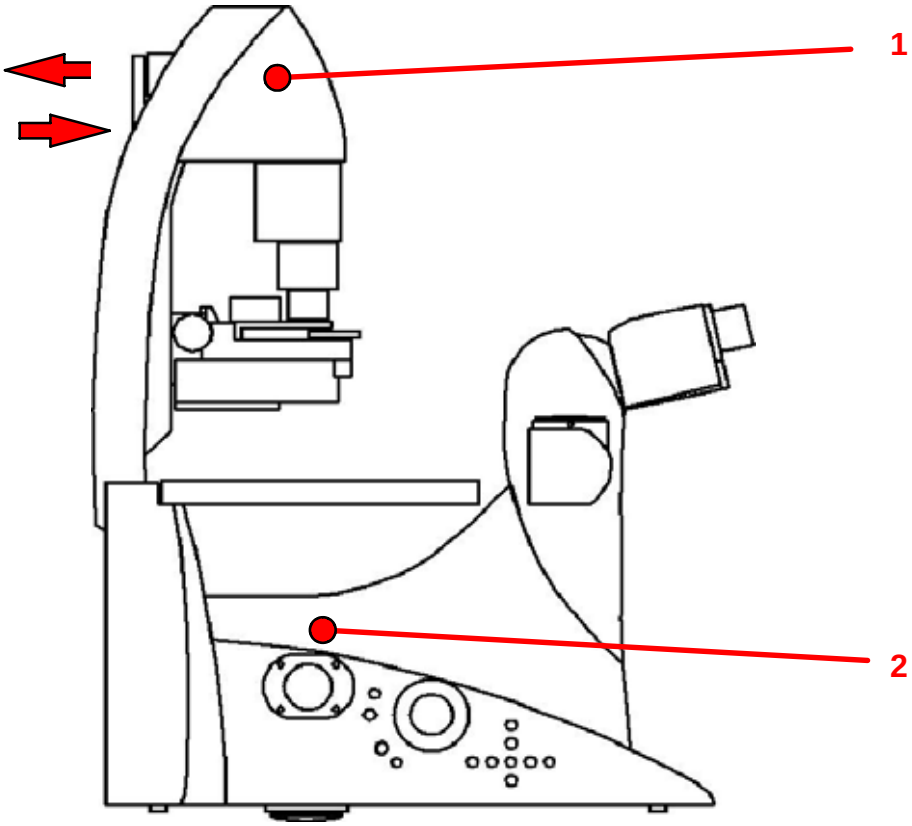
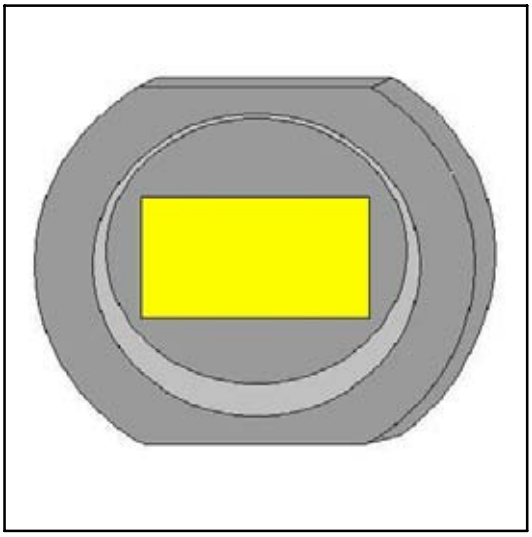


figure14. Position of the transmitted-light illumination arm (1) and switching from scan mode to eyepiece (2)

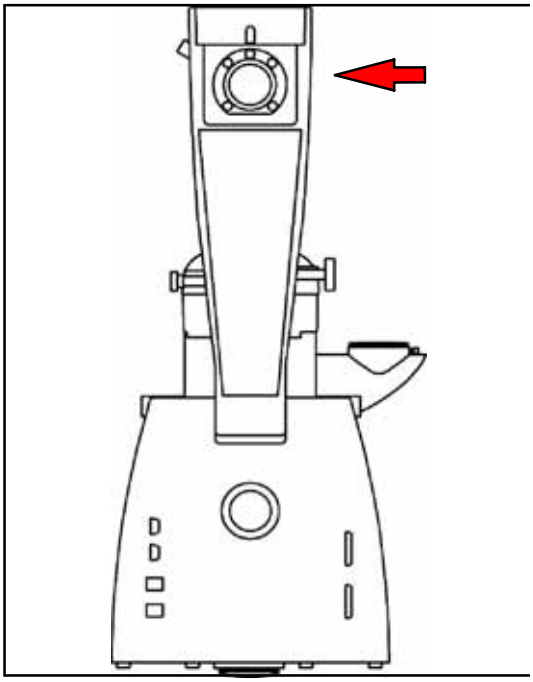
Position	Activated by:	Type of microscope	Activated if:	Function
1	Transmitted-light illuminator arm	Inverted stand DMI 4000 CS DMI 6000 CS	The illuminator arm is tilted (e.g. for working on the specimen).	Prevents laser light while working on the specimen.
2	Motorized changeover between scanning mode and eyepiece	Inverted stand DMI 6000 CS DMI 4000 CS	The deflection mirror to the scanner is motorized.	Prevents stray light if the user switches from confocal observation to eyepiece observation.

6.10.9 Transmitted-light lamp housing for inverted stands



During the time when no transmitted-light lamp housing is connected to the microscope stand, the opening must be tightly covered with the cap provided with the system to prevent laser radiation from exiting.

figure15. Cover for replacement flange



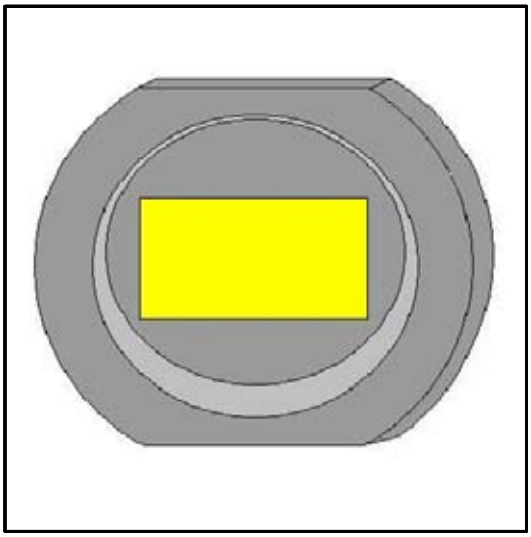
If your inverted stand features a transmitted-light housing that you would like to replace, proceed as follows:

- Switch off the lasers.
- Disconnect the lamp housing from the power supply.
- Remove the lamp housing.
- Perform the intended tasks at the lamp housing.
- After finishing the tasks, screw the new lamp housing back onto the microscope stand.

figure16. Connecting the transmitted light lamp housing

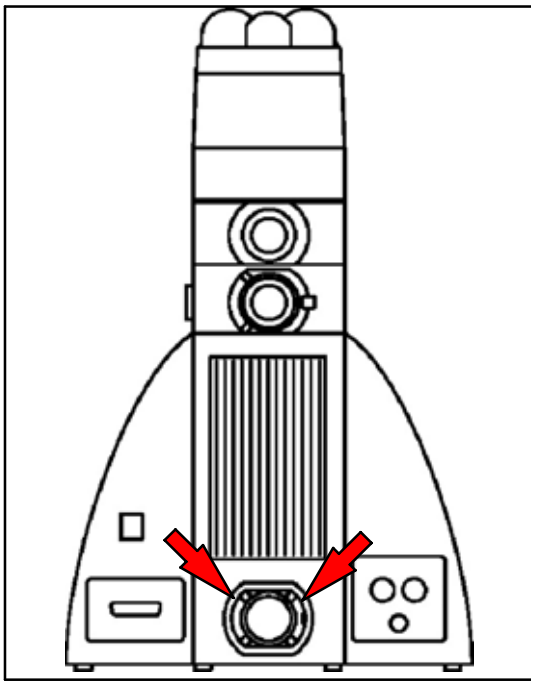
To prevent the emission of laser radiation, do not switch the lasers on without a lamp housing or cover on the microscope stand.

6.10.10 Transmitted-light lamp housing for upright stands



During the time when no transmitted-light lamp housing is connected to the upright microscope stand, the opening must be tightly covered with the cap provided with the system to prevent laser radiation from exiting.

figure17. Cover



If your upright stand features a transmitted-light housing that you would like to replace, proceed as follows:

- Switch off the lasers.
- Disconnect the lamp housing from the power supply.
- Remove the lamp housing.
- Perform the intended tasks at the lamp housing.
- After finishing the tasks, screw the new lamp housing back onto the microscope stand.

figure18. Connecting the transmitted light lamp housing

To prevent the emission of laser radiation, do not switch the lasers on without a lamp housing or mirror housing connected to the microscope stand, or if a cover is not present.

6.10.11 Mirror housing on upright stand

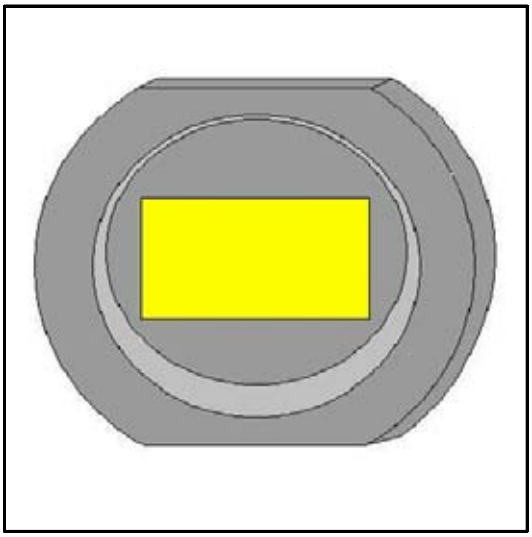


figure19. Cover

If a mirror housing is not connected to the upright microscope stand, the opening must be tightly covered using the cap provided with the system to prevent any laser radiation from escaping.

figure20. Mirror housing on upright stand

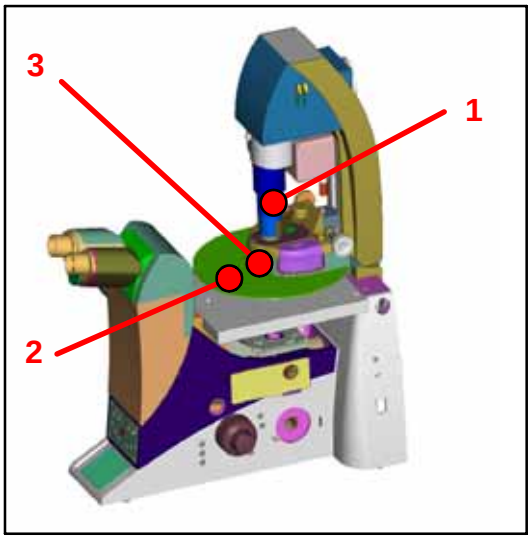
If your upright microscope stand is equipped with a mirror housing, note the following:

- The interlock connector on the mirror housing (see arrow) must be connected to the scan head at all times.
- The unused output on the mirror housing must be covered using the cover provided (1).
- If the mirror housing is removed, place a cover on the adapter left on the stand.

To prevent the emission of laser radiation, do not switch the lasers on without a mirror housing or cover on the microscope stand.

6.10.12 Special laser safety equipment

6.10.12.1 Safety beam guide and beam collector



The safety beam guiding and beam collector are used with inverted microscopes to protect against laser radiation and are located between condenser base and transmitted-light detector.

1 Safety beam guide
2 Beam collector
3 Condenser base

figure21. Inverted stand

When subsequently ordering condenser bases (3), be aware that condenser bases are supplied without beam collectors (2). The existing beam collector (2) must be reinstalled.

When using a condenser base with filter holder, always make sure that unused filter holders are swung out of the beam path, and that the safety beam guide covers the beam path. When equipping multiple filter holders with filters, do so from bottom to top so that the safety beam guide can cover the beam path to the greatest possible extent.

Do not swing in the filters during the scanning process.

6.10.12.2 Eye protection for MP systems

System with inverted microscope stand:

It is not necessary to wear eye protection.
If the device is used as prescribed and the safety notes are observed, the limit of the laser radiation is maintained so that eyes are not endangered.

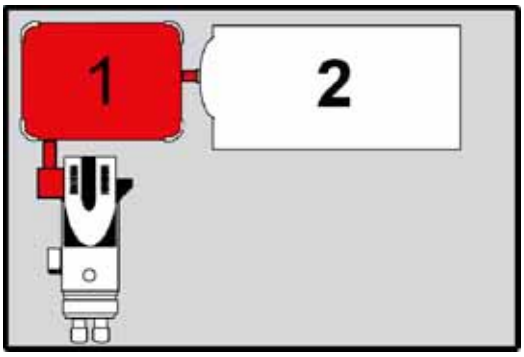
System with upright microscope stand:

The IR laser beam can be deflected or scattered by the specimen or objects moved into the specimen area.
Therefore, it is not possible to completely eliminate hazards to the eye from IR laser radiation.
Using protective eyewear (specification: 680-990 DIR L5 / 990-1064 DIR L4) is required.
Appropriate safety goggles for IR laser radiation are provided with the system when delivered.

The supplied eye protection only provides safe protection against the infrared lasers supplied by Leica Microsystems CMS GmbH.

6.10.12.3 Shielding in MP systems (UV laser)

The light of all employed VIS lasers (wavelength range 400-700 nm, visible spectrum
) and UV lasers (
wavelength range < 400 nm, invisible) is fed through an optical waveguide and, therefore, completely shielded until it leaves the microscope objective and reaches the specimen.



For systems with infrared laser (wavelength range > 700 nm), the beam is passed through a safety beam guide and, if necessary, also passed through an optical waveguide.
This shields the laser beam until it leaves the microscope objective and reaches the specimen.

figure22. Safety beam guiding (1) and IR laser (2)

6.11 Overview of usable VIS/UV lasers

The laser scanning microscope features a combination of the lasers listed below.

Laser type	Wavelength [nm]	Maximum luminous power at laser output [mW]	Maximum luminous power in focal plane [mW]	Pulse duration
Ar-UV	351, 364	< 60	< 4	Continuous wave (cw)
Diode 405	405	< 60	< 6	Continuous wave (cw)
Diode 405	405	< 5 (mean power)	< 0.3 (mean power)	pulsed, 60 ps
DPSS 442	442	< 25	< 5	Continuous wave (cw)
Ar	458, 476, 488, 496, 514	< 200	< 30	Continuous wave (cw)
Ar, external	458, 476, 488, 496, 514	< 500	< 125	Continuous wave (cw)
Solid state 488	488	< 500	< 150	Continuous wave (cw)
HeNe	543	< 1.5	< 0.5	Continuous wave (cw)
DPSS 561	561	< 12	< 4	Continuous wave (cw)
HeNe	594	< 4	< 1	Continuous wave (cw)
HeNe	633	< 15	< 4	Continuous wave (cw)

Table 1 Table of usable lasers (without MP)

6.12 Overview of usable MP lasers (IR)

Each MP system contains only one of the MP lasers listed below.

In addition, the MP system may also contain additional VIS/UV lasers (see the table for usable VIS/UV lasers).

Laser type	Wavelength [nm]	Maximum luminous power at laser output [W]	Maximum luminous power in focal plane [W]	Pulse duration
TiSa*	780-920	< 1.2	< 0.6	pulsed, 1.0 -1.5 ps
TiSa*	710-920	< 2.5	< 1.2	pulsed 1.0-1.5 ps
TiSa*	710-990	< 2.5	< 1.2	pulsed 1.0-1.5 ps
TiSa*	720-930	< 2.0	< 1.0	pulsed 1.0-1.5 ps
TiSa*	720-980	< 2.5	< 1.2	pulsed 1.0-1.5 ps

Table 2 Table of usable MP lasers

* Modified picosecond version

6.13 Safety label on TCS SP5 system

The corresponding safety labels are selected dependent on the laser configuration (VIS, UV, MP) and attached in the following locations.

