



Leica TCS SP5

User Manual

Published by:

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

<http://www.leica-microsystems.com>
<http://www.confocal-microscopy.com>

Responsible for contents: Leica Microsystems CMS GmbH

Copyright © Leica Microsystems CMS GmbH.
All rights reserved.

Table of Contents

1.	General	7
1.1	Copyright.....	7
1.2	About this Operating Manual	8
2.	The Leica TCS SP5	9
2.1	Important Notes	9
2.1.1	System Overview	9
2.1.2	Intended Use.....	10
2.1.3	Conformity	10
2.1.4	Patents	11
2.1.5	Serial Number	11
2.2	Specifications.....	12
2.2.1	Dimensions	12
2.2.1.1	System with Inverted Stand.....	12
2.2.1.2	System with Upright Stand	12
2.2.2	Electrical Connection Requirements	13
2.2.3	Requirements Regarding Ambient Conditions	13
2.2.4	Permitted Ambient Conditions.....	14
2.2.5	Waste Heat/Required Cooling Performance	14
2.3	Features.....	15
2.3.1	Overview of Usable VIS/UV Lasers	15
2.3.2	Overview of Usable MP Lasers (IR Lasers).....	16
2.3.3	Which Laser Class Does the Product Have?	17
2.3.4	Required Laser Safety Measures.....	17
3.	Safety Instructions and their Meanings	19
4.	General Safety Instructions	21
4.1	Laser Class for VIS and UV Systems	21
4.2	Laser Class for MP Systems.....	21
4.3	What Should the Owner/Operator of the Laser Scanning Microscope Observe?.....	21
4.4	Safety Instructions for the User.....	23
4.5	Operational Reliability	23
4.6	Maximum Current Load of the Multiple Socket Outlet at the Supply Unit	24
5.	Safety Devices	25

5.1	Disconnecting the Power Supply.....	25
5.2	Detachable-key Switch.....	26
5.3	Emissions Warning Indicators.....	26
5.4	Remote Interlock Connector on the Supply Unit.....	27
5.5	Remote Interlock Connectors on External Lasers.....	28
5.6	Remote Interlock Connector on the Scanner.....	29
5.7	Function and Position of Safety Switches.....	30
5.8	Special Laser Safety Equipment.....	31
5.8.1	Safety Beam Guide and Beam Stop.....	31
5.8.2	Shielding in MP Systems (IR Lasers).....	32
5.9	Safety Labels on the TCS SP5.....	33
5.9.1	Inverted Stand DMI 6000 CS.....	33
5.9.2	Upright Stand DM 5000/6000 CS.....	35
5.9.3	Scan Head.....	37
5.9.4	Supply Unit.....	38
5.9.5	MP Beam Coupling Unit.....	39
5.9.6	Cover (for Replacement Flange).....	40
5.9.7	Mirror Housing.....	41
6.	Safety Instructions for Operating the System.....	43
6.1	Requirements Related to the Installation/Storage Location.....	43
6.2	General Safety Instructions for Operation.....	43
6.3	Eye Protection.....	44
6.3.1	MP System with Upright Microscope Stand.....	44
6.3.2	MP System with Inverted Microscope Stand and VIS or UV Systems.....	44
6.3.3	VIS and UV Systems with Inverted or Upright Stand.....	44
6.4	Specimen Area.....	45
6.5	Changing Specimens.....	46
6.6	Changing Objectives.....	47
6.7	Changing the Transmitted-light Lamp Housing.....	48
6.8	Mirror Housing on Upright Stand.....	50
6.9	Changing Filter Cubes, Beam Splitters or Condenser.....	52
6.10	Piezo Focus with Upright Microscope Stand.....	53
6.10.1	Objective Change with Piezo Focus Configuration.....	54
7.	Starting Up the System.....	55
7.1	Switching On the System.....	55

7.2	Starting the LAS AF	59
7.3	Setting Up Users.....	61
8.	Switching Off the System	63
9.	Introduction to LAS AF	65
9.1	General	65
9.2	Online Help	65
9.2.1	Structure of the Online Help.....	65
9.2.2	Accessing the Online Help	66
9.2.3	Full-text Search with Logically Connected Search Terms.....	67
9.3	Structure of the Graphical User Interface	68
9.3.1	General Structure of the Graphical User Interface.....	68
9.4	Key Combinations.....	69
10.	Introduction to Confocal Work	71
10.1	Preparation	71
10.1.1	The Objective	72
10.1.2	Conventional Microscopy	72
10.1.3	Why Scan?.....	75
10.1.4	How Is an Optical Section Created?	76
10.2	Acquiring Optical Sections	78
10.2.1	Data Acquisition	78
10.2.2	Illumination	80
10.2.3	Beam Splitting.....	81
10.2.4	Emission Bands	82
10.2.5	The Pinhole and Its Effects	83
10.2.6	Image Detail and Raster Settings	85
10.2.7	Signal and Noise.....	89
10.2.8	Profile Cuts.....	91
10.3	Multiparameter Fluorescence	92
10.3.1	Illumination	93
10.3.2	Beam Splitting.....	94
10.3.3	Emission Bands	94