6.13.1 Inverted stand DMI 4000/6000 CS

Angled rear view of right side of stand

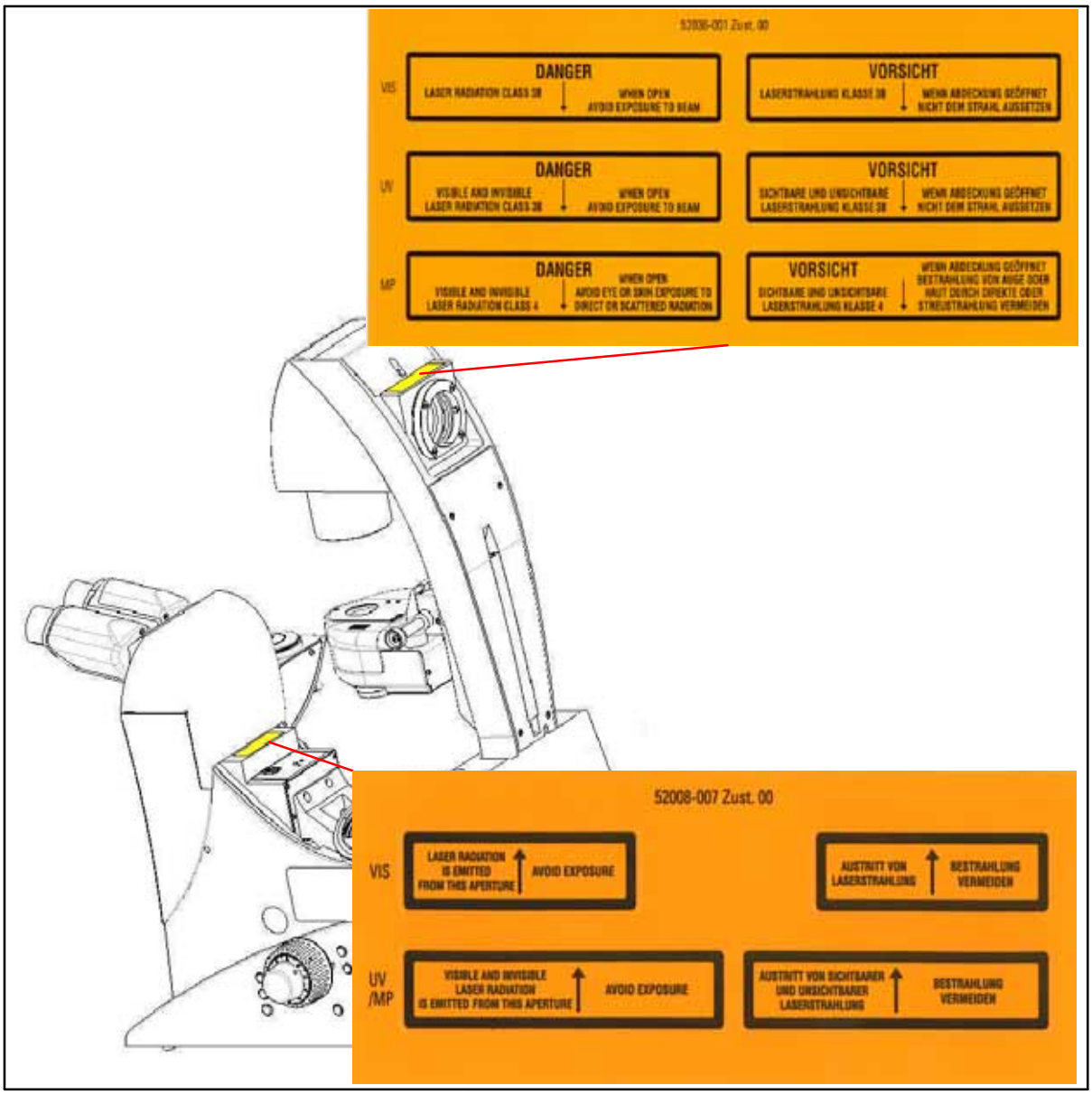


figure23. Safety label for DMI 4000/6000 CS inverted stand

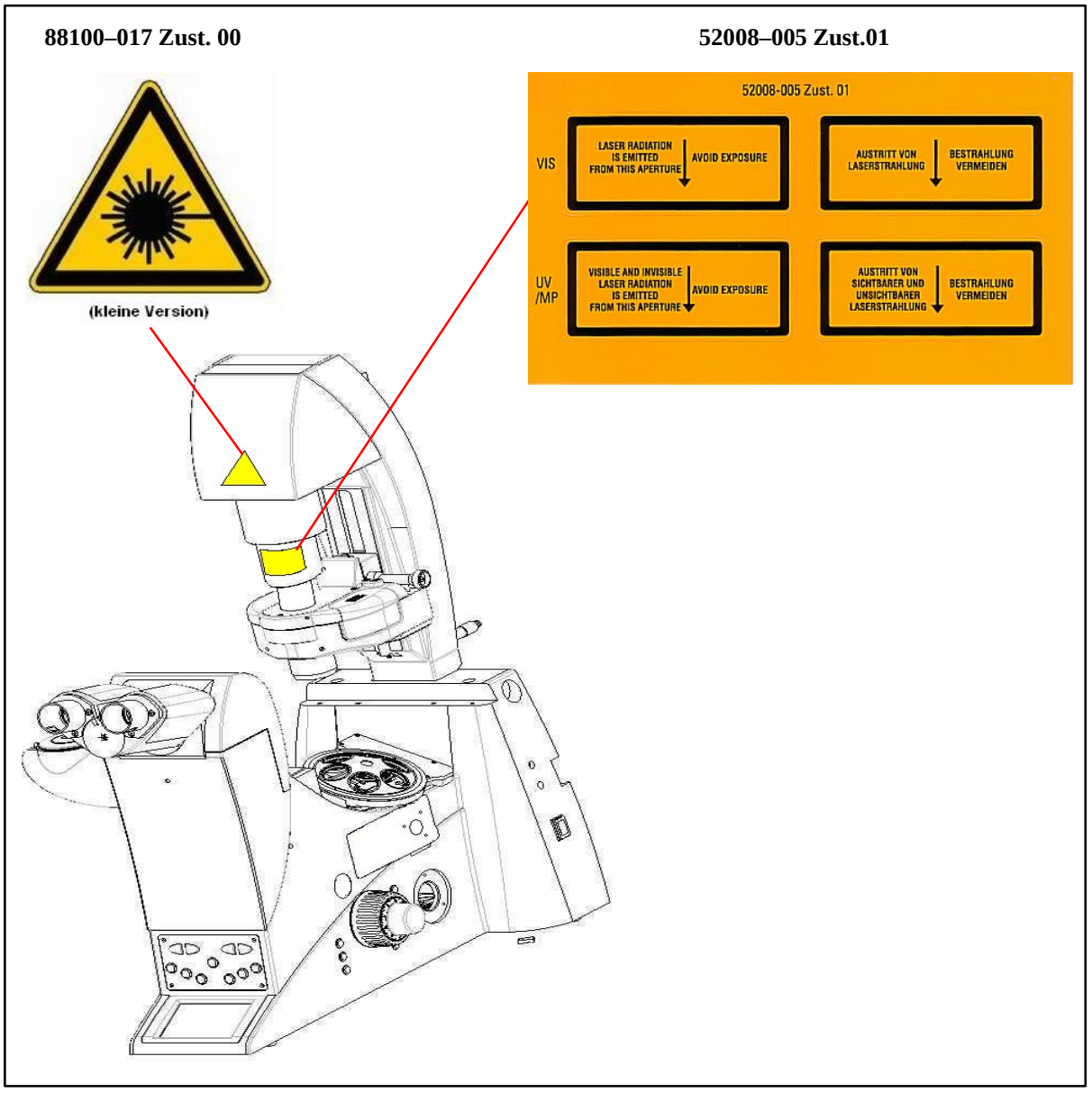


figure24. Safety label for DMI 4000/6000 CS inverted stand

6.13.2 Upright stand DM 5000/6000 CS

Angled front view of right side of stand

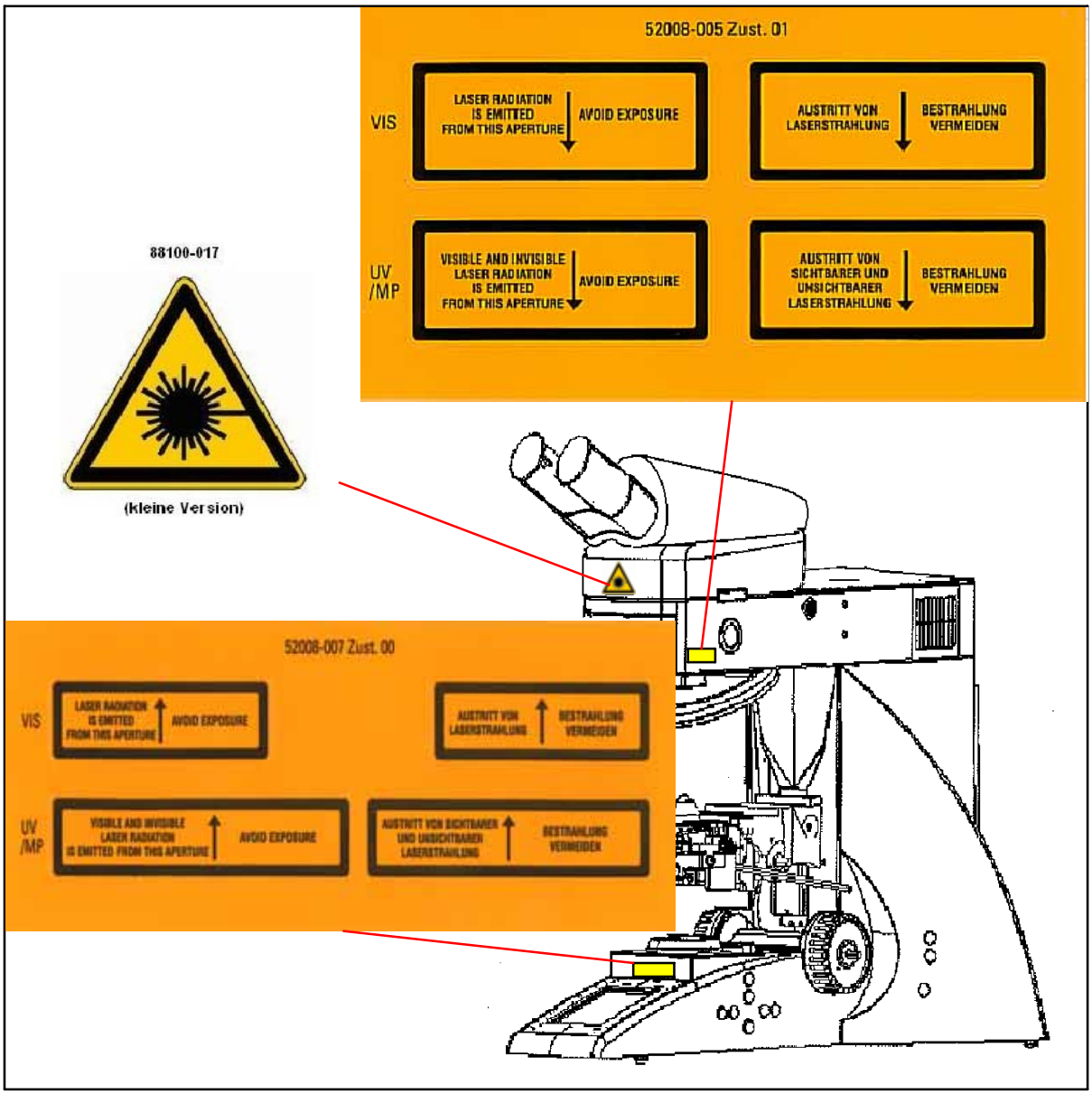


figure25. Safety label for DM 5000/6000 CS upright stand

Rear view of stand

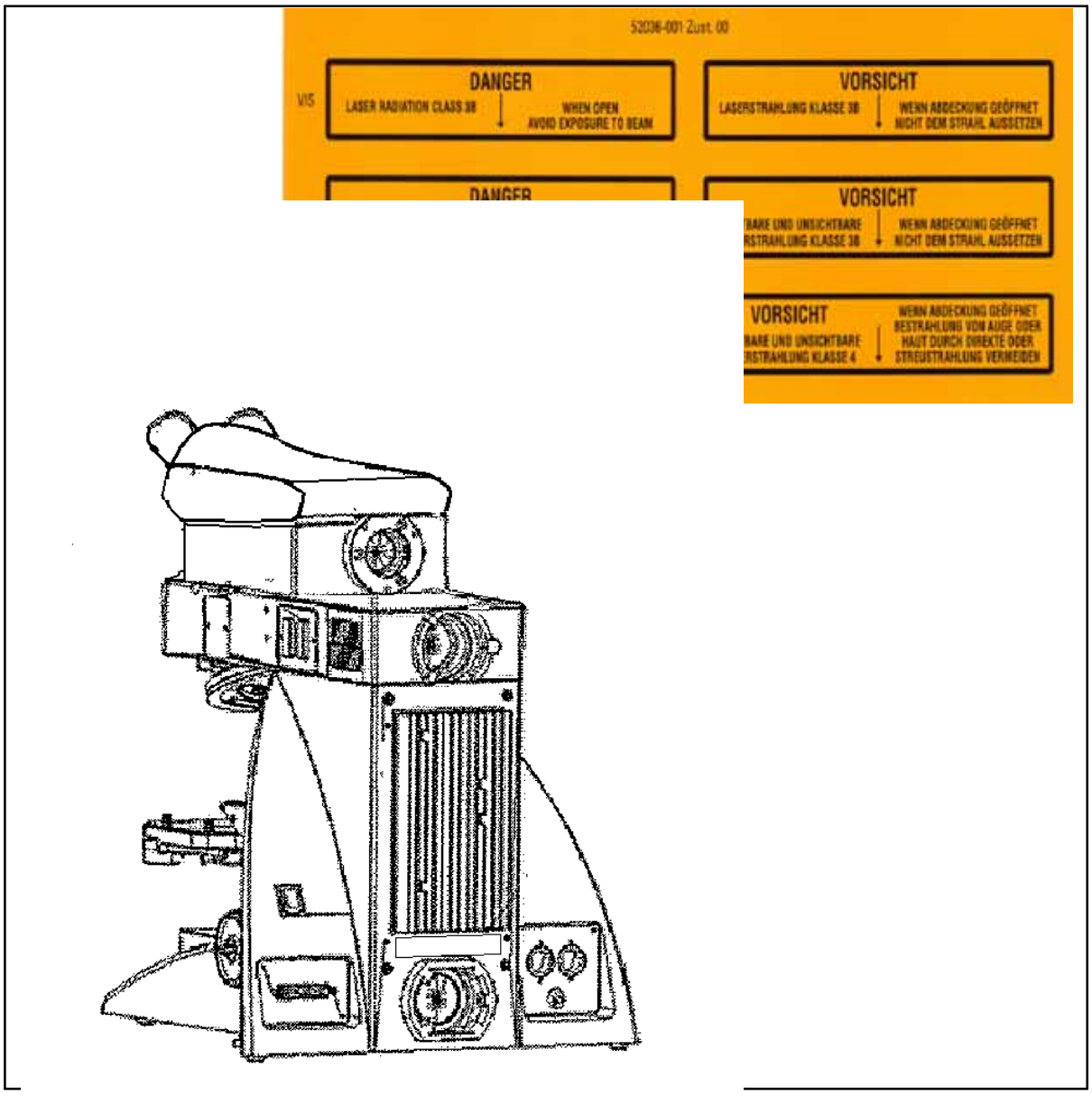


figure26. Safety label for DM 5000/6000 CS upright stand

6.13.3 Scan head

Angled front view of left side of scan head

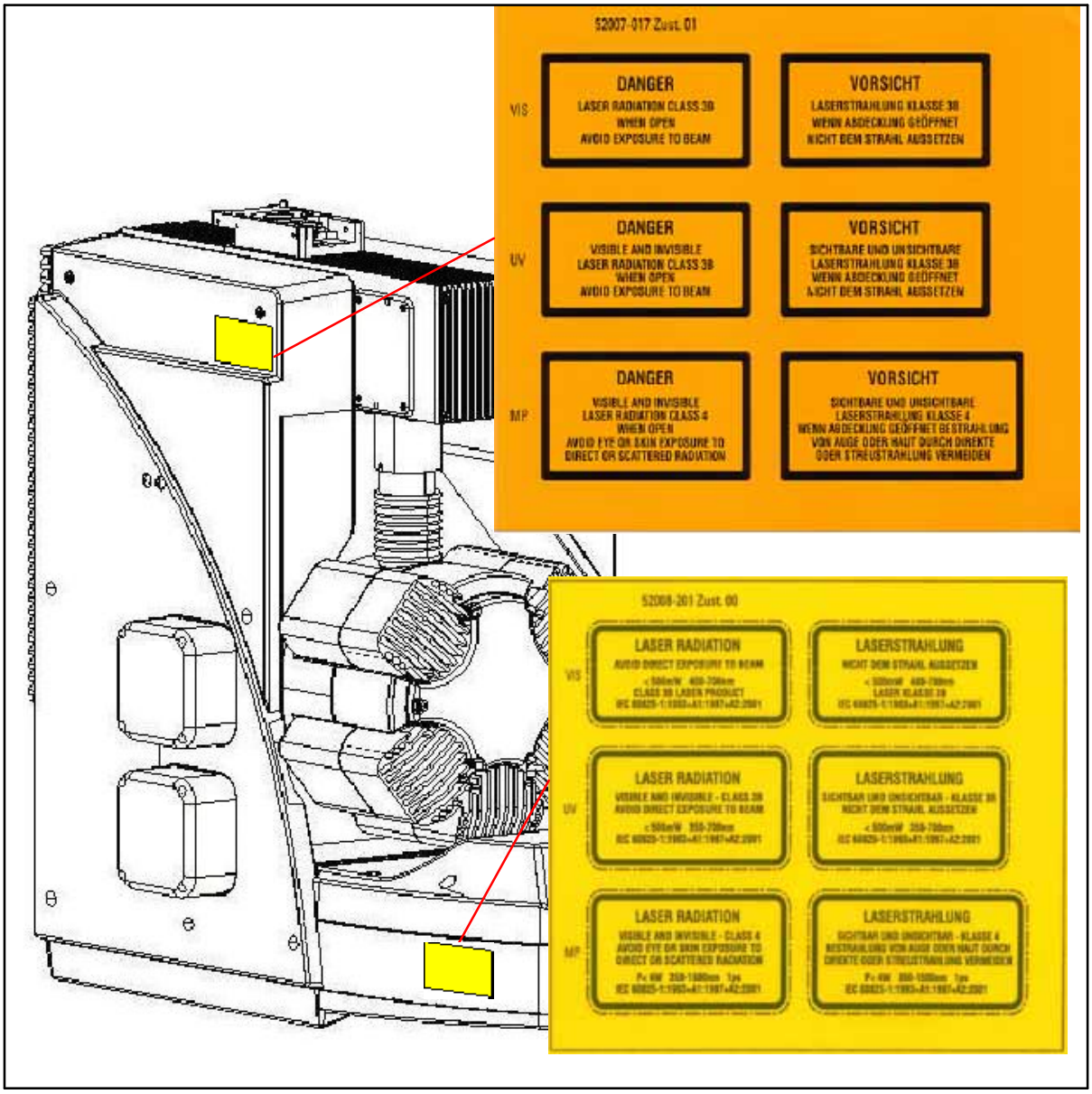


figure27. Safety label for the scanner

6.13.4 Supply unit

View of TCS SP5 supply unit.

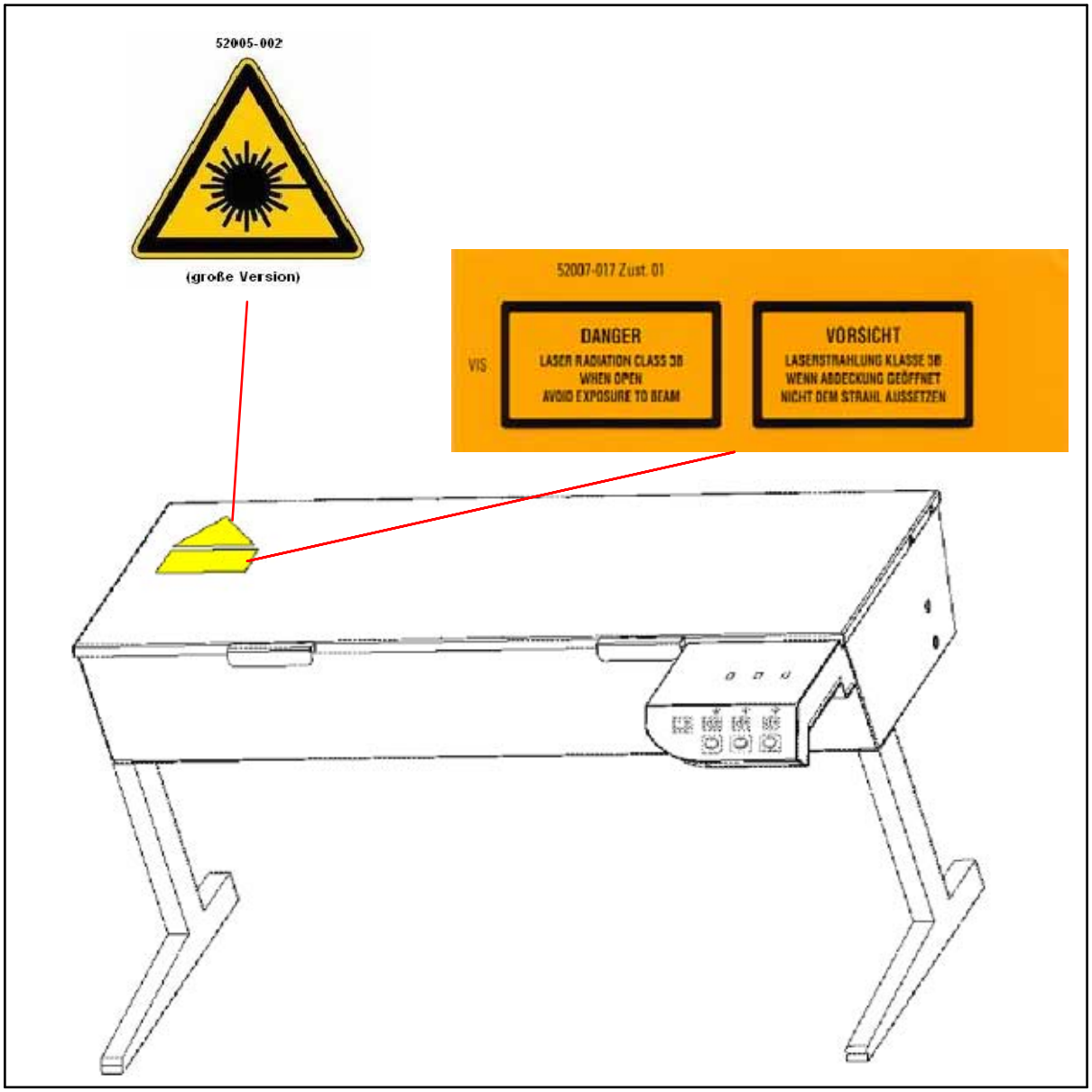


figure28. Safety label for the supply unit TCS SP 5 (front side)

6.13.5 External 405-nm laser Advance / 405-nm imaging laser

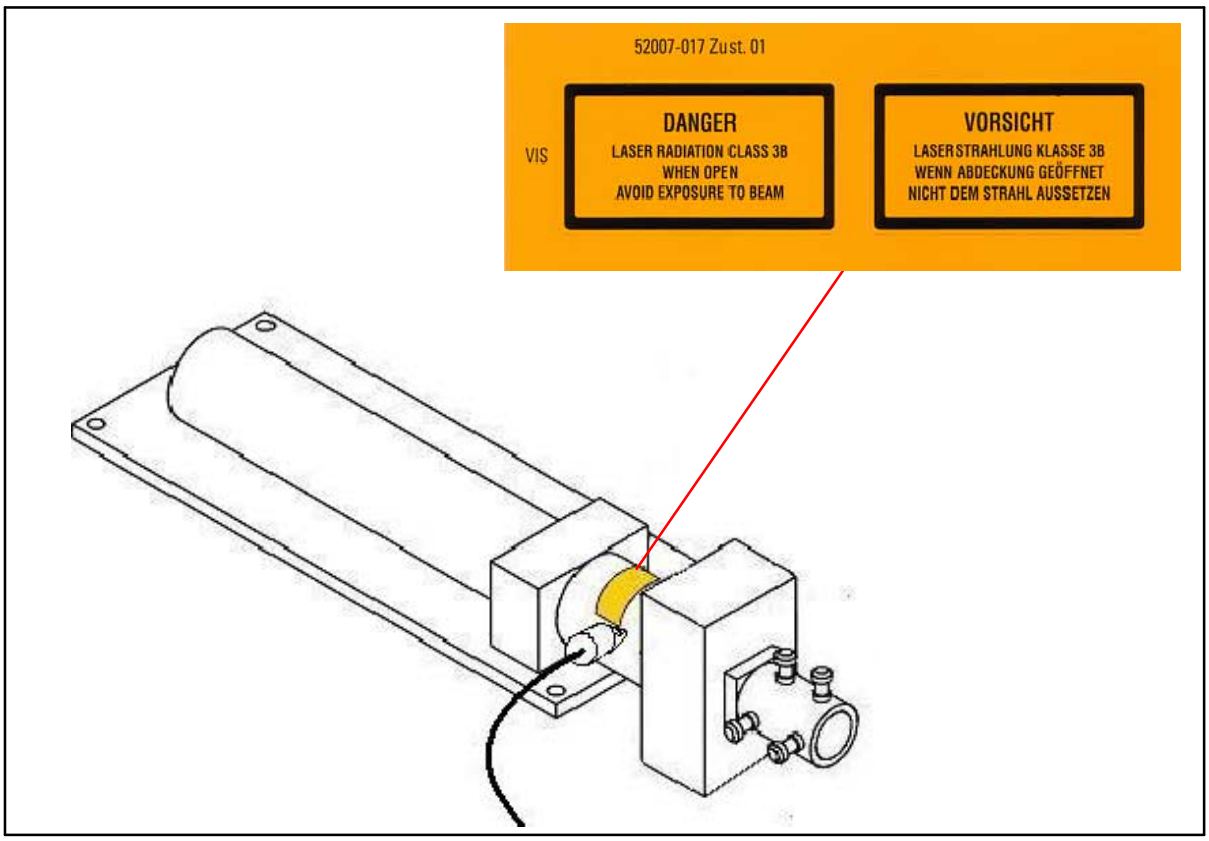


figure29. Safety label for the external 405-nm laser / safety label for the 405-nm imaging laser

6.13.6 External 488-nm laser

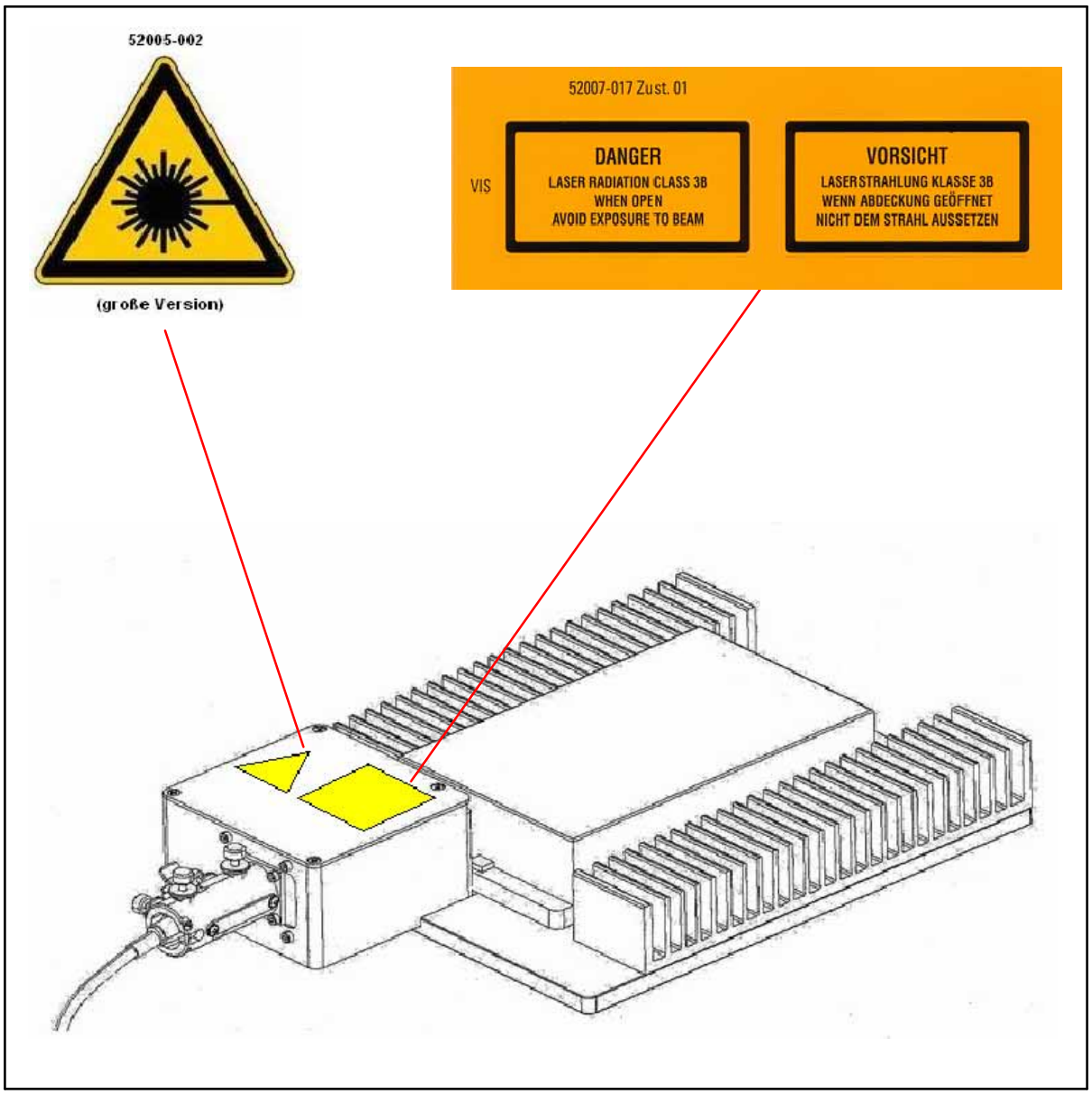


figure30. Safety label for the external 488-nm laser

6.13.7 MP beam coupling unit

Angled front view of the right side of the beam coupling unit MP.

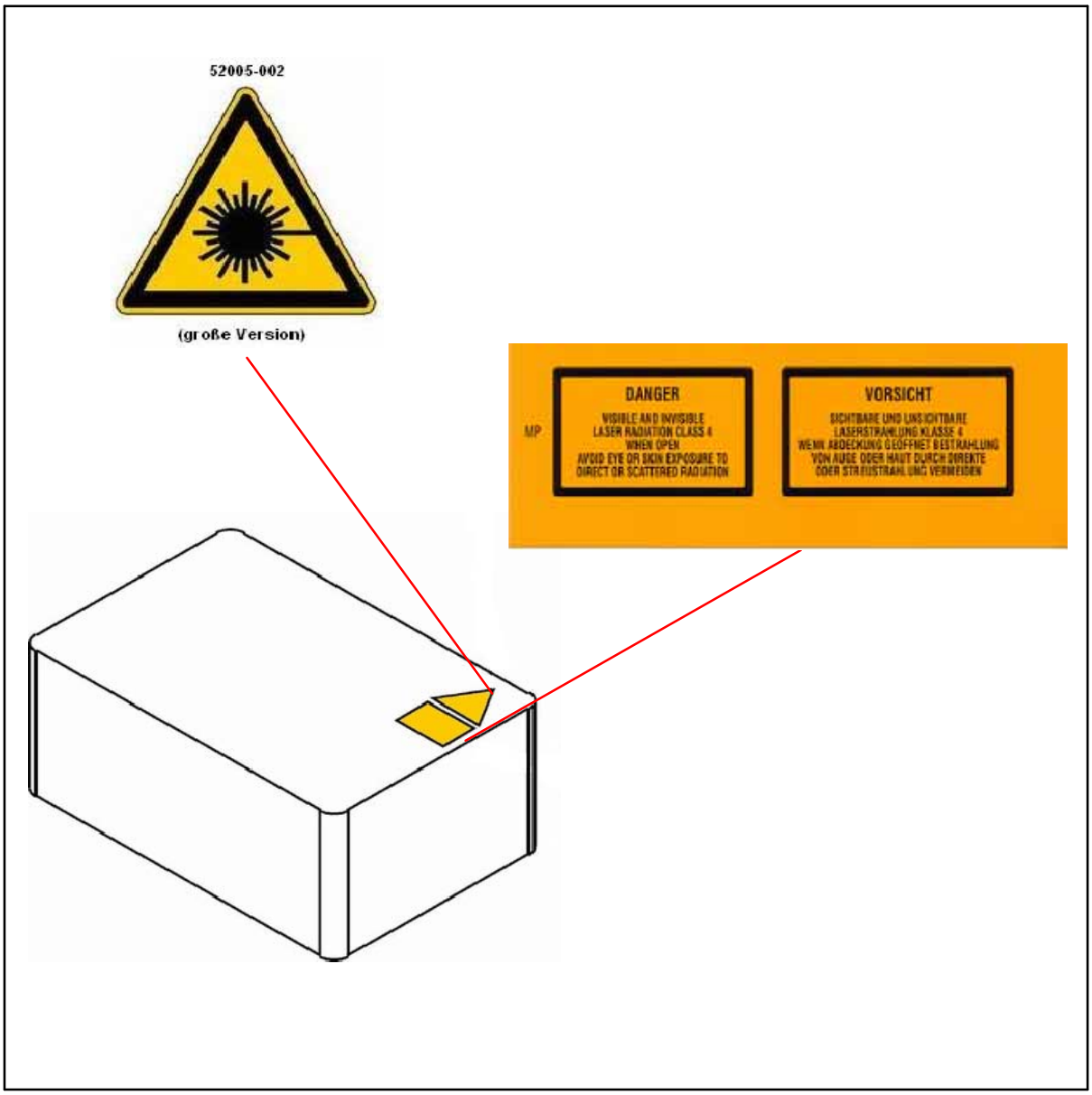


figure31. Safety label for the beam coupling unit MP (top side)

6.13.8 Cover (for replacement flange)

View from front on the cover.

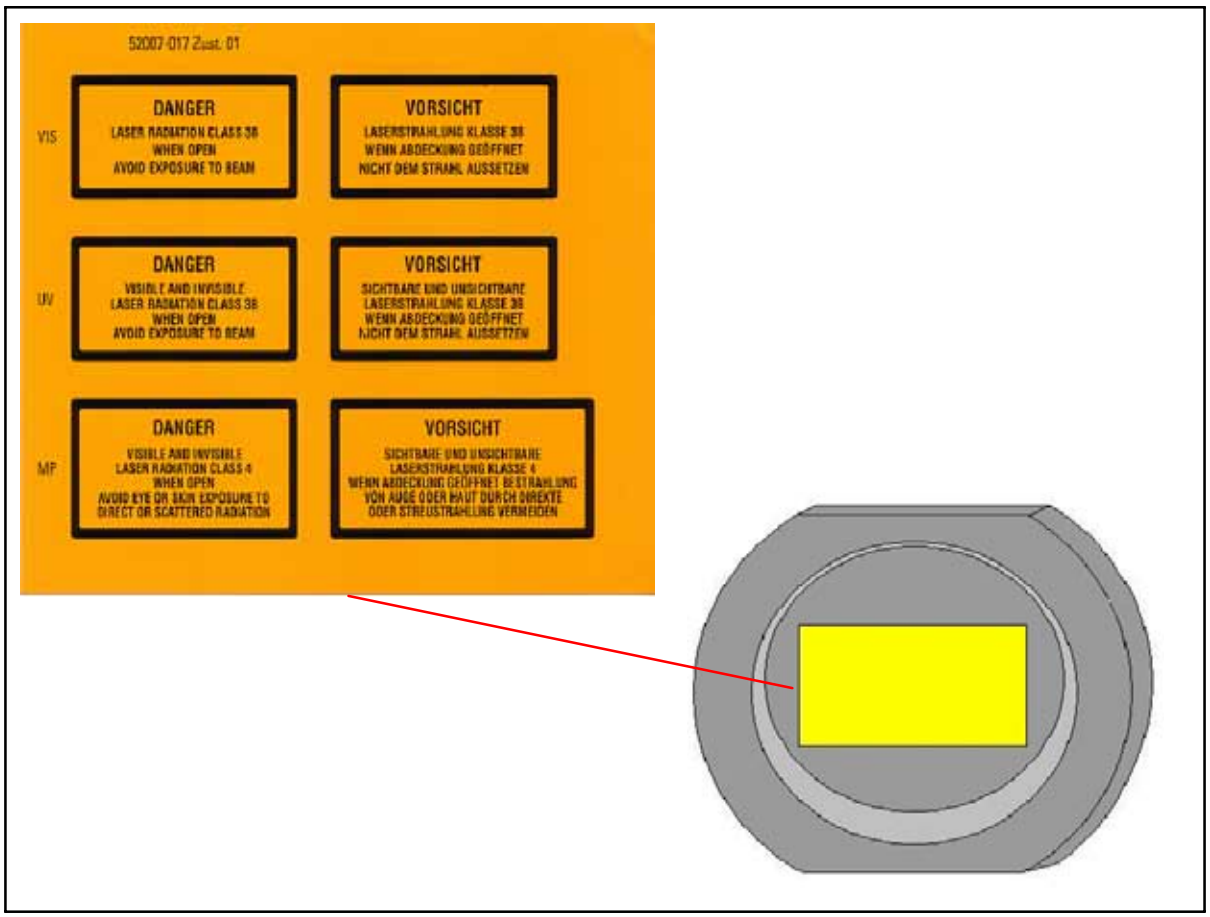


figure32. Cover for replacement flange

If the replacement flange for transmitted light is not equipped with a functional module such as a lamp housing, place a cover over the opening for laser safety reasons.

6.14 Requirements related to the installation/storage location

This device was designed for use in a lab and may not be set up in areas with medical devices serving as life-support systems such as intensive-care wards.

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

To avoid the risk of fire hazard and electrical shock, do not expose the unit to rain or humidity.
Do not open the cabinet. Do not allow any liquid to enter the system housing or come into contact with any electrical components.
The instrument must be thoroughly dry before connecting it to the power supply or turning it on.

6.15 Changing the installation site

Before moving the laser scanning microscope, 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.

6.16 Scanner cooling

The scanner of the TCS SP5 is liquid-cooled.

Observe the attached safety data sheet provided by the manufacturer, Innovatek, regarding the coolant used.

In case of a coolant leak, switch the power off immediately!
Inform Leica or a Leica-approved service facility immediately.

The coolant contains an irritating substance. Avoid eye and skin contact.

The scanner cooling system must be serviced by Leica or a Leica-approved service facility every two years.

7 Care 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 confocal system are summarized below.

Protect 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.

Exercise 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.

7.1 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 lint-free cloth, moistened with distilled water.

Persistent dirt can be removed from glass surfaces by means of pure alcohol or chloroform.

If an objective lens is accidentally contaminated by unsuitable immersion oil or by the specimen, please contact your local Leica branch office. 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. Repeat the process until the lens is completely clean. Use a clean piece of lens tissue each time.

7.2 Cleaning the microscope surface

Use a lint-free linen or leather cloth (moistened with 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.

8 Startup of the system

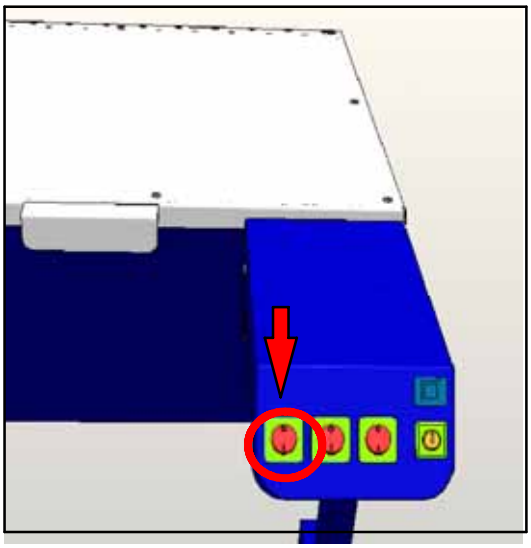


figure33. Switching on the workstation

Proceed as follows to start your TCS SP5 system:

1 Switch on the TCS workstation.

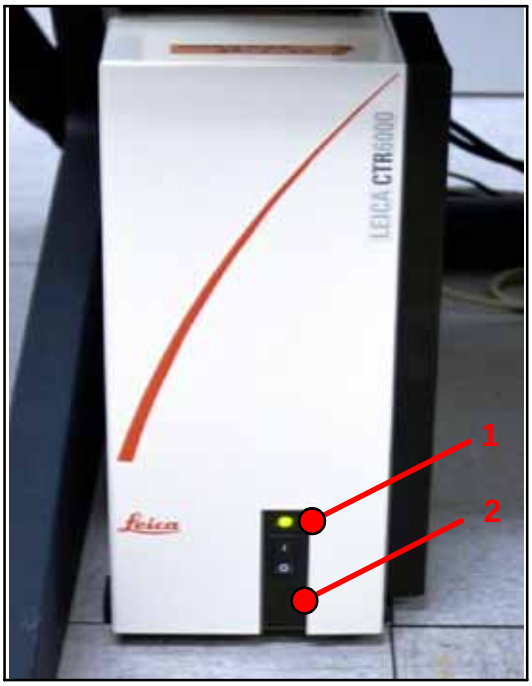


figure34. Switching on the microscope

Check whether the microscope stand is switched on.

If the readiness indicator (Figure 31/1) on the MIC box is lit, the stand is operating.

If the readiness indicator is not lit, activate the toggle switch (Figure 31/2) of the MIC box.

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.

1 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.

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

The default user name for the Leica TCS SP5 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 AF software is based on the user administration of the operating system, separate files are created for managing user-specific profiles of the LCS AF software. For information about setting up users, please refer to the chapter ”Setting Up Users” in this manual.

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

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

5 Type your current password in the ”Old Password” field (passwords are case sensitive, so be sure you use the right case).

6 Then press the Tab key. Pressing the Tab key moves the cursor to the next field.

7 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.

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

8.1 Setting Up Users

1 Log on as administrator. Us the ID ”Administrator” and the password ”Admin”

2 Open the User Manager. Select Start / Programs / Administrative Tools / User Manager.

3 Define a new user. Enter at least the following information in the open dialog window:

D User name

D Password (must be entered again in the next line for confirmation purposes)

4 Select the following two check boxes:

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)

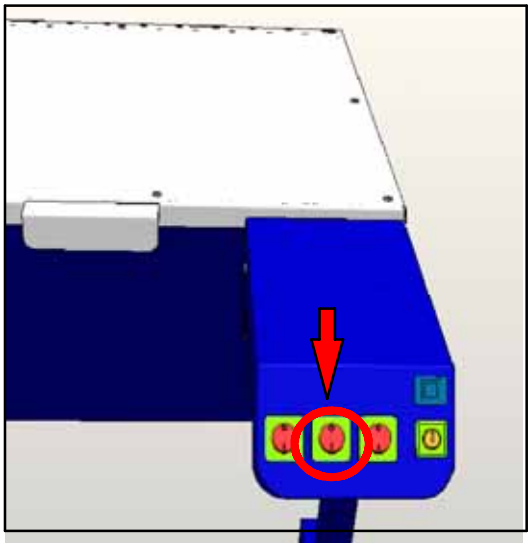
5 Select the ”Profiles” option in the bottom section of the dialog. In the ”Local path” field, enter the following path for storing the user-specific file: d:\users\username (”username” is a wildcard which must be replaced by the currently defined user name.)

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

figure35. The ”booted” system

6 Wait until the boot process of the TCS workstation is completed.

7 If not yet done, create a user and log on.



8 Turn on the TCS SP5 scanner.

figure36. Turning on the scanner

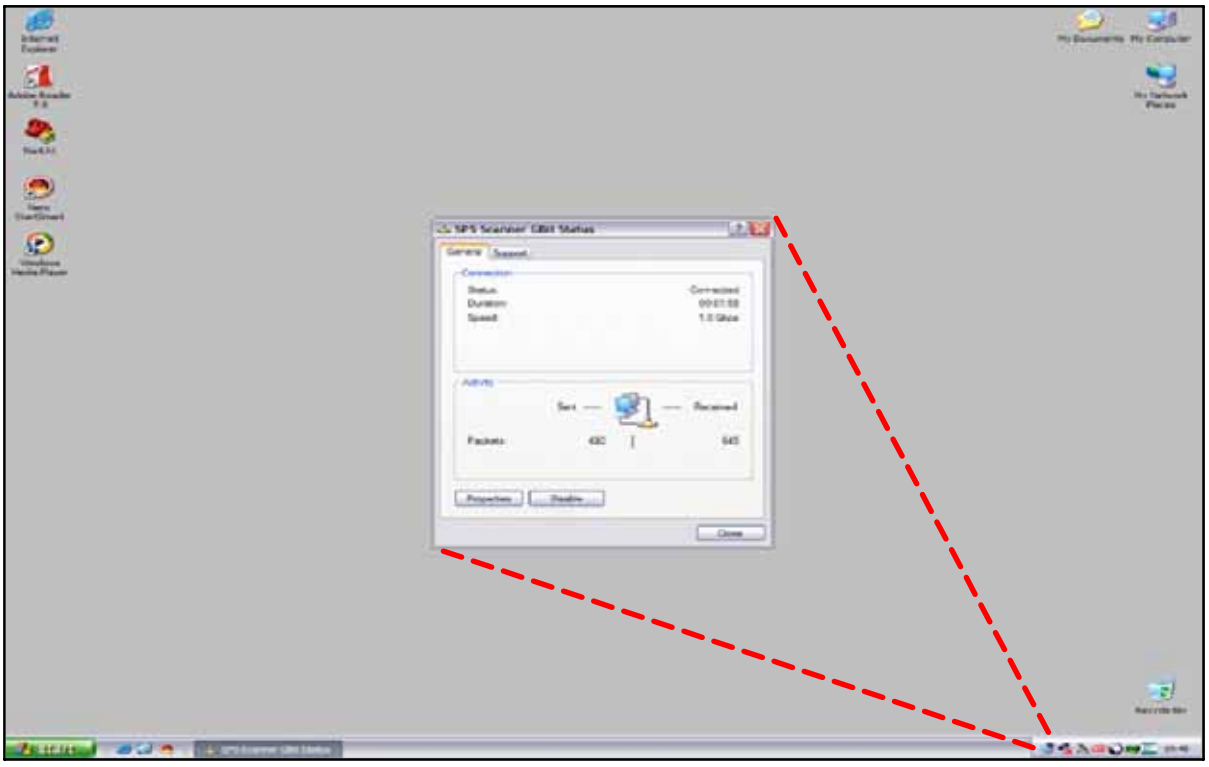


figure37. Turning on the scanner

The Windows status bar must show the ”SP5 Scanner Gigabit Interface” as connected!

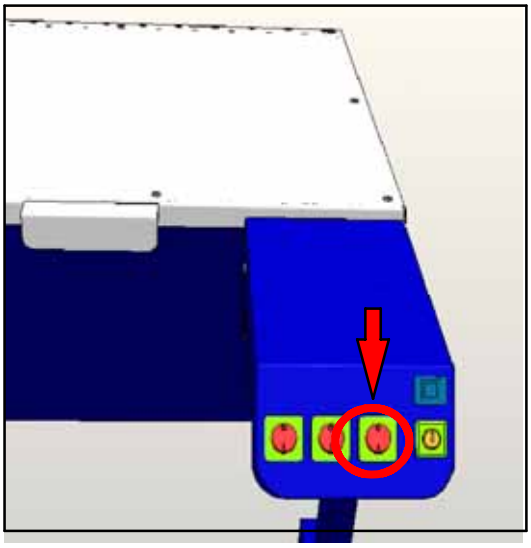


figure38. Switching on the laser

9 Switch on the lasers.

The power supplies and fans of the system start first.

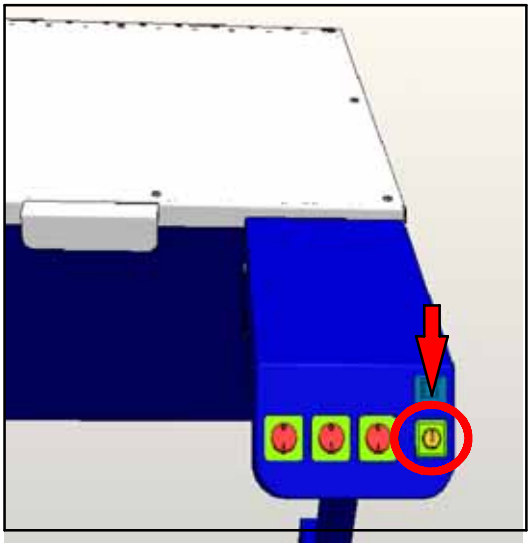


figure39. Activating the detachable key switch

10 Activate the detachable key switch.

Laser radiation may be present in the specimen area as of this time. Follow the safety instructions given in Chapter 6.

Please follow the instructions in Chapter 14 to switch off the TCS SP5 system.

9 Starting the LAS AF

1 Start the LAS AF by double-clicking the program icon.

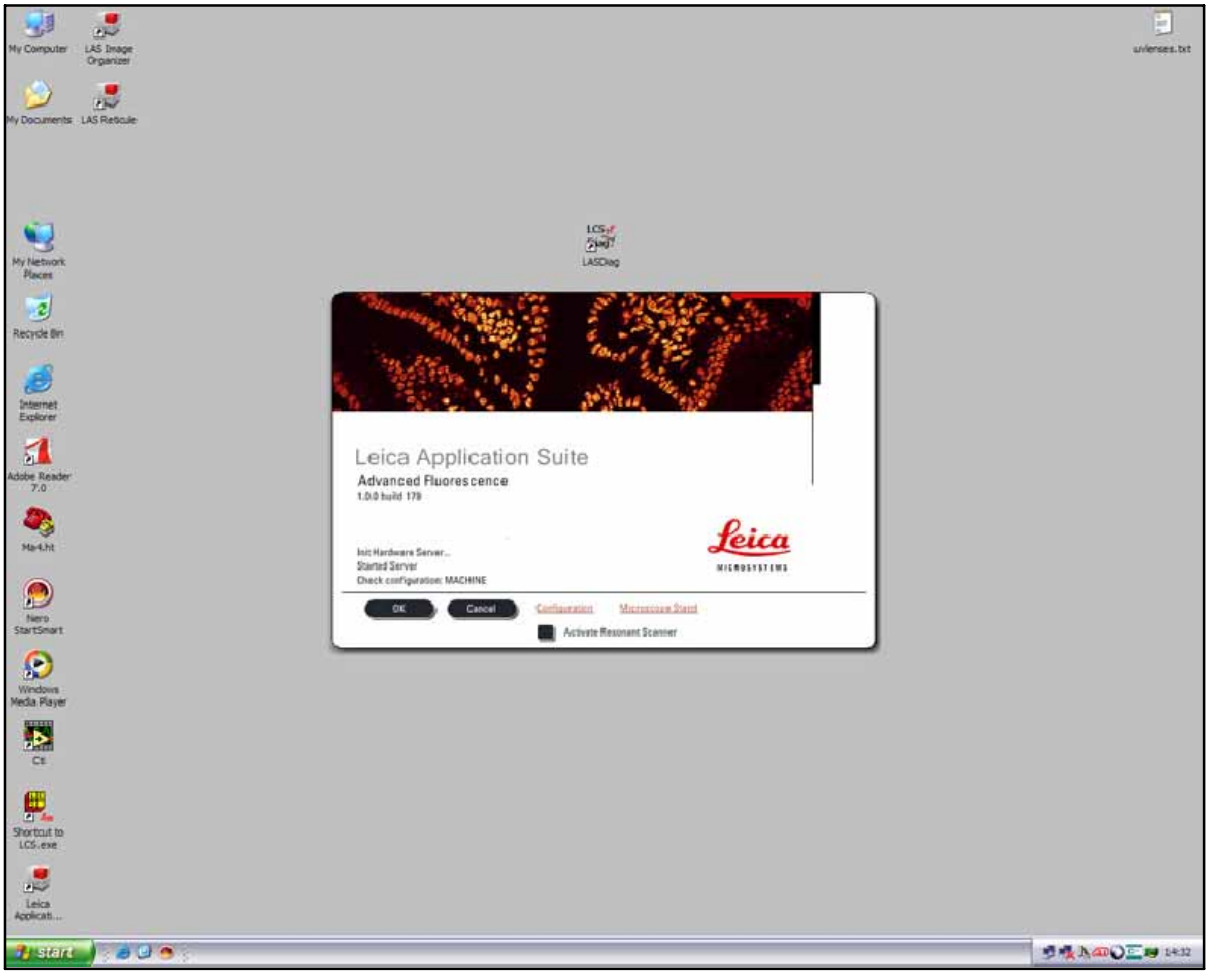


figure40. Starting the LAS AF

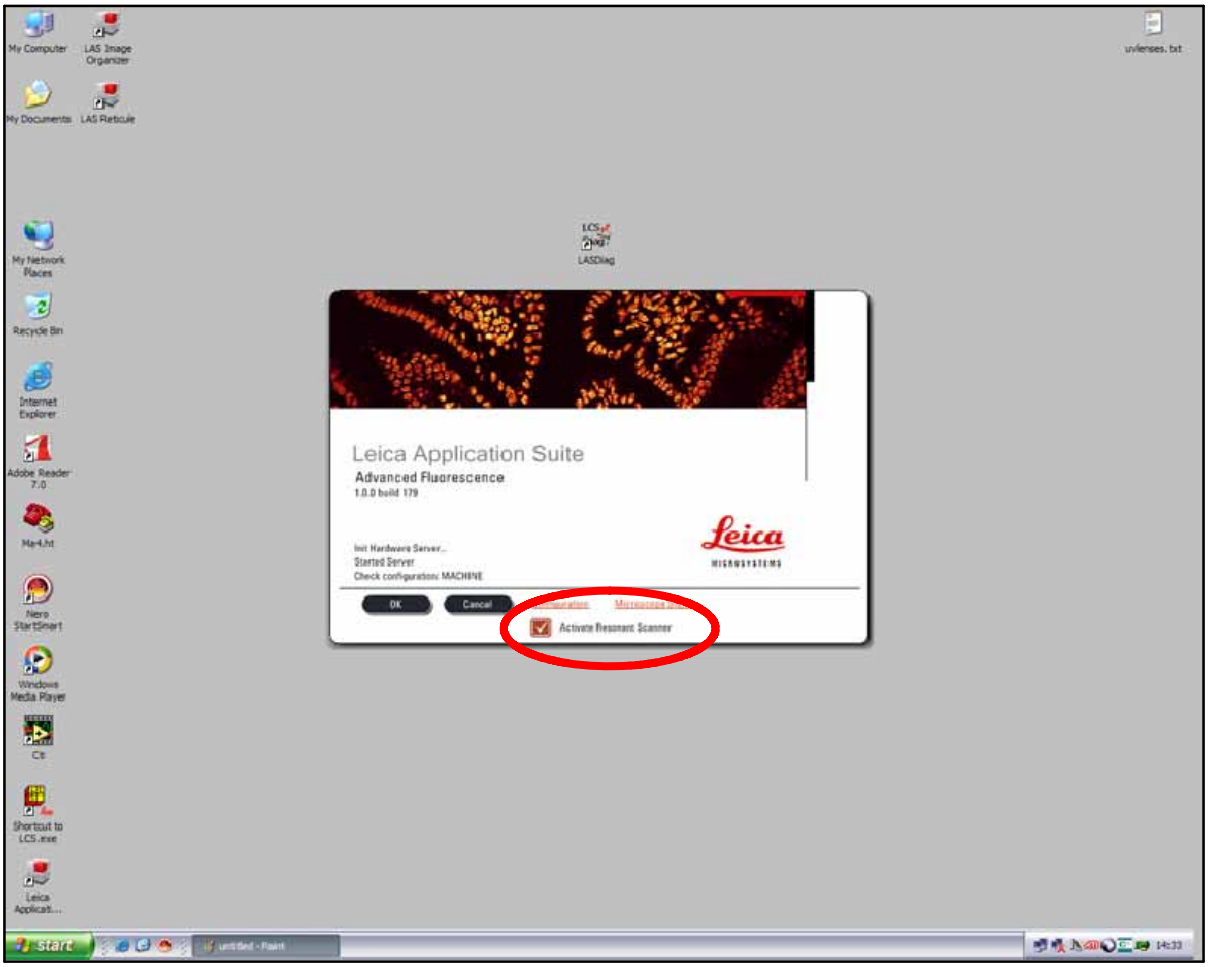


figure41. Resonant or non-resonant

If you purchased this option, you can start the system in resonant or non-resonant mode at this point.

2 Select whether the TCS SP5 system should be operated in resonant or non-resonant mode.

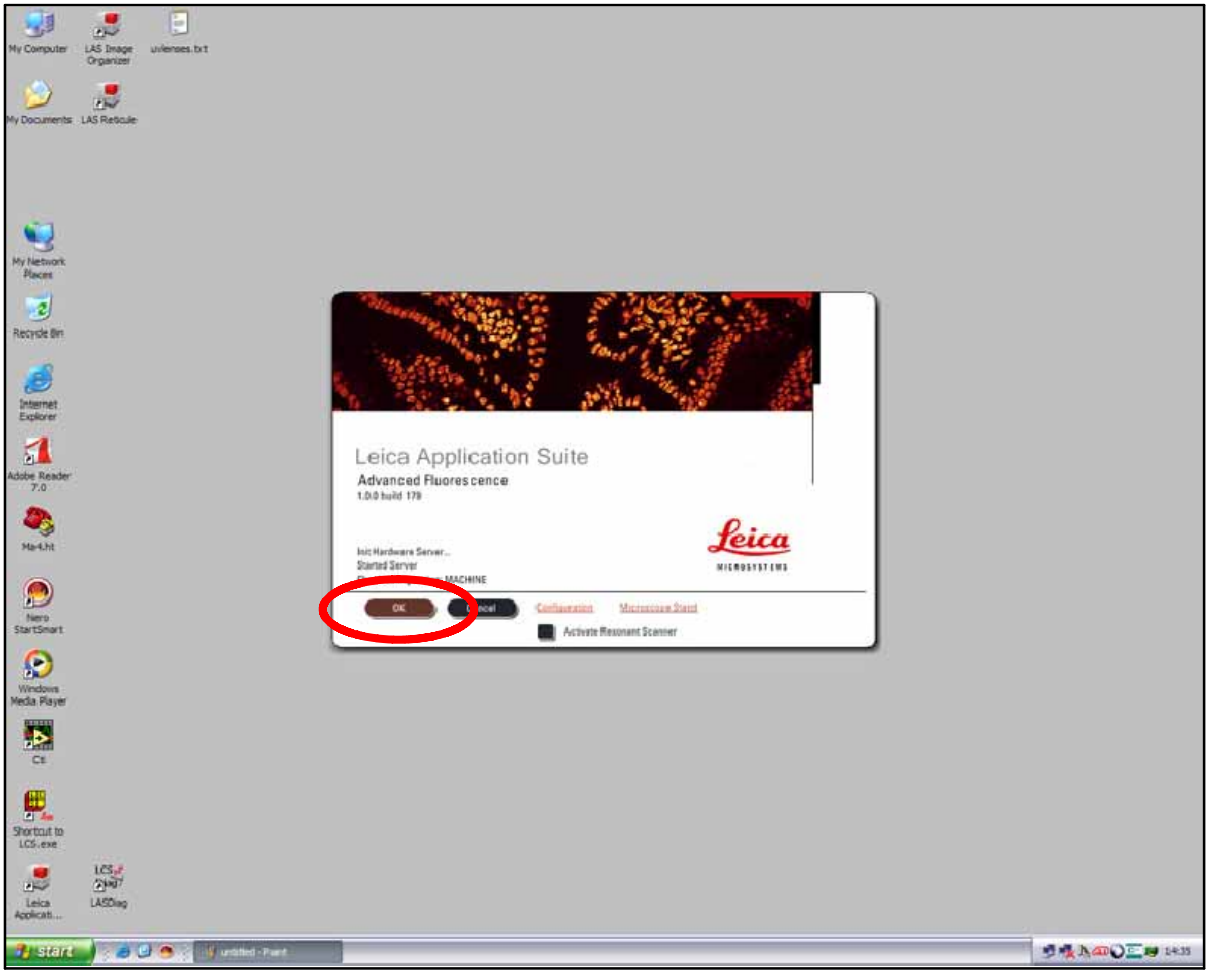


figure42. LAS AF start window

3 Start the LAS AF by clicking on ”OK”.

figure43. LAS AF base view

You are now in the base view of the LAS AF.

4 Start the LAS AF help with the F1 key.

10 Introduction to LAS AF – Help

10.1 General

The LAS AF software is used to control all system functions and acts as the link to the individual hardware components.

The experimental concept of the software allows for managing the logically interconnected data together. The experiment is displayed as a tree-structure in the software and features export functions to open individual images (JPEG, TIFF) or animations (AVI) in an external application.

10.1.1 Calling Online Help

The LAS AF software features a context-sensitive help system that explains the different functions of the system.

The online help can be called in two ways:

- In the respective context (context-sensitive)
- Via the Help menu

10.1.2 Structure of the online help

The online help is divided into 6 different books:

Books	Contents
General	This book contains general information about online help and the contact information of the manufacturer.
Structure of the user interface	This book describes the structure of the LAS AF user interface and features topics about the various menus, registers, operating steps and symbols.
Dialog descriptions	This book describes each individual dialog window of the user interface in individual topics.
Learning the basics	The fundamental steps for performing an experiment are described here.
Guidelines	This book contains step-by-step guidelines for performing certain applications. They are divided into two categories: for **beginners** and for **advanced users**.
Additional information	This book contains detailed descriptions about certain topics of biology, image editing, filter...

10.2 Imprint

Online help LAS AF; Version: 1.0

Address	Leica Microsystems CMS GmbH Am Friedensplatz 3 68165 Mannheim
Phone	+49-621-7028-0
Fax	+49-621-7028-2980
Internet	http://www.confocal-microscopy.com.

10.2.1 In the respective context (context-sensitive)

1 Click on the small question mark located in the top right corner of every dialog window.

2 Online help opens directly to the description for the corresponding function.

10.2.2 Via the Help menu

1 Click on the **Help** menu on the menu bar. The menu drops down and reveals the following search-based options:

Contents	This dialog field contains the table of contents in form of a directory tree that can be expanded or collapsed. Double-click an entry of the table of contents to display the corresponding information.
Index	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. Select a keyword. View the corresponding content pages by double-clicking the key word or selecting it and then clicking the Display button.
Search	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.

10.2.3 Full-text search with logically connected search terms

Click on the triangle to the right of the input field on the Search tab to view the available logical operators.

1 Select the desired operator.

2 Enter the second search term you would like to associate with the first search term behind the operator:

Examples	Results
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”.

10.3 Key combinations

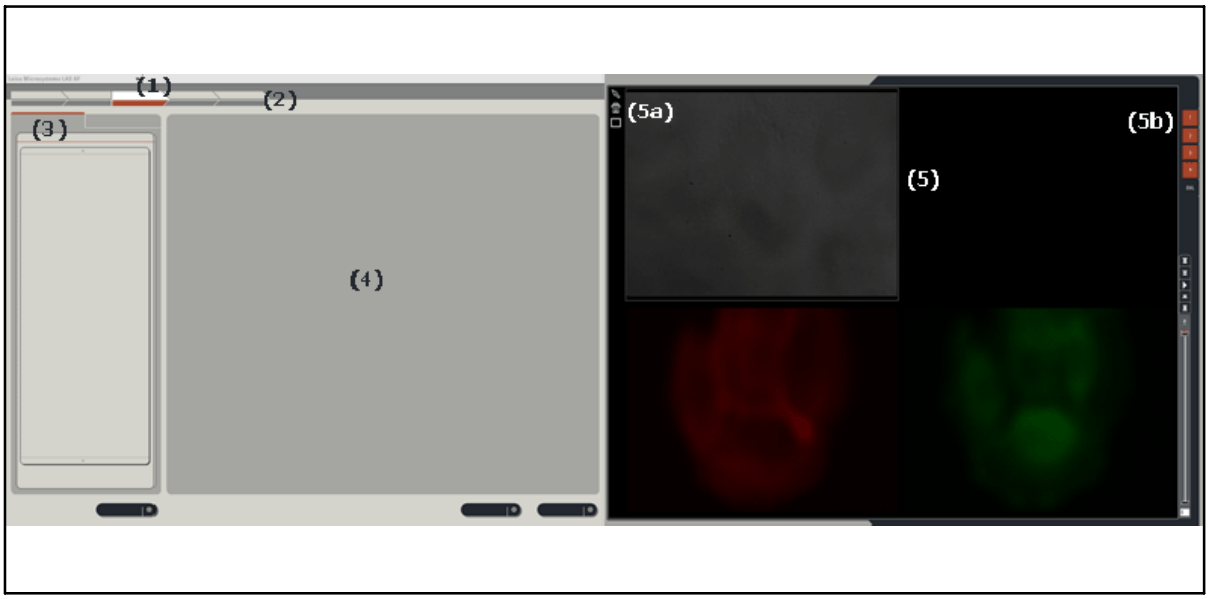
In order to accelerate recurring software functions, special key combinations have been defined:

CTRL + N	Opens a new experiment
CTRL + O	Starts the Open dialog window to open an existing file.

11 Structure of the user interface

11.1 General structure of the user interface

The user interface of the LAS AF is divided in five areas:



1 **Menu bar**: The different menus for calling functions are available here

2 **Arrow symbols:** Operating step with the individual functions. These operating steps mirror the typical sequence of an image recording and subsequent image processing. The functions are arranged appropriately in these operating steps.

D Configuration

D Acquire

D Process

D Quantify

D Application

3 **Tab area:** Different tabs belong to every operating step (arrow symbol) in which the settings for the experiment can be made.

Acquire	Experiments: Directory tree of opened files
	Setup: Hardware settings for the current experiment
	Acquisition: Parameter settings for the image recording
Process	Experiments: Directory tree of opened files
	Tools: Directory tree with all the functions available in the respective operating step
Quantify	Experiments: Directory tree of opened files
	Tools: Tab with the functions available in this operating step
	Graphs: Graphical display of values measured in regions of interest (ROI)
	Statistics: Display of statistical values that were determined in the drawn in regions of interest (ROI)

4 **Working area:** This area provides the **Beam Path Settings** dialog window in which the control elements for setting the recording parameters are located.

5 **Viewer window** : Displays the recorded images. In the standard setting, the Viewer window consists of the image window in the center and the buttons for image editing **(5a)** and channel display **(5b)**.

12 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.

12.1 Setting the Köhler Illumination

1 **Focusing:** 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.

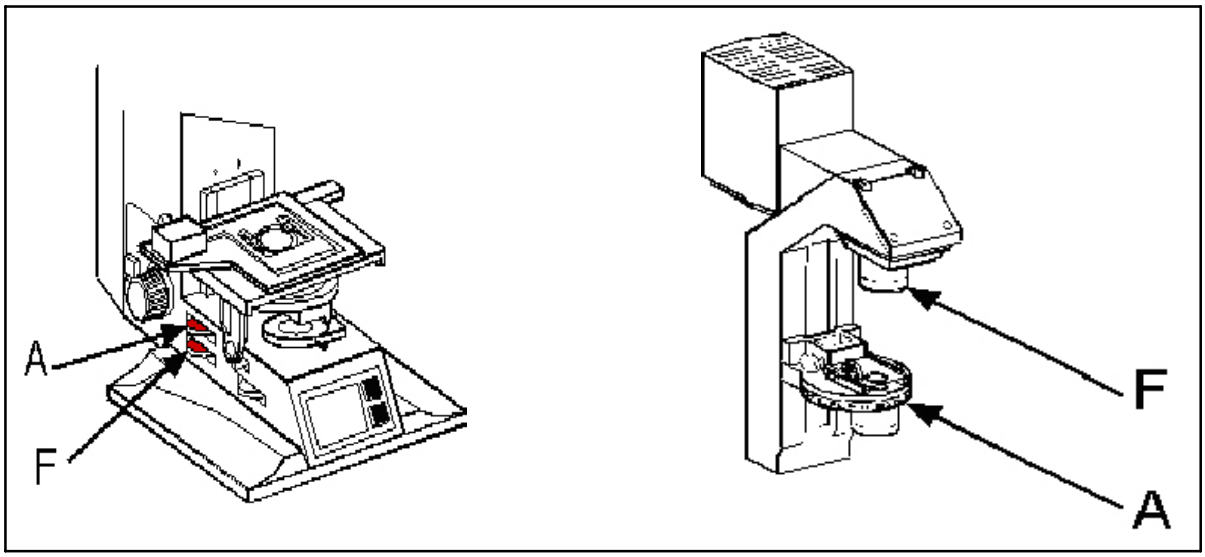


figure44. DMxxxx (left) / DMIxxxx (right)

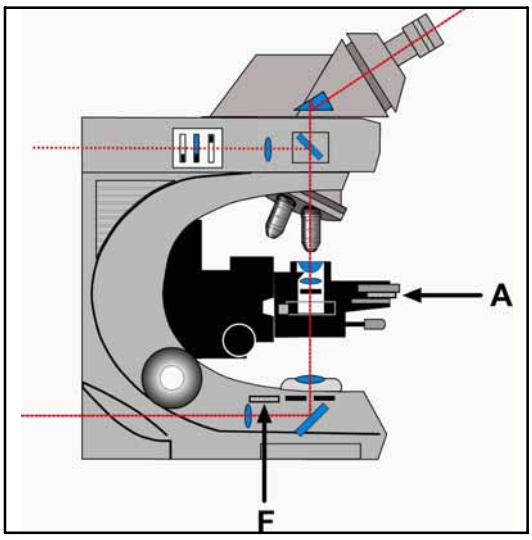


figure45. Field diaphragm

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 **Focusing**

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.

13 Introduction to confocal work

13.1 Preparation

The following sections describe a number of basic procedures that cover most of the tasks related to the instrument.

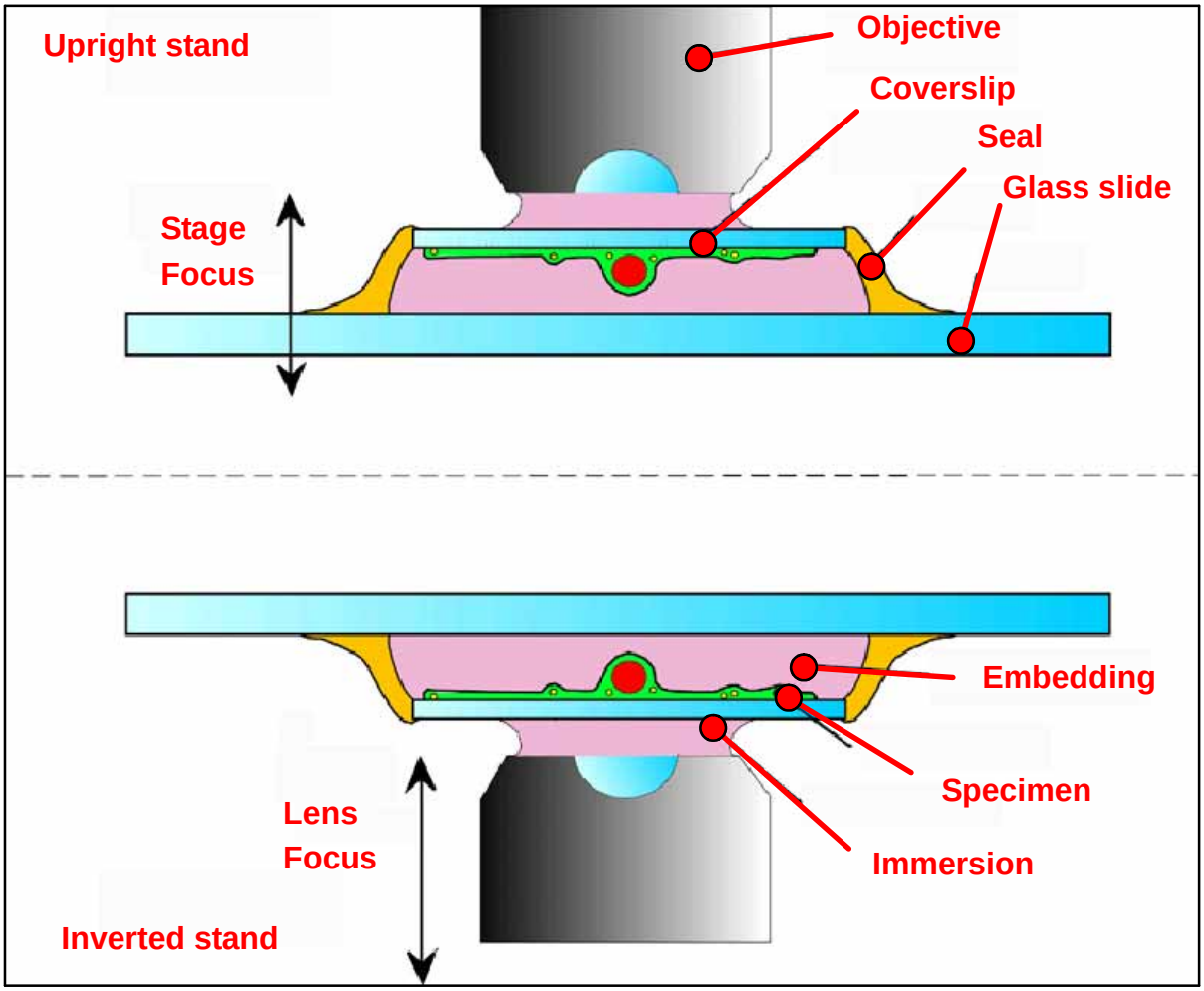


figure46. Arrangement of coverslip and specimen on an upright microscope (top) and inverted microscope (bottom). When using objectives with coverslip correction, ensure that the coverslip (i.e. the top side of embedded specimens) is facing down.

Background information has also been provided to explain the reasons behind various settings. These are not descriptions of the

individual functions and controls of the instrument and graphical user interface, but an informative tour of the essential tasks that is designed to remain valid even if future upgrades change the specific details of operating the instrument.

The very first step is to place a specimen in the microscope, of course. When placing specimens in an inverted microscope, ensure that fixed specimens on slides are inserted with the coverslip facing down (Fig.43). Failing to do so is a frequent reason for not being able to find the specimen or focus on it in the beginning.

13.1.1 The objective

Select the objective with which you want to examine the specimen initially.

Medium		Refractive Index
Water	Imm	1,333
PBS	Emb	1,335
Glycerol 80% (H_2O)	Imm	1,451
Vectashield	Emb	1,452
Glycerol	Imm	1,462
Moviol	Emb	1,463
Kaisers Glycerol Gel	Emb	1,469
Glass	Mat	1,517
Oil	Imm	1,518
Canada Balsam	Emb	1,523

Table 3 Immersion media

When using immersion objectives, ensure that an adequate quantity of immersion medium is present between the front lens of the objective and the specimen. Immersion oil, glycerol 80% and water may be used as immersion media (Table 3). Apply the immersion medium generously, but be sure that it does not flow into the stand of inverted microscopes.

13.1.2 Conventional microscopy

To view the specimen conventionally through the eyepieces, select ”VISUAL” operating mode. ”SCAN” is for use with the laser scan image process. Select a suitable position and focus on the specimen.

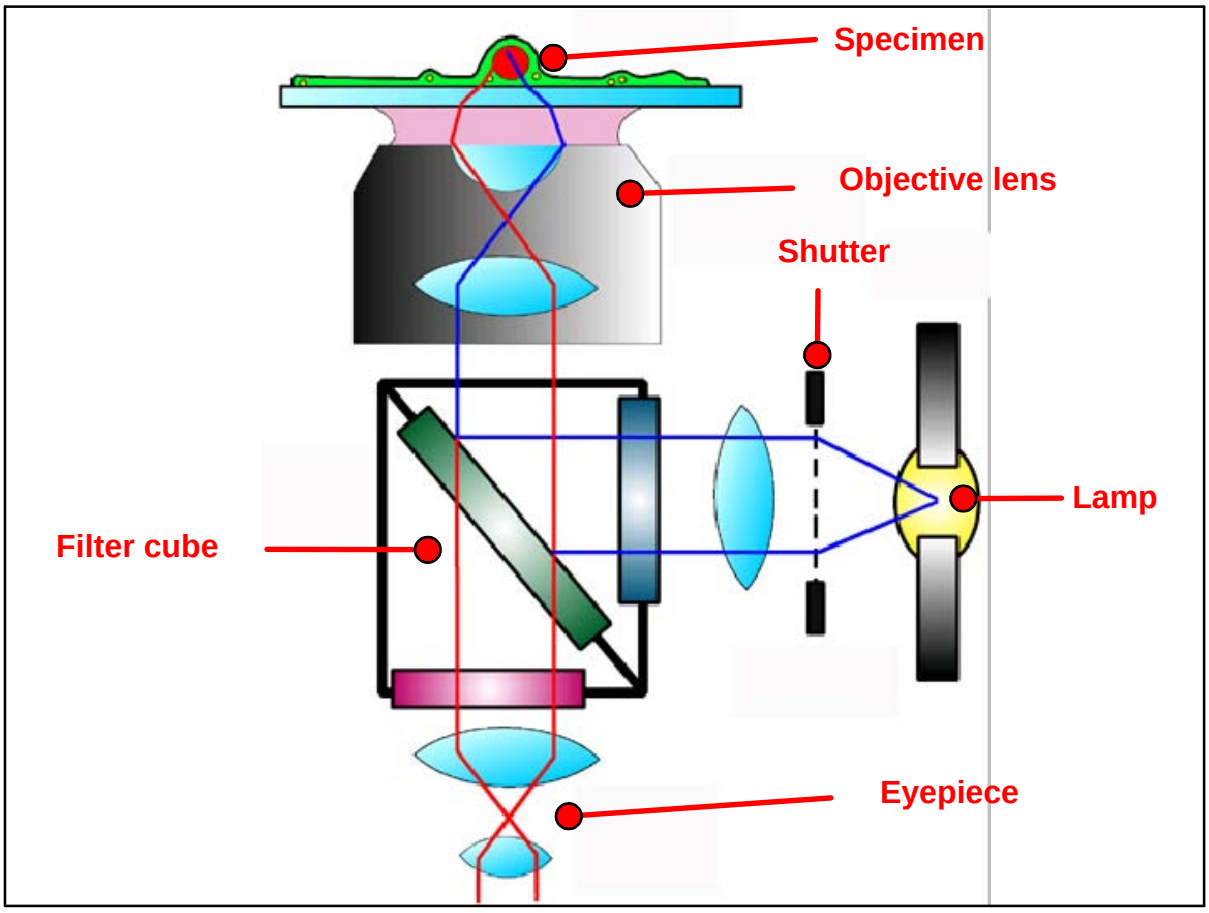


figure47. Incident light fluorescence scheme: light from a mercury lamp is collimated, selected spectrally via an exciter filter and applied to the specimen via a color splitter mirror. A shutter permits the specimen to be darkened. The emission (longer wavelength than the excitation) is visible through the color splitter mirror and emission filter via the eyepiece. The exciter filter, color splitter mirror and emission filter are grouped in a filter cube.

Optical sections are created using a transmitted-light process. Your specimen must therefore reflect or fluoresce. Fluorescent specimens are most common. In many cases, specimens with multiple stains will be examined. Reflective specimens can also provide interesting results, however.

Filter cubes **(Fig. 44)** suitable to the fluorescence must be positioned in the beam when

viewing the specimen via the eyepieces. For more information on selecting fluorescence filter cubes, please refer to the Leica fluorescence brochure or contact your Leica partner. For a selection of filter cubes, see Table 4 below.

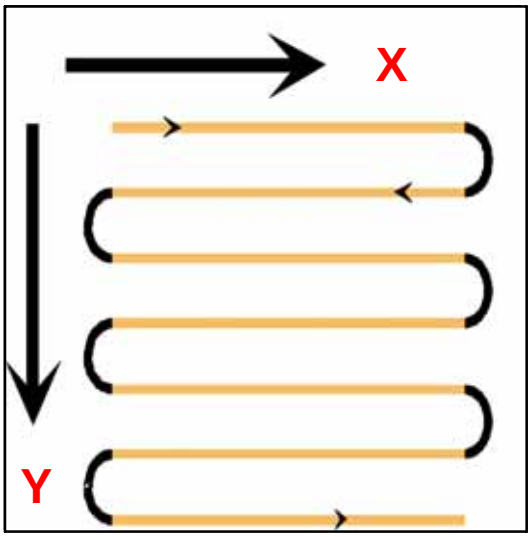
As specimen fluorescence can fade quickly, always close the shutter of the mercury lamp when you are not looking into the microscope.

To switch to scan mode, press the appropriate buttons on the stand or use the switching function in the software. The switching function may vary according to the motorization of the microscope. Please consult help for more information.

Filter cube	Excitation filter	Dichroic mirror	Emission filter
A		400	
A/G		430;500	
B/G/R		415	
B/R		435;565	
BFP/GFP		420	
CFP		455	
D		455	
E4		455	
FI/RH		500	
G/R		505	
GFP		500	
H3		510	
I3		510	
K3		510	
L5		505	
M2		580	
N2,1		580	
N3		565	
Y3		565	
Y5		560	
YFP		515	

Table 4 Selection of filter cubes for Leica research microscopes and associated filter specifications.

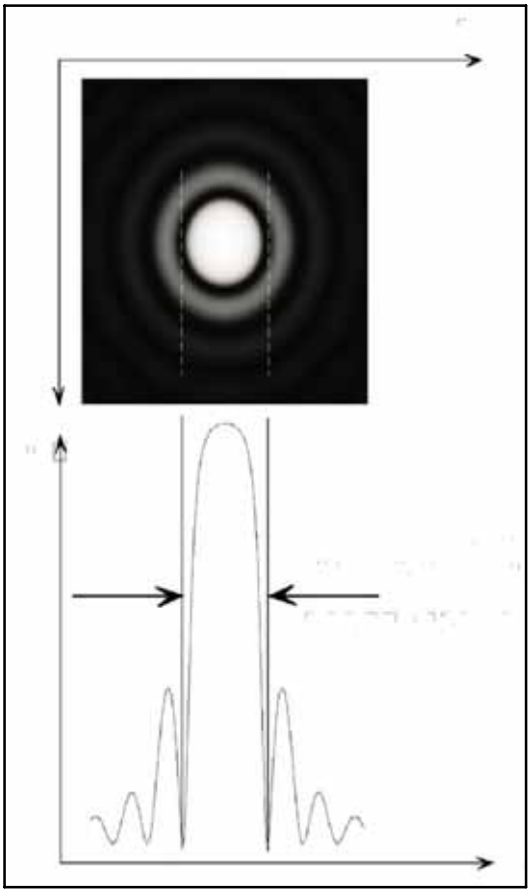
13.1.3 Why scan?



Specimens must be illuminated over the smallest possible area to achieve a true confocal image – this is essential to attaining truly thin optical sections.

That has been achieved when the illumination spot is diffraction limited; i.e. it cannot be made physically smaller. The diameter of such a diffraction limited spot corresponds to d_B=1.22*ë/NA, with ë representing the excitation wavelength and NA the numerical aperture of the objective used (Fig. 46).

figure48. Illustration of the raster scan. Two mirrors move the illumination spot in x and y directions across the specimen in rows so that the entire image can be reconstructed in parallel.



To create a two-dimensional image, the spot must be moved over the entire surface and the associated signal recorded on a point-by-point basis.

This is performed in a raster process similar to that of SEM instruments or the cathode ray tubes still used in computer monitors and televisions (Fig. 45). In a confocal microscope with point scanners, the movement is realized by two mirrors mounted on so-called galvanometric scanners. These scanners have the same design as electric motors; their rotors are fixed at their base to the housing. Applying power to the scanner turns the axis to the point at which the torsional force and the electromagnetic force balance. The mirror can thus be moved quickly between two angles by applying an alternating voltage.

figure49. Smallest possible, diffraction-limited illumination spot (Airy disk). Below: an intensity profile.

To scan a line, the mirror must travel across the field of view. The y mirror is then moved a small amount, after which the x mirror then scans the next line. The signals from the specimen are written to an image memory and can be displayed on the monitor.

13.1.4 How is an optical section created?

The term ”confocal” is strictly technical and does not describe the effects of such an arrangement. That will be described in greater detail here.

As already described in 13.1.3, the illumination of the specimen is focused on the smallest possible spot. The confocal design also involves an observation point. The sensitivity distribution of the detector is reduced to a point by focusing light from the specimen on a very small opening, the so-called pinhole. This pinhole cuts off all information not coming from the focal plane (Fig. 47).

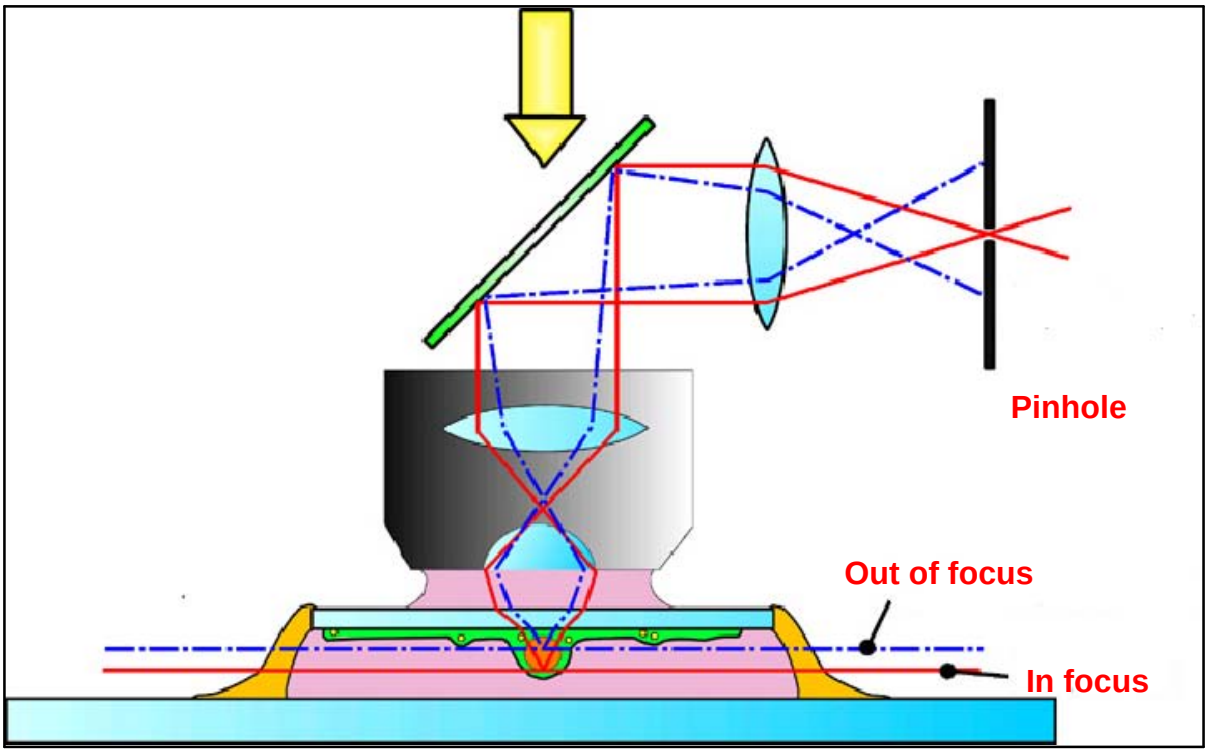


figure50. Creating an optical section using an incident-light process. Light not originating from the focal plane is cut off by a spatial filter (here, a pinhole). Only information from the focal plane can reach the detector.

The diaphragm thus acts as a spatial filter, only when used with the correct, i.e. point-shaped illumination.

As a rule, the optical section becomes thinner when the size of the pinhole is reduced. This effect is reduced near the wavelength of the light used, and at a pinhole diameter of

zero one would theoretically receive the thinnest optical section for the wavelength and numerical aperture used. A range apparently exists at 1 Airy which does not yet offer the thinnest optical sections, but which is nevertheless very close to the theoretical limit. As the intensity of the passing light increases roughly in proportion to the square of the pinhole diameter, it is advisable not to close the pinhole too far to avoid excessive image noise. A value of 1 Airy is a very good compromise and is selected automatically by the Leica TCS SP5. A dialog is available to set smaller or larger diameters if required. Playing with this parameter to study its effects can be very worthwhile when you have the time.

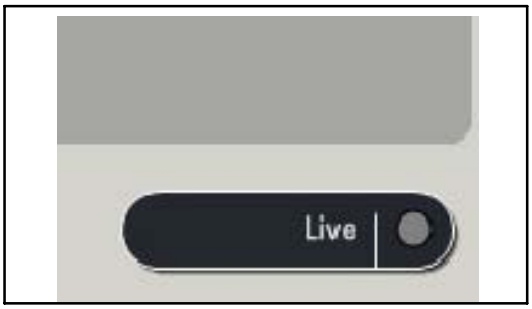
13.2 Acquiring optical sections

figure51. Use the ”Acquire” arrow button to acquire data in all Leica LAS AF applications.

The Leica TCS SP5 contains many functions in its user interface that reflect its wide range of potential applications. The functions not needed for a given application are disabled, however, to ensure efficiency and ease of use. Select the task at hand from the row of arrow buttons at the top. The functions required for data acquisition (and that is the focus of this section) are grouped under ”Acquire” (Fig. 48).For descriptions of the individual functions, please see the online help.

This section will describe the aspects affecting the configuration of the most important acquisition parameters and special points that must be taken into consideration.

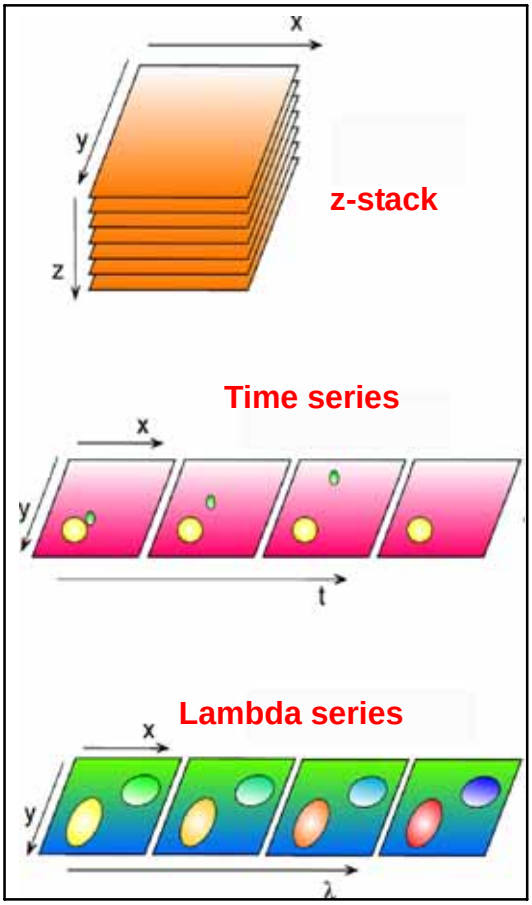
13.2.1 Data acquisition



Press the ”Live” button to begin data acquisition (Fig. 49). Data will be transferred continuously to video memory and displayed on the monitor. Initially, the data will not be stored in a manner suitable for subsequent retrieval.

figure52. The ”Live” button starts data acquisition in all Leica LAS AF applications.

This is a preview mode suitable for setting up the instrument. Stopping data acquisition will also immediately stop the scan process, even if the image has not been fully rendered.



Alternatively, a single image can be captured. This image is then stored in the experiment and can be retrieved later or stored on any data medium. Individual image capture has the advantage of only exposing the specimen once, but is less convenient if additional setup work is required. Once all parameters are correctly set up, an image of the result may be captured. Functions such as accumulation and averaging are supported.

The third data acquisition situation is the acquisition of a series in which the preselected parameters are changed incrementally between the capture of the individual images. Time series, lambda series and z stacks can be acquired in this manner (Fig. 50).

When using the instrument in ”LiveData-Mode”, all captured images are automatically stored with the time of capture. A preview mode is not available in that case (Fig. 51).

figure53. Stack acquisition for 3D, time and lambda series

This method is especially suitable for the observation of living objects over time while changing the medium, applying electrical stimuli or executing changes triggered by light.

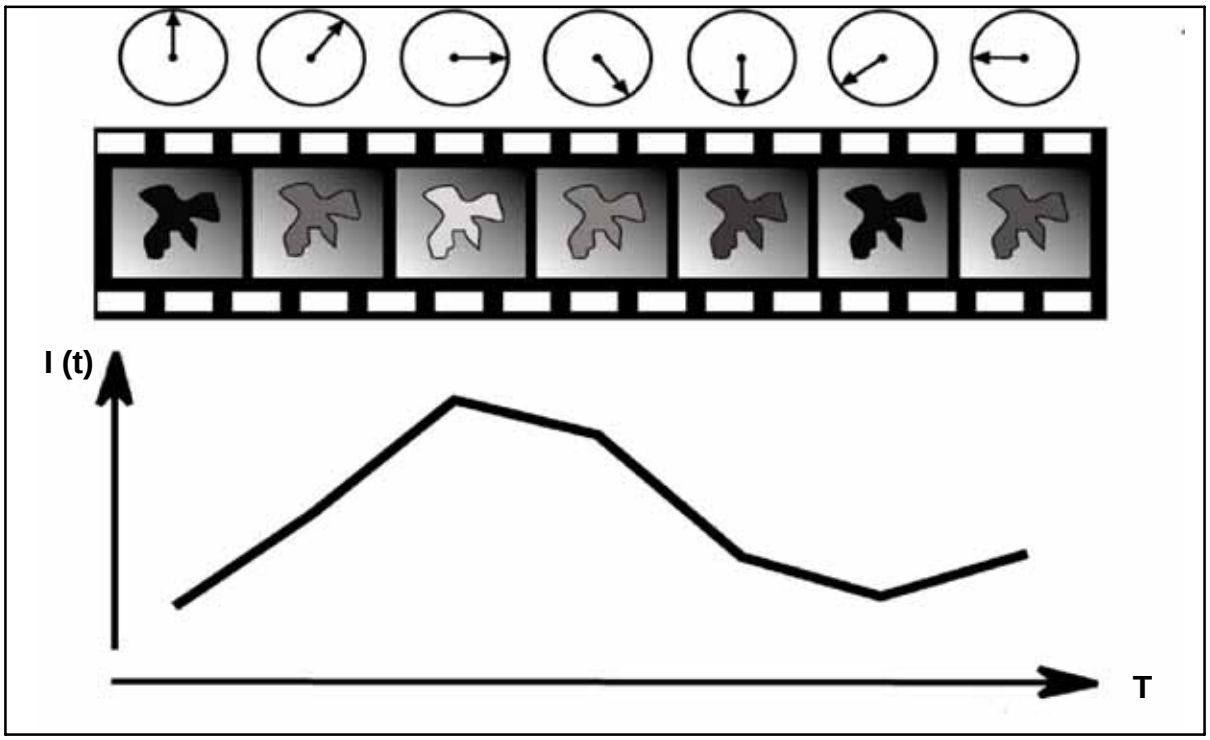
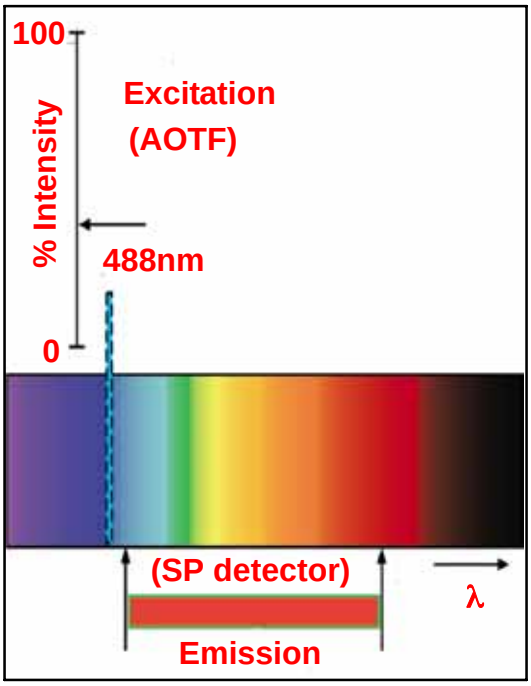


figure54. LiveDataMode supports the continuous acquisition of data while changing setting parameters, manipulating the specimen or performing bleaching sequences between the individual captures. The clock continues running throughout the experiment and intensity changes in interesting areas can be rendered graphically online.

The setting parameters for a simple optical section are described and discussed below. These settings are identical for all work with the instrument. Preconfigured parameter sets have been stored in the software for typical specimen situations. You may also store and recall custom parameter sets. The description below is based on the assumption that you are using a specimen similar to the included standard specimen. The standardspecimen is a Convallaria majalis rhizome section with a histological fluorescent stain. The specimen can be used for a wide range of fundamental problems and has the advantage that it practically does not bleach.

13.2.2 Illumination



Laser lines suitable for the excitation of fluorescence may be selected as illumination. The intensity of the laser line can be adjusted continuously using the line's slider. Moving the slider all the way down disables the line. The intensity setting of the slider is realized steplessly via an acousto-optic tunable filter (AOTF). The intensity at which a sufficiently noise-free image of the specimen can be obtained must be determined to reduce deterioration of the specimen. Factors affecting this are the fluorescence stain, the line used, the density of the stain in the specimen, the location and width of the selected emission band, the scanning speed and the diameter of the emission pinhole.

figure55. Selecting the illumination intensity via acousto-optic tunable filter (AOTF, above) and selection of the emission band in the SP detector (below).

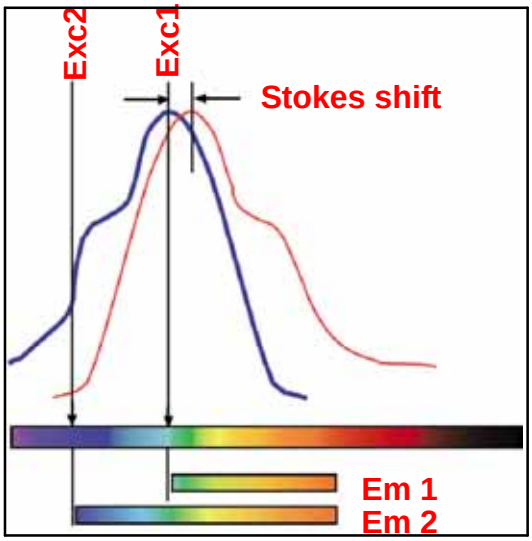
When selecting the "FITC" parameter set, the 488nm argon line and a suitable band between 490nm and 550nm is set.[12]

The entire beam path is represented graphically on the user interface. A spectral band with the settings for the emission bands is located on the emission side. The laser line is visible at the appropriate location in the spectrum as soon as a line is activated. When viewing the specimen through the microscope, the light in the selected color will become lighter or darker according to the position of the slider. This does not involve any danger for the viewer's eyes, despite the fact that this is laser light. As the light is focused on the specimen through the objective, the beam is strongly divergent and is harmless at a distance of a few centimeters. Nevertheless, please read the safety notes in this manual.

If all of the other settings are in order, darker and lighter images will be visible on the mo-

nitor when moving the slider for the illumination.

13.2.3 Beam splitting



The simplest case would involve the selection of a laser line roughly at the maximum of the excitation spectrum of a given fluorescence stain. This would achieve the best yield. In general, however, lasers deliver much more light than necessary, and attenuation to 10% is generally sufficient for good images (although that depends very strongly on the specimen's stain, of course). One can thus also excite the fluorescence on the blue side of the excitation maximum, which has the advantage of providing a broader band for the collection of the emission (Fig. 53).

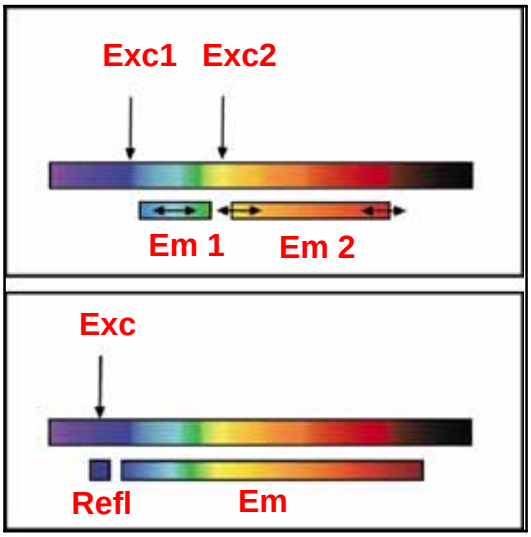
figure56. Excitation spectrum of a fluorescence stain (blue) and emission spectrum (red). An excitation in the maximum (Exc1) would result in only a narrow band to be collected on the emission side (Em1). A significantly broader emission band (Em2) is available from an excitation in the blue range, at which point the intensity of the laser can be increased without detrimental effects.

Experimenting a bit is worthwhile here. An acousto optical beam splitter (AOBS®), permits all available laser lines to be conveniently added or removed without devoting attention to the beam splitter characteristics or the spacing of the lines.

13.2.4 Emission bands

Once the excitation light has reached the specimen via the AOBS® and the objective, an emission is generated in the fluorescent molecules, the light of which is shifted toward longer (redder) wavelengths. This is known as "Stokes shift", and its degree depends on the fluorochrome. As a rule, the excitation and de-excitation spectra overlap, and the Stokes shift is the difference between the excitation maximum to the emission maximum.

It is, of course, advantageous for good separation and yield if the Stokes shift is very high. Typical stains have a Stokes shift between 10nm and 30nm. Shifts of more than 100nm also occur, in natural chlorophyll for example, an excellent stain for curious experimenters.



The emission characteristics of stains can be displayed on the spectral band graphic of the user interface. It is therefore very easy to choose where an emission band should begin and end. If an emission curve has not been stored, it is possible to record and save such a curve directly with the system.

An adjustable bar under the spectral band has been assigned to each confocal detector. The limits to the left and right indicate the limits for the selected emission band.

figure57. SP detector setting options for two fluorescences with different excitations (above) or for fluorescence and reflection (below).

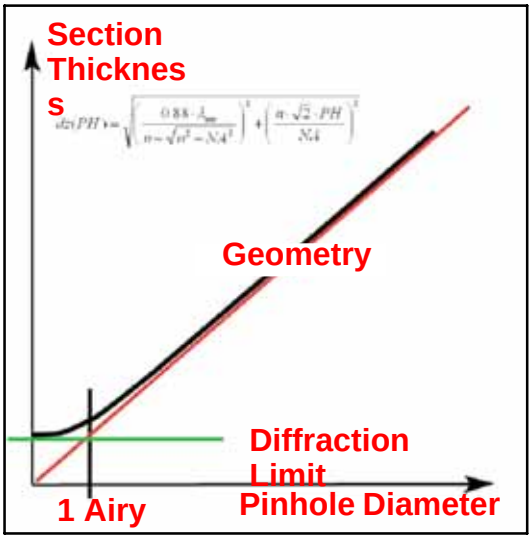
It is possible to move the entire bar back and forth to adjust the average frequency, or move the limits independently. Using the excitation lines and displayed emission characteristics for orientation and adapting the emission band using the Leica SP® detection system is thus very convenient. This is also true while images are being captured.The effects of settings on the images are immediately apparent and suitable values can thus be selected empirically (Fig. 54).

The reflected excitation light also appears in the image as soon as the emission band crosses under the excitation line. While this is naturally undesirable for fluorescence, it does provide a very simple way of creating a reflection image. The smallest band is 5nm, and such a 5nm band would generally be set under the excitation line for reflectometry applications.

To suppress interference from reflected excitation light, it generally suffices to set the start of the emission band to around 3 to 5nm to the red side of the excitation line. Naturally, this depends strongly on the reflective properties of the specimen. It is usually also necessary to maintain a greater distance when focusing close to the glass surface for this reason. That especially applies to specimens embedded in aqueous media. The further the refractive index of the embedding material deviates from 1.52, the more likely distracting reflections become. Greater caution would also be required for specimens containing a high number of liposomes, for example.

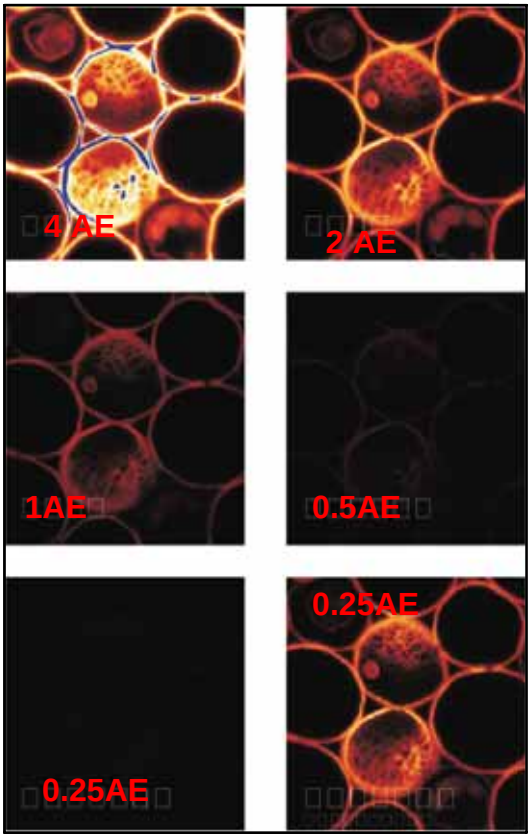
13.2.5 The pinhole and its effects

The reason for deploying confocal microscopy is its ability to create optically thin sections without further mechanical processing of the specimen. The essential component of the instrument that creates these sections is a small diaphragm in front of the detector – the so-called pinhole – as already described in 13.1.4. Ideally, the diameter of this pinhole would be infinitely small, but this would no longer allow lightto pass, preventing the capture of an image. However, the effect would be lost if the pinhole were too wide, as the image would contain excessive blurred shares of the specimen from above and below the focal plane.



The relationship of the thickness of the optical section to the diameter of the pinhole is linear for large diameters and approaches a limit value at smaller diameters, being roughly constant near zero (Fig. 55).The limit value is dependent on the wavelength of the light and the numerical aperture. As the section thickness changes little when initially opening the pinhole, but the passing light increases in proportion to the square of the pinhole diameter, it is advisable not to use too small a diameter.

figure58. Relation of optical section thickness to pinhole diameter



A good compromise is the point where the diffraction limitation
(constant dependence) transitions to geometric limitation (linear dependence). When depicted in the specimen plane at this point, the pinhole has roughly the size of the diffraction-limited light disk of a focused beam. This is known as the Airy diameter. The Airy diameter can easily be calculated from the aperture and wavelength. Setting the pinhole to roughly the size of the diffraction-limited spot thus results in sharp optical sections with a good signal-to-noise ratio (S/N) (Fig. 56).

Naturally, the instrument can calculate and set this value automatically. The objective used is known to fully automatic instruments and can be set when working with manual systems. The excitation lines used are also known to the system.

figure59. Optical sections with a variety of pinhole diameters (63x/1.4 objective). The strong loss of light can be seen clearly with the small diameters, as can the pronounced background in the images with very large diameters.

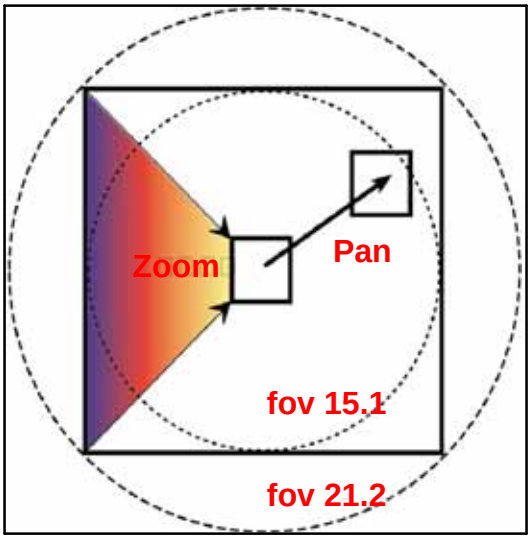
A pinhole diameter of 1 Airy is therefore the default setting. Switching objectives also adjusts the diameter of the pinhole accordingly.

A larger pinhole may be selected simply by adjusting a slider on the user interface for specimens with weak fluorescence or high sensitivity against exposure to light. Smaller pinhole diameters may be selected as well for very bright specimens. With reflecting specimens in particular, the pinhole may be reduced to 0.2 or even 0.1 Airy units (AU) for the thinnest possible sections.

13.2.6 Image detail and raster settings

Depending on the objective used, conventional microscopes show a circular excerpt of the specimen. The diameter of the circle, multiplied by the magnification of the objective, is the field number (FOV). The field number is therefore a microscope value which is independent of the objective, and which conversely can be used to calculate the size of the specimen being observed. A scanner always acquires square or rectangular excerpts, of course. If such a square is exactly circumscribed by the field of vision, than the diagonal dimension will correspond exactly to the field number, allowing the largest possible image to be displayed on the monitor without restrictions.

Unlike eyes or conventional cameras, scanners can simply be set to a smaller angle. A further-enlarged section of the field of view will then be displayed on the monitor. It is thus possible to zoom into details without the need for additional optics. As the scan angle can be adjusted very quickly and continuously over a wide range, magnification increases of around 40x can be achieved simply by moving a slider. As always in microscopy, the total



magnification must be appropriate, i.e. within a suitable range, to obtain good images. Other scales are important for overviews and bleaching experiments.

As errors can easily be made when interpreting captured data, the following is an example of how an appropriate total magnification can be calculated, as well as the information that is automatically provided to the user by the software.

figure60. Fields of view for conventional (21.2mm) and resonant scanners (15,1mm). A smaller scanning angle increases the magnification (zoom), while a scan offset shifts the image detail (pan) within the field of view.

The edge length of the displayed field of a conventional scanner corresponds to 15mm without magnification by the objective (1x scale). A field number of 21.2 is thus also fully utilized (Fig. 57).

That is suitable for most good research microscopes. How many points are now actually resolved optically in this dimension? That depends on the numerical aperture of the objective and the wavelength. According to Ernst Abbe's formula, two points can still be distinguished if the distance between them is not smaller than d=ë/2*NA. A line can thus contain a maximum of 15mm/d resels (resolved elements). When using an actual objective such as a plan apochromat 10x/0.4, the edge length corresponds to 1.5mm (15mm/10) and d=0.625 µm when using blue-green light with a wavelength of 500nm. Such an image would thus contain 1500/0.625=2400 optically resolved elements along each edge.

Rendering this resolution in a digital pixel image would require working withtwice the resolution to prevent losses (Nyquist theorem). That would be an image with 4800x4800 pixels. Some purists require 3x oversampling, i.e. 7200x7200 pixels, or 52

megapixels. Image formats for x and y can be adjusted independently and in very fine steps, with the Leica TCS SP5 supporting capture formats of up to 64 megapixels (8000x8000 pixels) (Table 5).

Magnification		63	40	10
Numerical Aperture		1,4	1,25	0,4
Optical Resolution (400nm)	µm	0,14	0,16	0,5
Intermediate Image (Edge)	mm	15	15	15
Field (Edge)	µm	238	375	1500
# Resel (Field / Resolution)		1667	2344	3000
2x Oversampling		**3333**	**4688**	**6000**
3x Oversampling		**5000**	**7031**	**9000**

Table 5 Table of resolved elements at 400nm for a variety of objectives over the entire scan field. It becomes apparent here that a resolution of 64 megapixels (8000x8000 pixels) is appropriate for quality microscopy applications.

It is thus possible to capture truly all of the image information resolved by the microscope in a single image at that setting. This naturally results in large data volumes, which are especially undesirable for measurements with a high temporal resolution. Zoom is the correct solution here. When capturing data in the standard 512x512 format, this means limiting the image to a field 10-15 times smaller to avoid a loss of information. Zoom factors of 10x and higher provide usable data, but from rather small fields of view. The information required will determine what constitutes an acceptable compromise here.

Such an image is initially an excerpt of the center of the scan field. That is notalways desirable, as it can be difficult to center the interesting structures with such precision. However, the scan field excerpt can be moved across the entire scan field to the actual points of interest, a method called ”panning”.

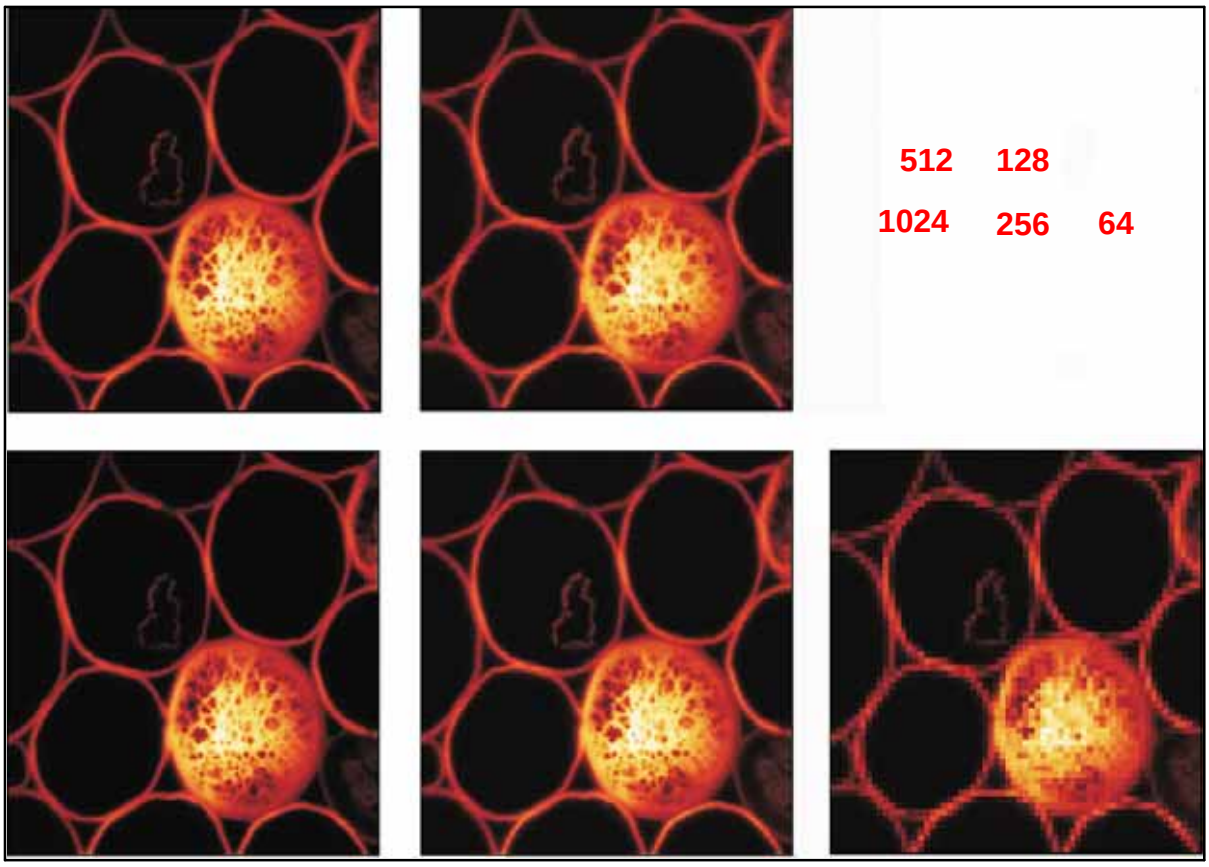


figure61. The same image detail in a variety of pixel resolutions. Please note that the printing medium may not be capable of reproducing the full detail of high resolutions. You may therefore have difficulty detecting the differences between the top two images, despite the enormous differences in the optical resolution. This must also be taken into consideration in publications.

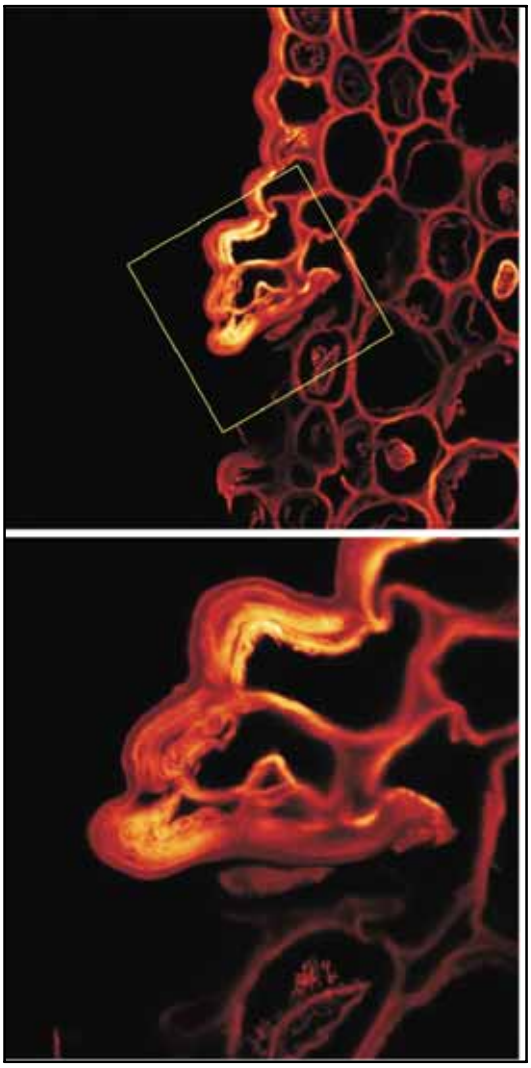
The simplest solution is to combine both methods in the so-called ”Zoom In” function. Simply select a square on the monitor that encloses the structures of interest, and the instrument will automatically select the appropriate zoom and pan values. This function is very fast and thus easy on the specimen. An ”Undo Zoom” function returns you to your starting point – for quickly concentrating on a different cell in the field of view, for example.

The size of the grid spacing used can be found in the image properties. The spacing of the image points in x, y and z can be found under ”Voxel Size”. At Zoom 1, the pictures calculated above would have a grid spacing between 200nm and 300nm. Larger spacings would lead to a loss of resolution when using an objective with an aperture of 0.4 (Fig. 58).

Rectangular formats are important for higher image capture rates. An additional parameter is required here: the rotation of the scan field. As field rotation is performed optically in the Leica TCS SP5, rotation by +/- 100° does not have any effect on the speed and possible grid formats (Fig. 59).

Finally, it must be pointed out in this section that a good microscopic image in a scientific context must always contain a scale. Such a scale can simply be added to the image and adjusted in its form, color and size as required. Scales were not added to the images in this document for the sake of clarity.

13.2.7 Signal and noise



The gain of the capture system must be matched to the signal intensity when capturing data. Signal strengths can vary by several orders of magnitude, making such an adjustment necessary to ensure a good dynamic range for the capture. The goal is to distribute the full range of intensity over the available range of grayscale values. 256 grayscale values (from 0 to 255) are available for images with 8-bit encoding. If the gain is too low, the actual signal may only correspond to 5 grayscale values, causing the image to consist solely of those values. If the gain is too high, parts of the signal will be truncated – i.e. they will always be assigned the value 255, regardless of the information they originally contained. This image information is then lost (Fig. 60).

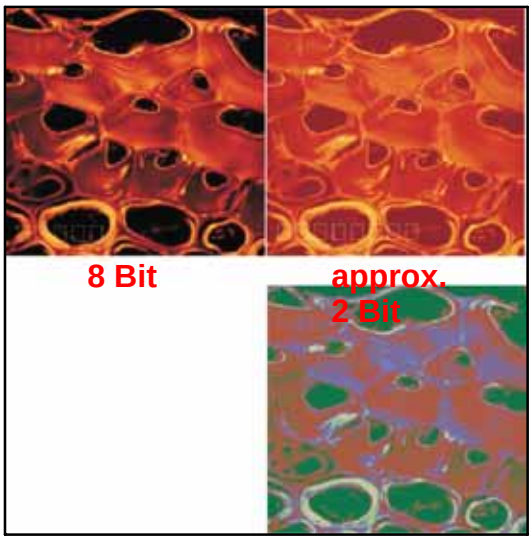
Correctly setting the zero point is also important. This can be accomplished by shutting off the illumination via the AOTF and setting the signal to zero with ”Offset”.

figure62. Zoom, Pan and Rotation combined in one example.

Turn the illumination back on and adjust the gain to prevent distortion.

This configuration work is simplified by special color tables such as ”Glow-over/Glow-under” – a table that initially uses yellow and red for intensities in steps to indicate the signal strengths. The grayscale value zero is always shown as green, value 255 as blue. Both values can thus be identified immediately. The zero point is set correctly when around half the pixels are zero – i.e. green. To be safe, the offset can be set one or two grayscale values higher to ensure that the lower signal values are not truncated. The

loss of dynamic range is negligible (approx. 0.4% per grayscale value at 8 bits).



The electronic deviations from the zero point will generally be negligible; nevertheless, occasional testing is advisable. The actual significance of adjusting the offset value is to compensate for unspecific fluorescence in the specimen at the time of capture. Simply set the offset value in such a manner that the background fluorescence is no longer visible. Please note that this may also truncate signals containing image information.

figure63. Top left. An 8-bit image (256 gray steps). At the right, the same specimen with a considerably smaller dynamic range. Around 6 gray steps can be made out in the false-color image at the bottom. That corresponds to less than 3 bits.

Such settings must always be verified by a careful examination of the results.

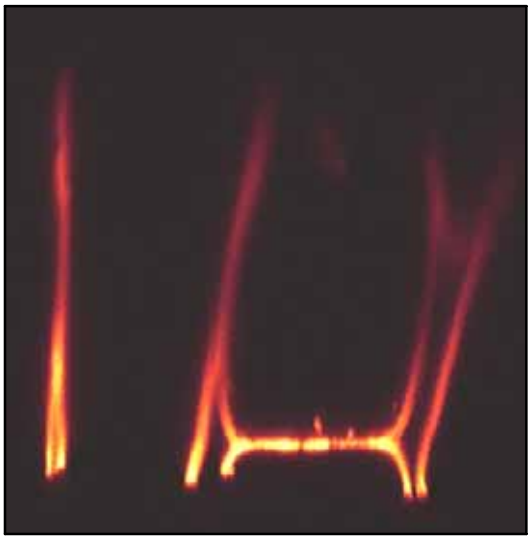
The amplification of the signal must be performed after the offset correction. This operation is quite simple with the described color table: adjust the high tension at the PMT until no more blue pixels are visible. We recommend focusing to ensure that truly the brightest signals in the field of view are used for the adjustment. This is also the right time to check whether the intensity of the excitation light is correctly set. The intensity of the illumination can be increased at the AOTF to reduce image noise. However, it must be taken into consideration here that a higher illumination intensity is detrimental to the specimen. In the case of extremely sensitive specimens and in situations in which rapid changes in intensity in living specimens is of interest, images with more noise can be acceptable. This compromise depends on the specimen and the application, however.

The signal-to-noise ratio may be influenced by a number of other factors in addition to illumination intensity: the speed at which data is captured. The actual speed of the

scan, which can be adjusted via the horizontal frequency (1 Hz - 1,400 Hz, conventional scanners only), and the averaging process offer additional options for enhancing the signal. The change of the scan speed itself leads to averaging in the pixels, as data is recorded for each pixel over a longer period. When averaging lines, each line is scanned several times and the result of the averaging displayed. In the case of image averaging, an entire image is captured and then averaged with the subsequent image captured at the same location.

All processes have their advantages and disadvantages – as always. Temporal correlation is important for moving objects, calling for a slower scan. On the other hand, triplet phenomena speak for longer times between averages, i.e. for the averaging of entire images. Averaging lines represents a compromise here. The averaging of complete images is the gentlest method, but has the disadvantage of not immediately showing the quality of the results; this can be evaluated easier with the other methods. On the other hand, the averaging operation can also be aborted manually when averaging images once the quality impression is adequate. A general rule of thumb for all application situations thus does not exist. Choosing the best method is a matter of experimentation and experience.

13.2.8 Profile cuts



So far, we have always assumed that images are captured parallel to the focal plane That is both correct and appropriate for conventional microscopy. A confocal point scanning system offers interesting new options for capturing data, however. For example, profile cuts through the specimen can be realized by always moving the light spot along the same line, and instead of making an incremental y movement, moving it between the lines of the focal plane (using the fast, precise SuperZ galvanometer stage, for example).

figure64. Profile cut through the Convallaria majalis specimen, indicating a thickness of approx. 30mm.

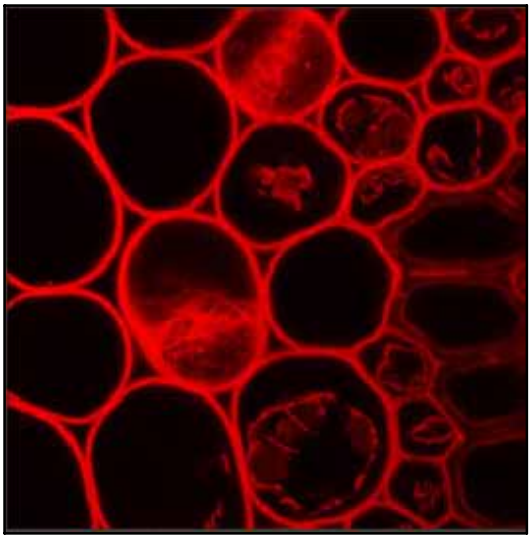
This is similar to slicing through a cake, permitting impressions to be gained online about the contents of the specimen.

Camera-based systems (also ”confocal” systems) can only compute such profiles out of complete stacks (Fig. 61).

13.3 Multiparameter fluorescence

In many cases today, specimens are used that contain more than one fluorescent stain. Multiple stains are achieved using hybridization of various linked fragments (fluorescence in situ hybridization, FISH), through differently marked antibodies or with fluorescence proteins with differing spectral properties. Traditional histological fluorescent stains and autofluorescence are also usable parameters (Fig. 62).

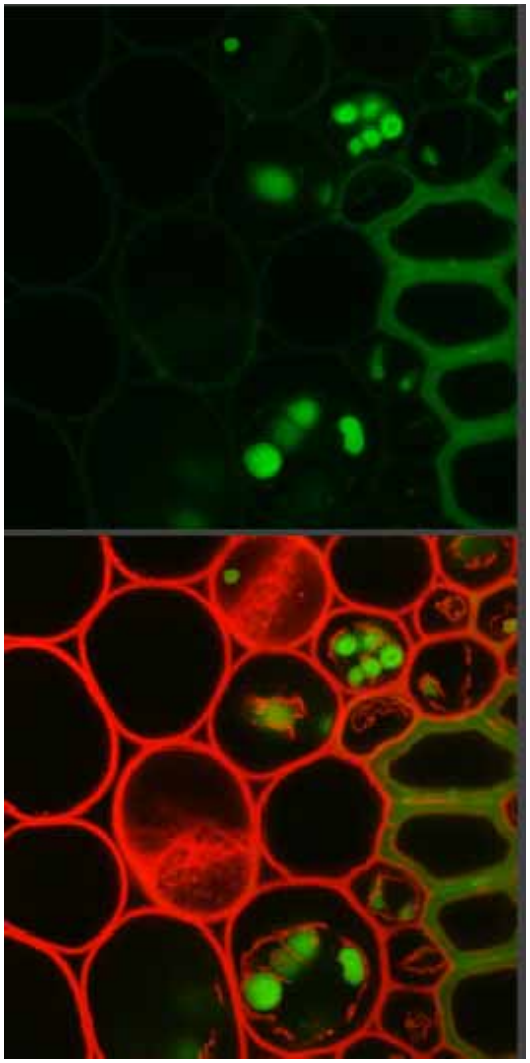
13.3.1 Illumination



Specimens with multiple stains generally require illumination with multiple colors (in this case: laser lines). That is not always the case, however: there are naturally also stains with differing emissions that can be excited by the same wavelengths. A distinctive example would be a botanical specimen with a FITC stain and blue excitation. The emission of FITC would then be visible in the blue-green range of the spectrum. The same excitation can also be applied to chlorophyll, however, which would respond with emission in the deep-red range.

figure65. Simultaneous capture of two fluorescences, in this case excited by a single laser line. The depiction in the colors green and red is arbitrary.

Fluorescence and reflection images can also be rendered at the same time. Using a single excitation, this merely requires observing a second ”emission band” below the laser line.



Under normal circumstances, stains will be used that require different excitation wavelengths, however. A variety of lasers are usually installed in the instrument for this purpose. To activate a second excitation line, simply set the desired slider for the second wavelength as described in 13.2.2 for simple excitation. Additional excitation wavelengths can be added just as easily. It is frequently helpful for the bleaching experiments described below to activate multiple Ar lines, even if you are not capturing a signal or are using only one channel. This provides additional intensity.

Experimenting a bit with laser combinations is always beneficial. It frequently becomes apparent that one does not need all of the lines initially selected for the stains, or a different line turns out to be a better compromise. Default configurations for illumination, beam splitting and emission band settings can be selected from a list for most typical stain combinations.

figure66. Simultaneous capture of two fluorescences, in this case excited by a single laser line. The depiction in the colors green and red is arbitrary.

13.3.2 Beam splitting

Beam splitting is very easy to describe in AOBS® systems: there is no need to give it any thought. The AOBS automatically switches a narrow band for the selected lines to ensure that the excitation is applied to the specimen. Such bands have a width of around 2nm. Everything else is available to capture the emission.

A suitable beam splitter must be selected when using instruments with traditional beam splitters. In this regard, it is important to

know that not only simple, but also double and triple beam splitters are available (DD and TD for double-dichroic and triple-dichroic).

Lines in close proximity to one another cannot be served with dichroic splitters. For example, no usable splitters are available for the simultaneous use of 594nm and 633nm HeNe lines. In these cases, an AOBS is a significant advantage: thanks to the very small bands (approx. 1 to 2nm), both lines can be used for excitation, while capturing an emission band of 35nm in between with the SP detector.

13.3.3 Emission bands

Naturally, the same boundary conditions apply for the emission bands as described in 13.2.4 – with the difference that two laser lines limit the band for all stains except the reddest, and that precautions must be taken to ensure that the excitation light does not reach the detector. In addition, the suppression of crosstalk can have a strong effect on the choice of band limits. The following section will cover this in greater detail. Setting the bands is described in Section 13.2.

13.3.4 Crosstalk

The emission spectra of stains (including those that are responsible for autofluorescence) typically have a rather simple characteristic with a maximum emission and a blue flank that drops more steeply than the red side. The emission extends quite far, but with low amplitude, on either side. Especially the red side can be a problem. Crosstalk or bleed-through refers to the fact that the emission of a stain not only contributes to the signal in one channel, but in other detection channels as well. This should naturally be avoided, as it leads to the display of incorrect

images and falsifies the determination of correlations. The reliability of separation – and thus the avoidance of crosstalk – is therefore an important issue.

Several parameters must be considered for this purpose: illumination intensity, laser selection, sequential capture, emission bands and unmixing methods. Initially, we will be covering illumination and emission parameters.

Crosstalk is frequently caused by strong differences in the concentration of the fluorochromes used. Even illumination will then result in a very good signal from the more highly concentrated stain, yet it is very likely that the signal will also bleed into other channels. This can be compensated by setting the various laser intensities in such a manner that stains with weak concentrations are excited with higher intensities while the higher concentrations receive less-intense excitation. Balancing in this manner already eliminates a significant crosstalk problem. Thanks to the continuously adjustable intensity via AOTF, the results can be monitored directly on the display and can thus be adjusted online with suitable feedback.

It may be useful to try a variety of laser lines for excitation in order to obtain sufficient leeway to adjust the emission bands. This parameter can also be used for balancing: if a stain is very dominant, the selection of a different excitation line can reduce the intensity of the stain (and thus improve the separation against the other stain) while increasing the spacing to the other excitation, permitting larger emission bands and thus enhancing sensitivity. Every improvement in this regard permits a reduction of excitation energy, which in turn reduces bleaching.

A further option for the reduction of crosstalk is the selection of suitable emission bands. The emission characteristics of the stains used can be displayed in the user interface, and a lot can be gained if the emission bands are restricted to ranges that do not

overlap in the graphic on the monitor. Naturally, the stored characteristics are not necessarily identical to the actual emissions, as many factors (e.g. polarity, metabolic products) can affect the spectrum. However, in this case it is also possible to change and optimize the settings during data capture.

13.3.5 Sequential capture

A further way to reduce crosstalk is to capture the information for the various stains sequentially. This has two advantages: Whenever different laser lines are used for excitation (and this is generally the case), sequential capture provides significantly improved separation, as only one stain is excited at a time and the emissions are thus solely from that stain, regardless of the spectral range in which the signals are captured. This is naturally the ideal state – in practice other stains may also be excited slightly, but the separation is nevertheless clearly better than that from a simultaneous capture. Generally, crosstalk can be virtually eliminated this way.

A further advantage of the sequential method is that the emission bands of the individual stains can be set rather widely. This improves sensitivity and is thus easier on the specimen.

An obvious disadvantage is that the capture of two stains also takes twice as long. In the case of physiological measurements, this is frequently not permissible.

13.3.6 Unmixing

As in most cases, a software solution is available to deal with crosstalk whenever a physical separation is not possible. However, we recommend optimizing separation with the means provided by the instrument (see 3.4 and 3.5) to the greatest extent possible and

to use the software only in those cases in which the results are still not satisfactory.

The unmixing method determines the share of a stain's emissions distributed across the various capture channels. This process is applied to each of the stains. The result is a distribution matrix that can be used to redistribute the signal strengths so that they correspond to the stains. This is described for two stains in the following figures, but it is equally valid for any number of stains. The precondition is that the number of channels used is at least the same as the number of stains. The shares can then be correctly redistributed with the simple methods of linear equation systems.

The actual objective for effective unmixing is to determine the required coefficients of the matrix. This is also covered by a variety of methods available in the Leica software. It is advisable to experiment a bit to determine the best method for the task at hand. As all measurement data contains certain error and noise components, there is no perfect recipe for the ultimate truth.

The simplest approach for the user is to determine the coefficients on the basis of the statistical data of the captured images. In this process, the coefficients of the scatter diagrams of both channels are determined using statistical methods. ”Hard” and ”soft” separation methods are available, leaving the degree of separation at the user's discretion.

If the coefficients are known from other experiments, the data can be entered into a matrix manually. This method is also suitable for trial-and-error work – when manually compensating for background interference or autofluorescence, for example.

The method that delivers the most accurate results is channel dye separation. In it, the distribution of stains in the various channels is determined directly using individual stain reference data. When using this method it is important to ensure that the parameter set-

tings of the instrument are not altered, as the laser intensity and gain at the PMT also affect these coefficients.

In the spectral dye separation method, the emission spectra of the individual stains known from literature or determined by measurements directly at the instrument are used to calculate the relative intensity of the stains. This method is especially suited for situations in which the stains do not significantly change their emission in situ and in which the related data is well-known.

13.4 3D Series

Altering the position of the focus between captures permits a whole series of optical sections to be captured and their structure rendered as a 3D data record. Naturally, such a three-dimensional ”image” cannot be observed directly, but it contains spatial information related to the observed structures, and – in the case of multiple stains – their local connections.

13.4.1 z-stack

To capture such a 3D series (”z stack”), set the upper and lower limits simply by traveling to the top of the specimen, marking the location, and doing the same for the bottom. Next, determine the number of steps to be captured between the two positions and the rest will be handled automatically by the instrument.

13.4.2 About section thickness...

As described in sections 13.1.4 and 13.2.5, the thickness of the optical section depends on the wavelength, the numerical aperture of the objective, and the diameter of the pinhole. The relationship of these parameters is expressed by the formula described there. The aperture should be as high as possible to obtain truly good (thin) sections. Confocal microscopes use objectives with large apertures for this reason. The wavelength of the emission will generally be between 450nm and 600nm, so 500nm would be a suitable value for a rough estimate. Selecting the pinhole diameter 1 Airy will result in section thicknesses between 0.5µm and 2.5µm for apertures from 0.7 to 1.4. These are typical values in practice. In the literature – especially in advertising materials – the thickness

is often stated for sections in reflection at pinhole diameter zero. Although this value is much smaller and thus looks better, it is not relevant for practical applications in fluorescence microscopy.

13.4.3 ...and spacing

The thickness of the optical sections is important when capturing z stacks. If the spacing between the sections is too large (greater than the thickness of the section), this will result in gaps in the data record and a loss of information. A reconstruction then can no longer be calculated correctly. On one hand, there is little point in taking as many sections as possible, as a very tight spacing will result in reduced differences between the individual sections and an unnecessarily high data volume. This relates to the z axis in the same way as ”empty magnification” in a conventional microscope. For a dense data record without gaps, but also without superfluous oversampling, set the spacing between the sections to around one half to one third of the optical section thickness. In practice, this will be around 0.7 to 0.2 µm. This means capturing between 1 and 5 sections per micron in z, depending mainly on the aperture of the used objective.

13.4.4 Data volumes

A further factor that must be considered when capturing a series is that it may result in very large volumes of data that in some cases may not be suitable for processing or which can only be processed very slowly. A ”normal” image with 512x512 pixels, one channel and a standard 8-bit grayscale resolution weighs in at 0.25MB. One hundred such images (i.e. a 20µm thick specimen at high resolution) already require 25MB, which only a couple of years ago amounted to a very cumbersome volume of data. If 5 chan-

nels are captured in parallel with an image format of 1000x1000 pixels, the stack will have a volume of 500MB, almost filling a standard CD. Using 16-bit grayscale and 8000x8000 pixels would result in a data record of 64 GB, a volume that most modern computers cannot handle with ease. A critical assessment of the data capture parameters to be used is definitely called for here.

13.4.5 Depictions

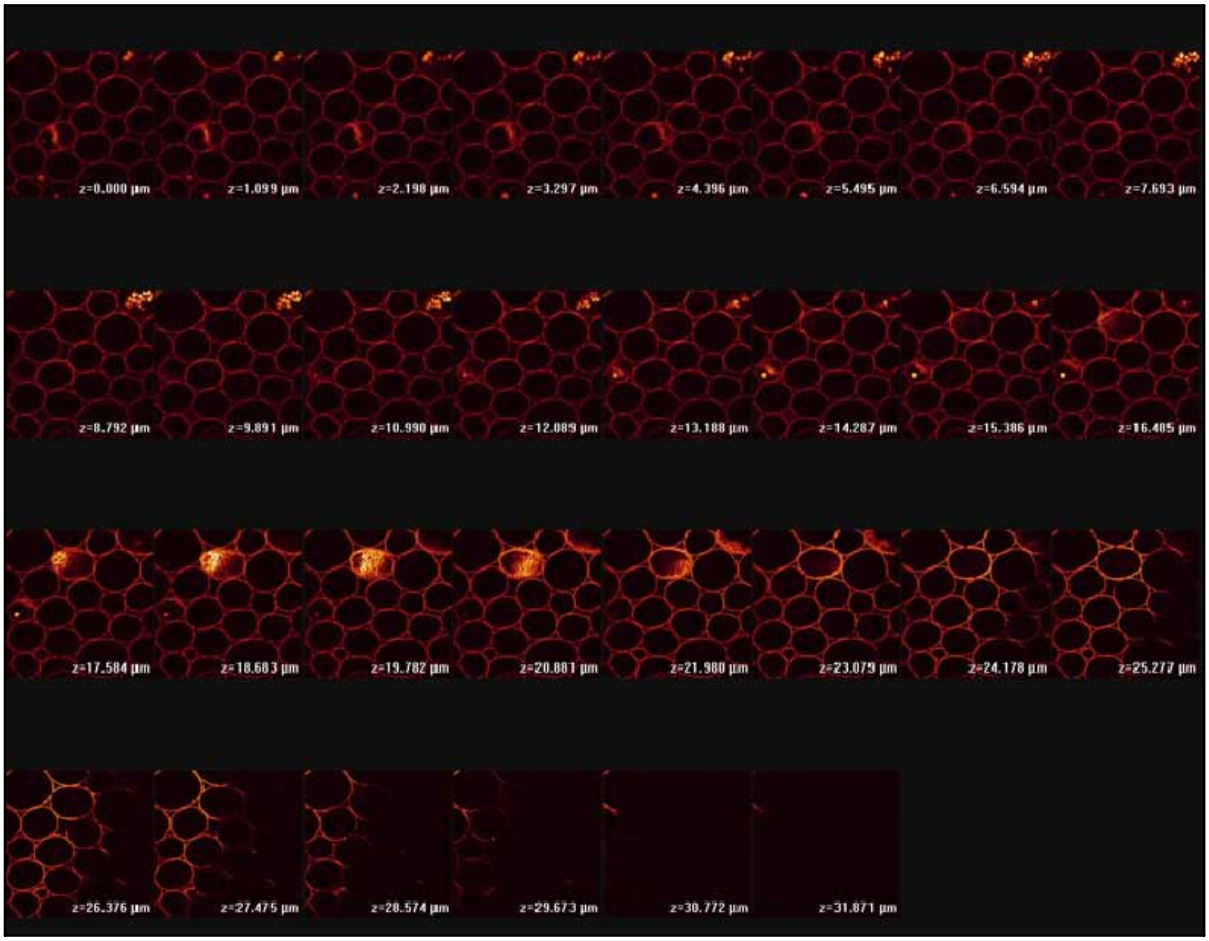


figure67. Gallery of a z stack. This ”stamp collection” is well-suited for monochrome publications.

As mentioned earlier, a three-dimensional image cannot truly be displayed on a two-dimensional monitor. A variety of methods are therefore available for presenting this information.

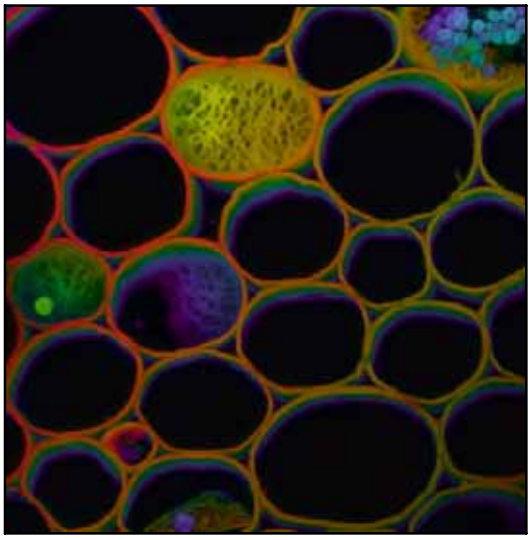
13.4.5.1 Gallery

The simplest of these is to display all of the sections of a series in a gallery – rather like a stamp collection (Fig. 66). Changes from section to section can thus be analyzed and the images printed in periodicals.

13.4.5.2 Movie

Many publications today are available via the Internet, making it possible to publish movies presenting such sequences at a convenient speed. These movies provide the impression of focusing directly through the specimen at the microscope. Both methods are suitable for monochrome (black and white) and multi-channel captures.

13.4.5.3 Orthogonal projections



A further option for displaying the full range of information (with losses) compressed into two dimensions is to compute projections of the entire series. The most common method is the so-called maximum projection. The brightest value along the z axis is determined for each pixel and entered into the resulting image at this point. The result is an image consisting solely of the sharply focused values, but distributed over the entire distance of the image in the z direction.

figure68. Color-coded relief of the series shown above

The operation also increases the depth of focus over the entire height of the z stack. Such projections are therefore called ”extended depth of focus” images. This method is also suitable for multichannel captures.

Coloring each layer differently, for example by mapping the colors of the rainbow to the z axis, permits the z positions of structures to be identified immediately in this projection. This is only possible with one channel, of course, as the color is used for the height. This representation is known as ”height-color coded extended depth of focus” (Fig.65).

The SFP (simulated fluorescence projection) method uses a more complex approach to

achieve impressive images with shadow projections. The quantification must always be checked with care when using this method, however.

13.4.5.4 Rotated projections

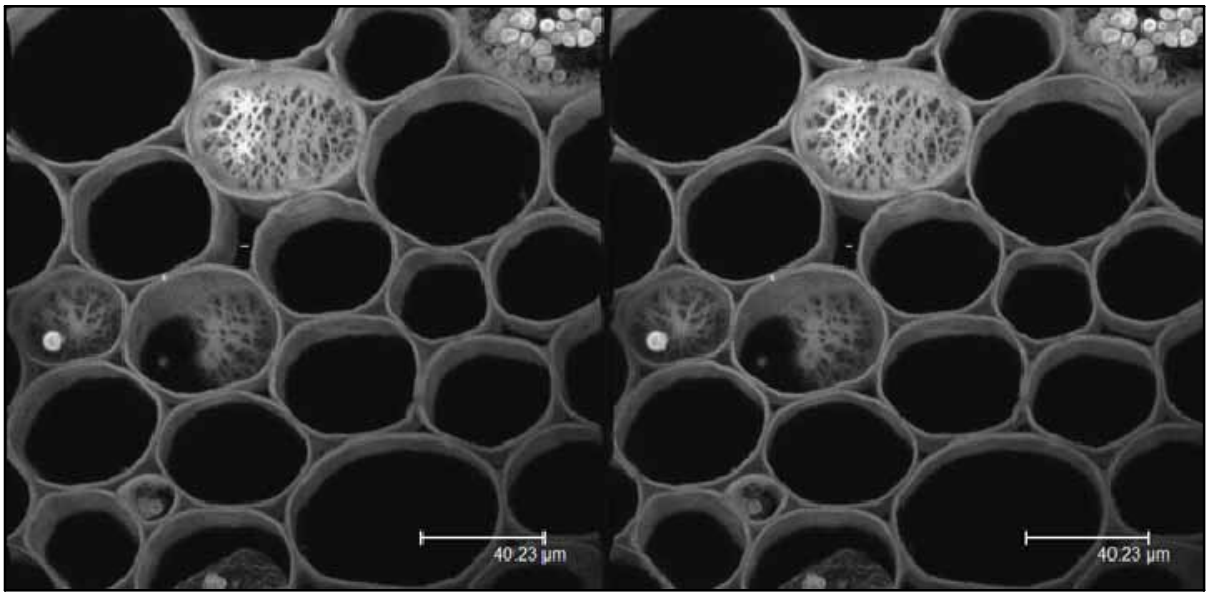


figure69. Stereo image of the same 3D data. A bit of practice is required, but this nevertheless represents a worthwhile exercise for any confocal microscope user.

The methods described in 13.4.5.3 initially assume that the projection will be performed along the visual axis. The computer data is available in a spatially homogeneous state, however, permitting projections from any direction.

In the simplest case, two projections from slightly different angles can be displayed next to one another and superimposed by ”unaided fusion”, or squinting. We then mentally generate a three-dimensional image in the same way as we would of any other objectviewed with both eyes (Fig. 66).

If only one channel is used, it is possible to display both views in different colors and view them through spectacles containing filters for those specific colors (red-green anaglyph). This is simpler for most users, but cannot be applied to multiparameter data.

Like the sections themselves series of projections can be observed with increasing angles and presented as movies. 3D movies of this type are today the most common and convincing means of displaying three-dimensional data.

13.5 Time series

A confocal scanning microscope records images like a camera. It can therefore also be used to record a time series – essentially a z stack without altering z. Such time lapse experiments are an important tool in physiology and developmental biology, whenever interest is focused on dynamic processes.

13.5.1 About scan speeds

Temporal resolution is an important parameter in dynamic processes, especially those related to kinetic studies of cellular biophysical processes. Unfortunately, are imposed here by a number of factors such as the mechanical speed of the scanner, the bandwidth of the data line, and the simple volume of photons that can be expected from the specimen during the period of observation. While mechanical and data bottlenecks can be resolved in principle and great progress has been made in this regard in recent years, limitations related to light are a hurdle that cannot be overcome. Little light leads to a poor signal-to-noise ratio, and thus to poor resolutions and poor image quality. It is therefore necessary to verify the parameters that truly require measurement. A central difference between various measurements is the dimensionality that attempts to compensate for mechanical limits.

13.5.2 Points

The highest temporal resolution can be achieved when the mechanical elements of the scanner do not move at all. This amounts to measuring the changes in light intensity at a fixed, preselected point in the Leica TCS SP5 with a temporal resolution of 40MHz (corresponding to 25ns). Naturally, that particular spot in the specimen can be expected to bleach within a very short time.

13.5.3 Lines

Less fast, but nevertheless suitable for many highly dynamic processes, is the restriction to images consisting of a single line. The data can be displayed as an xt image, with one dimension being the location (the selected line) and time as the second dimension. An 8 kHz resonant scanner thus supports a resolution of 16 kHz (63µs) in bidirectional mode.

13.5.4 Planes

The standard scenario is the capture of xy images as a t series. In this case, the temporal resolution depends on the speed of the scanner and the number of lines per image. When limiting the capture to a band-shaped image of 16 lines, a resonant scanner can capture up to 200 images per second (5ms).

This standard capture process (generally at 512x512 pixels) is also used for long-term experiments in which the image of the specimen is captured repeatedly over the course of hours or days, for example when recording the development of embryos or cell cultures. In these cases, mechanical and photonic limitations play a subordinate role; the system must be extremely stable, free of drift and climate controlled, however.

13.5.5 Spaces (time-space)

The three-dimensional development of structures in biology is naturally of great interest. The broad application field of 4D microscopy has established itself here. This is realized by recording a series of z stacks and processing them into 3D movies. This is a field in which many innovations and exciting results can be expected in the future.

13.5.6 FRAP measurements

A completely different field of application for laser scanning microscopy involves dynamic studies in which a system is subjected to interference to disturb its equilibrium and studied as the restoration of its equilibrium progresses. The FRAP method (fluorescence recovery after photobleaching) is very well-known in this regard.

Such experiments can be used to make deductions about membrane permeability, diffusion speeds and the binding behavior of molecules. The capture of a time series is always integral to such measurements.

13.6 Spectral series

Section 2.4 described how the Leica SP® detector is capable of selecting emission bands over a continuously variable range. Incremental shifts of the emission band can also be used as the basis for an image series. The Leica SP® detector was thus the first instrument with which a spectral image series could be captured using a confocal microscope. Experience has shown that its technology is the most efficient; all other spectral microscopes that have arrived on the market since its introduction have significant weaknesses with regard to their signal-to-noise ratio.

13.6.1 Data acquisition and utilization

The capture of a Lambda series does not differ significantly from that of a z series or a time series. The emission band for the beginning and the end of the measurement must be specified, as well as the number of steps for the spectrometer to cover the specified range.

Sections of the image are then chosen interactively for evaluation. Their average intensity is then graphed as a function of the wavelength, a spectrum at the selected point.

13.6.2 About spectral resolution

The debate has developed recently in conjunction with spectral series related to the technology that offers the best spectral resolution, i.e. technology capable of detecting the finest differences in the spectrum. The TCS SP5 supports the adjustment of emission bands in 1nm steps,which corresponds to a formal resolution of one nanometer. The optical spectral resolution is dependent on

the wavelength, however, and amounts to roughly 0.5nm in the blue and 2nm in the red range. This resolution is far better than required in practice: in typical specimens that are in a liquid or gel state at room temperature, fluorescent emissions are never sharper than roughly 20nm.

13.7 Combinatorial analysis

Many of the methods described above can be combined and deliver new insights in biology, with both fixed and living specimens. The term ”multidimensional microscopy” has been coined to describe this form of combinatorial analysis. However, a certain inflation in this regard has become apparent recently. Stitching together a large number of dimensions (measuring parameters) does not in itself make a good experiment, and it is definitely not conducive to sound results. The synthesis of a broad range of measurements is often difficult and always requires a solid intellectual overview to avoid the creation of data graveyards and incorrect conclusions.

14 Switching off the system

1 Save your image data

On the menu bar, select

File->Save

to save the data record.

2 Close the LAS AF

On the menu bar, select

File->Exit

Exit the LAS AF.

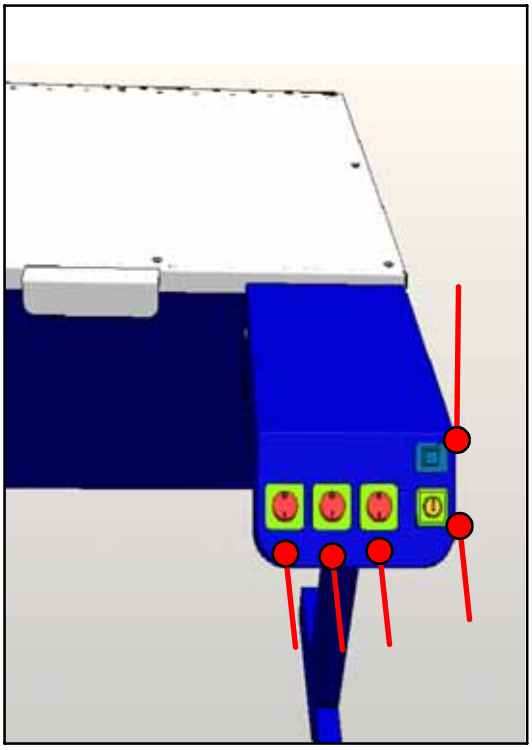


figure70. Control panel

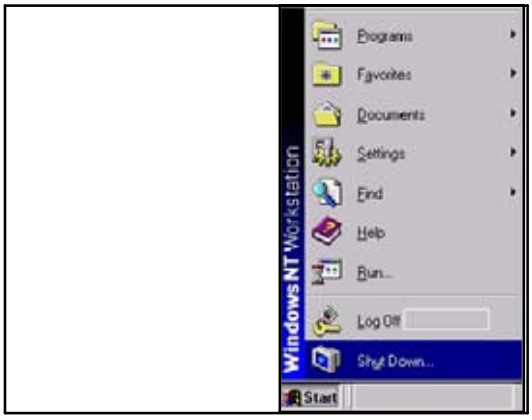
3 Switch off the lasers with the key switch (Fig. 67/2).

The emission warning indicator (Fig. 67/1) will go out.

4 Next, turn off the switches on the control panel for the TCS workstation and the TCS SP5 (Fig. 67/4/5) scanner.

5 The external fan of the argon laser will switch off automatically after several minutes. Also set the switch for the lasers (Fig. 67/3) to ”O” at this point.

The delayed shutdown of the laser cooling system ensures the operational reliability of the TCS SP5 system.



6 Shut down the computer

On the toolbar, select

Start->Shutdown

to shut down the TCS Workstation.

figure71. Shutting down the computer

7 Switch off the microscope and any activated fluorescence lamps

If your system features external lasers (IR, UV or others), switch them off in accordance with their respective manuals.

15 Contact

If you have any further questions related to your TCS SP5, please contact the Leica branch office in your country.
Please refer to the country list below for contact information.
If your country is not listed below, please use the area selector at http://www.confocal-microscopy.com.

Country	City	Phone	Fax
Australia	Gladesville	+61 2 9879 9700	+61 2 9817 8358
Austria	Vienna	+43 1 486 80 50 0	+43 1 486 80 50 30
Canada	Richmond Hill Ontario	+1 905 762 2000	+1 905 762 8937
Denmark	Herlev	+45 4454 0101	+45 4454 0111
France	Rueil-Malmaison	+33 1 473 285 85	+33 1 473 285 86
Germany	Bensheim	+49 6251 136 0	+49 6251 136 155
Italy	Milan	+39 0257 4861	+39 0257 40 3273
Japan	Tokyo	+ 81 3 5421 2800	+81 3 5421 2896
Korea	Seoul	+82 2 514 65 43	+82 2 514 65 48
Netherlands	Rijswijk	+31 70 4132 100	+31 70 4132 109
PRC	Hong Kong	+852 2564 6699	+852 2564 4163
Portugal	Lisbon	+351 21 388 9112	+351 21 385 4668
Singapore		+65 6779 7823	+65 6773 0628
Spain	Barcelona	+34 93 494 95 30	+34 93 494 95 32
Sweden	Sollentuna	+46 8 625 45 45	+46 8 625 45 10
Switzerland	Glattbrugg	+41 1 809 34 34	+41 1 809 34 44
United Kingdom	Milton Keynes	+44 1908 246 246	+44 1908 609 992
USA	Bannockburn/ Illinois	+1 847 405 0123	+1 847 405 0164

16 Declaration of conformity

Leica MICROSYSTEMS

EC Declaration of Conformity

Manufacturer:	Leica Microsystems CMS GmbH
Address:	Am Friedensplatz 3 Germany, 68165 Mannheim
Product:	**TCS SP5 Confocal Laser Scanning Microscope**

We declare that the product described herein complies with the following European Directives:

89/336/EEC Directive on Electromagnetic compatibility

73/23/EEC Directive on Low-voltage equipment

The product conforms to the standards:

EN 61326: 1997 + A1: 1998 + A2:2001 + A3: 2003
EMC requirements for Class A electrical equipment for measurement, control and laboratory use

EN 61000-3-2: 2000
Electromagnetic compatibility (EMC)
Part 3-2: Limits – Limits for harmonic current emissions

EN 61000-3-3: 1995 + A1:2001
Electromagnetic compatibility (EMC)
Part 3: Limits – Section 3: Limitation of voltage fluctuations and flicker in low-voltage supply systems for equipment with rated current ≤ 16A

EN 61010-1: 2001
Safety requirements for electrical equipment for measurement, control and laboratory use; Part 1: General requirements

EN 60825-1: 1994 + A2: 2001 + A1:2002
Safety of laser products
Part 1: Equipment classification, requirements and user's guide

Manager Research & Development

Mannheim, Germany
October 19, 2005

Dr. Rafael Storz

17 Glossary

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

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.

Apochromatic

Describes a correction class for objectives. For objectives of this type, the chromatic aberration is corrected for three wavelengths (usually 450 nm, 550 nm and 650 nm) and the sine condition is met for at least two colors. The image curvature aberration is not corrected.

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.

Instrument parameter setting

An instrument parameter setting (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.

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).

Refraction index

The factor by which the light velocity in an optical medium is less than in a vacuum.

Chromatic aberration

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.

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 example, with a short-pass filter RSP 510 (reflection short pass), excitation light below 510 nm is reflected and above this value it is transmitted. The transmission values are generally between 80% and 90% and the reflection values between 90% and 95%.

Digital phase-true filter

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).

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. With a double dichroite DD 488/568, for example, the excitation light at 488 nm and 568 nm is reflected and above these values it is transmitted. The transmission values are generally at 80% and the reflection values are between 90% and 95%.

Experiment

A file with Leica-specific data format (*.lei) that consists of one ore more individual images or image series. Images recorded with different scan parameters or result images from image processing can be combined here.

Fluorescent dye

A dye used for analysis that reacts with the emission of light of other wavelengths upon excitation with light energy (Stokes shift). e.g. Fluorescein, Rhodamin, Eosin, DPA.

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, i.e.objectives whose degree of correction falls between achromatic and apochromatic.

Frame

A frame corresponds to the acquisition of a single optical section. For example, if a single optical section is acquired four times (to average the data and to eliminate noise), then frames are created for this optical section.

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.

IR-Laser

Laser with a wavelength > 700 nm, invisible light. (infrared).

Confocal subprocedure

Methods for examining microstructures that are derived from the classical contrast methods (bright field, interference contrast, phase contrast, polarization) in conjunction with a confocal system. These procedures each define a certain configuration of optical elements (filter cubes, ICT prisms, phase rings). In addition, some of them are dependent upon the selected objective.

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 optical resolution diameter of the detection pinhole, the wavelength and the numerical aperture of the selected objective determine the axial range of an optical section (optical resolution).

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).

Lambda series

Stack of individual images of a single optical plane that were each detected at a specific wavelength.

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).

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.

MP laser

Multi-photon, the designation for infrared lasers with a high photon density (generated by pulsed lasers).

Neutral filter

Neutral filters are semi-reflective glass plates. They are used to distribute the light path independent of wavelength. The incoming 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. For example, for a neutral filter RT 30/70, 30% of the excitation light is reflected and 70% transmitted.

Numerical Aperture

Aperture is the sine of the aperture angle under which light enters the front lens of a microscope objective; Symbol NA. The aperture influences both the light intensity and the resolution capacity of an objective optical system. Since different media can be located between specimen and objective (e.g. the embedding medium of the specimen), the numerical aperture (NA = n * sina) is generally used as the unit of measure for the luminous intensity and the resolution capacity.

Optical bleaching

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.

Phase visualization

The principle of phase visualization as used by Leica is an optimized alternative method to ratiometric displaying. The main area of application is the measurement of 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. For detailed information on phase visualization, please contact Leica Microsystems CMS GmbH directly.

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.

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.

Spherical aberration

An optical image distortion conditional on the varying spacing of parallel light rays of the same wavelength from the optical axis. Light rays that travel through outer lens zones have shorter focal distances than rays that travel through the lens center (optic axis).

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 455≥ The transmissivity or reflectivity of triple dichroites depends on three specific wavelengths of light. With a triple dichroite TD 488/568/647, for example, the excitation light at 488 nm, 568 nm and 633 nm is reflected and above these values it 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.

UV-Laser

Laser with a wavelength < 400 nm, invisible light.

VIS-Laser

Lasers of the wavelength range 400 - 700 nm, visible light.

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.

z-stack

Z-stacks are comprised of two-dimensional images that were recorded on different focal planes and displayed as three-dimensional.

18 Safety data sheets

The following are safety data sheets from third-party manufacturers.

EG-Sicherheitsdatenblatt
Gem. 91/155/EG; 2001/58/EG

Stand:
2.3.2004

Innovatek OS GmbH
www.innovatek.de
info@innovatek.de

1.) Stoff-/Zubereitungs- und Firmenbezeichnung

Handelsname: innovatekProtect IP
Firma: innovatek OS GmbH, Stadtweg 9, 85134 Stammham
Tel: 08405/92590
Fax: 08405/925921
Notfallauskunft: 08405/92590

2.) Zusammensetzung / Angaben zu Bestandteilen

Chemische Charakterisierung: Ethylenglykol. Inhibitoren.
Gefährliche Inhaltsstoffe:

Ethandiol	Gehalt (w/w): >90%	CAS-Nr.: 107-21-1
	EG-Nr.: 203-473-3	Gefahrensymbol Xn
	Index-Nr.: 603-027-00-1	R-Sätze: 22
2-Ethylhexansäure, Natriumsalz	Gehalt (w/w): 2%-3%	CAS-Nr.: 19766-89-3
	EG-Nr.: 243-283-8	Gefahrensymbol Xn
		R-Sätze: 63

Falls gefährliche Inhaltsstoffe genannt sind, ist der Wortlaut der Gefahrensymbole und R-Sätze in Kapitel 16 aufgeführt.

3.) Mögliche Gefahren

Besondere Gefahren für Mensch und Umwelt: Gesundheitsschädlich beim Verschlucken.

4.) Erste Hilfe Maßnahmen

Allgemeine Hinweise:	Verunreinigte Kleidung entfernen
Nach Einatmen:	Bei Beschwerden nach dem Einatmen von Dampf/Aerosol: Frischluft, Arzthilfe.
Nach Hautkontakt:	Mit Wasser und Seife gründlich abwaschen.
Nach Augenkontakt:	Mindestens 15 Minuten bei gespreizten Liedern unter fließenden Wasser gründlich ausspülen.
Nach Verschlucken:	Sofort Mund ausspülen und reichlich Wasser nachtrinken, Arzthilfe.
Hinweise für den Arzt:	Symptomatische Behandlung (Dekontamination, Vitalfunktionen) Gabe von 50ml trinkbaren Ethanol in trinkbarer Konzentration.

5.) Maßnahmen zur Brandbekämpfung

Geeignete Löschmittel:	Sprühwasser, Trockenlöschmittel, alkoholbeständiger Schaum, Kohlendioxid (CO^2).
Besondere Schutzausrüstung:	Im Brandfall umluftunabhängiges Atemschutzgerät tragen.
Weitere Angaben:	Gefährdung hängt von den verbrennenden Stoffen und den Brandbedingungen ab. Kontaminiertes Löschwasser muss entsprechend den behördlichen Vorschriften entsorgt werden.

6.) Maßnahmen bei unbeabsichtigter Freisetzung

Personenbezogene Maßnahmen:	Übermäßigen Haut- und Augenkontakt vermeiden, bei größeren Mengen durchtränkte Kleidung entfernen u. Körper mit Wasser abspülen. Handschutz. Wegen Rutschgefahr sofort aufnehmen.
Umweltschutzmaßnahmen:	Verunreinigtes Wasser/Löschwasser zurückhalten. Nicht in die Kanalisation/Oberflächenwasser/Grundwasser gelangen lassen.
Verfahren zur Reinigung/Aufnahme:	Ausgelaufenes Material eindämmen und mit großen Mengen Sand, Erde oder anderen absorbierenden Material abdecken; dann zur Förderung der Absorption kräftig zusammenkehren.

Gemisch in Behälter oder Plastiksäcke Füllen u. der Entsorgung zuführen. Kleine Mengen (Spritzer) mit viel Wasser fortspülen. Große Mengen: Produkt abpumpen, sammeln und der Entsorgung zuführen. Bei großen Mengen, die in Drainage/Gewässer laufen konnten, zuständige Wasserbehörde informieren.

7.) Handhabung und Lagerung

Handhabung:	Gute Be- und Entlüftung von Lager- und Arbeitsplatz.
Brand- u. Explosionsschutz:	Maßnahmen gegen elektrostatische Aufladung treffen. Elektrische Betriebsmittel müssen für die Temperaturklasse T2 (VDE 0165) geeignet sein (D). Durch Hitze gefährdete Behälter mit Wasser kühlen.
Lagerung:	Produkt ist hygroskopisch. Behälter dicht geschlossen an einem trockenem Ort aufbewahren. Die Lagerung in verzinkten Behältern wird nicht empfohlen.

8.) Expositionsbegrenzung und persönliche Schutzausrüstungen

Bestandteile mit arbeitsplatzbezogenen zu überwachenden Grenzwerten:

107-21-1: Ethylenglykol	MAK-Wert 26mg/m³: 10ppm (TRGS 900(DE)). Spitzenbegrenzung/Überschreitungsfaktor: =1= Ein Risiko der Fruchtschädigung braucht bei Einhaltung des MAK- und des BAT-Wertes nicht befürchtet zu werden. Hauteffekt (TRGS 900 (DE)). Der Stoff kann über die Haut aufgenommen werden.
Persönliche Schutzausrüstung	
Atemschutz:	Einatmen von Dampf/Aerosol vermeiden.
Handschutz:	Chemikalienbeständige Schutzhandschuhe (EN374), empfohlen: Nitrilkautschuk (NBR), Schutzindex 6. Wegen großer Typenvielfalt Gebrauchsanweisung der Hersteller beachten.
Augenschutz:	Schutzbrille mit Seitenschutz (Gestellbrille) (EN 166)
Allgemeine Schutz- u. Hygienemaßnahmen:	Die beim Umgang mit Chemikalien üblichen Vorsichtsmaßnahmen sind zu beachten.

9.) Physikalische und chemische Eigenschaften

Form:	flüssig	
Farbe:	farblos	
Geruch:	produktspezifisch	
PH-Wert (500 g/l, 20°C):	7-9	
Erstarrungstemperatur:	< -18°C	
Siedepunkt:	>= 165°C	
Flammpunkt:	>120°C	
Untere Explosionsgrenze:	3,0 Vol.-%	
Obere Explosionsgrenze:	15,0 Vol.-%	
Zündtemperatur:	>200°C	(DIN 51794)
Dampfdruck (20°C):	2 mbar	(DIN 51757)
Dichte (20°C):	ca. 1.12 g/cm³	
Mischbarkeit mit Wasser:	beliebig mischbar	
Löslichkeit (qualitativ) Lösemittel:	polare Lösemittel: löslich	
Viskosität (kinematisch, 20°C):	20-30 mm²/s	

EG-Sicherheitsdatenblatt
Gem. 91/155/EG; 2001/58/EG

innovatek

Innovatek OS GmbH
www.innovatek.de
info@innovatek.de

Stand:
2.3.2004

10.) Stabilität und Reaktivität

Zu vermeidende Stoffe:	starke Oxidationsmittel
Gefährliche Reaktionen:	Keine gefährlichen Reaktionen bei vorschriftsmäßiger Lagerung und Handhabung.
Gefährliche Zersetzungsprodukte:	Keine gefährlichen Zersetzungsprodukte, wenn die Vorschriften/ Hinweise für Lagerung und Umgang beachtet werden.

11.) Angabe zur Toxikologie

LD50/oral/Ratte: >2000 mg/kg
LD50/dermal/Kaninchen: Nicht reizend.
Primäre Hautreizung/Kaninchen: Nicht reizend.
Angaben zu Ethylenglykol:

Weitere Angaben:	Prüfungen an Mäusen und Ratten zeigten nach oraler Aufnahme hoher Dosierungen fruchtschädigende Wirkung, die in einer Studie an Kaninchen nicht auftrat.
Erfahrungen am Menschen:	Ethandiol: Tödliche Dosis beim Verschlucken ca. 1,5 g/kg Körpergewicht. Tödliche Dosis ca. 90-110 g bei Erwachsenen, entsprechend weniger bei Kindern. Geringe Dosierungen können zu Bewustseinsstörungen oder Schäden an Nieren oder Zentralnervensystem führen. Die angegebenen Symptome/ Diagnosen/ Befunde können bei geringen Dosierungen auftreten.
Zusätzliche Hinweise:	Die Aussage ist von den Eigenschaften der Einzelkomponenten abgeleitet. Ein Risiko der Fruchtschädigung braucht bei Einhaltung des MAK-Wertes nicht befürchtet zu werden. Gefahr der Hautresorption. Aus der Gesamtheit der vorliegenden Informationen ergeben sich keine Hinweise auf krebserzeugende Wirkung.

12.) Angaben zur Ökologie

Ökotoxizität:	Fischtoxizität: Leuciscus Idus/LC50 (96 h): >100 mg/l Aquat. Invertebraten: Daphnia magna/EC50 (48 h): >100mg/l Wasserpflanzen: Algen/EC50 (72 h): >100mg/l Mikroorganismen/Wirkung auf Belebtschlamm: Bei sachgemäßer Einleitung geringer Konzentrationen in adaptierte biologische Kläranlagen sind Störungen der Abbauaktivität von Belebtschlamm nicht zu erwarten
Persistenz und Abbaubarkeit:	Angaben zur Elimination: Versuchsmethode OECD 301 A (neue Version). Analysemethode: DOC-Abnahme. Eliminationsgrad: >70%. Bewertung leicht biologisch abbaubar.
Zusätzliche Hinweise:	Sonstige ökotoxikologische Hinweise: Nicht ohne Vorbehandlung in Gewässer gelangen lassen. Das

3

Produkt wurde nicht geprüft. Die Aussage ist von den Einzelkomponenten abgeleitet

13.) Hinweise zur Entsorgung

innovatekProtect IP muss unter Beachtung der örtlichen Vorschriften z.B. einer geeigneten Deponie oder einer geeigneten Verbrennungsanlage zugeführt werden. Bei Mengen unter 100l mit der örtlichen Stadtreinigung bzw. mit dem Umweltmobil in Verbindung setzen.

Ungereinigte Verpackung: Nicht kontaminierte Verpackungen können wieder verwendet werden. Nicht reinigungsfähige Verpackungen sind wie der Stoff zu entsorgen.

14.) Angaben zum Transport

Kein Gefahrengut im Sinne der Transportvorschriften.
(ADR RID ADNR IMDG/GGVSee ICAO/IATA)

15.)Vorschriften

Vorschriften der Europäischen Union (Kennzeichnung) / Nationale Vorschriften:

EU-Richtlinie 1999/45/EG (´Zubereitungsrichtlinie`):

Gefahrensymbol: Xn: Gesundheitsschädlich
R-Sätze: 22: Gesundheitsschädlich beim Verschlucken
S-Sätze: 2 Darf nicht in die Hände von Kindern gelangen
24/25: Berührung mit den Augen und der Haut vermeiden
46 Bei Verschlucken sofort ärztlichen Rat einholen und Verpackung oder Etikett vorzeigen

Gefahrenbestimmende Komponente zur Etikettierung: 1,2-Ethandiol
Sonstige Vorschriften: Klassifizierung nach VbF (Deutschland): keine
Einstufung nach TA-Luft (Deutschland) 3.1.7. Klasse III
Wassergefährdungsklasse (Anhang 4 der VwVwS (Deutschland) vom 17. Mai 1999): (1) Schwach wassergefährdend.

16.) Sonstige Angaben

Vollständiger Wortlaut der Gefahrensymbole und R-Sätze falls im Kapitel 2 unter ‚Gefährliche Inhaltsstoffe' genannt:
Xn: Gesundheitsschädlich
22: Gesundheitsschädlich beim Verschlucken
63: Kann das Kind im Mutterleib möglicherweise schädigen

Das Sicherheitsdatenblatt ist dazu bestimmt, die beim Umgang mit chemischen Stoffen und Zubereitungen wesentlichen physikalischen, sicherheitstechnischen, toxikologischen und ökologischen Daten zu vermitteln, sowie Empfehlungen für den sicheren Umgang bzw. Lagerung, Handhabung und Transport zu geben. Eine Haftung für Schäden im Zusammenhang mit der Verwendung dieser Information oder dem Gebrauch, der Anwendung, Anpassung oder Verarbeitung der hierin beschriebenen Produkte ist ausgeschlossen. Dies gilt nicht, soweit wir, unsere gesetzlichen Vertreter oder Erfüllungsgehilfen bei Vorsatz oder grober Fahrlässigkeit zwingend haften. Die Haftung für mittelbare Schäden ist ausgeschlossen.
Diese Angaben sind nach bestem Wissen und Gewissen angefertigt und entsprechen unserem aktuellen Kenntnisstand. Sie enthalten keine Zusicherung von Produkteigenschaften.

Leica Microsystems CMS GmbH
Am Friedensplatz 3
D-68165 Mannheim (Germany)

Tel.: +49 (0)621 7028 - 0
Fax: +49 (0)621 7028 - 1028
http://www.leica-microsystems.com

Order No.: 156500002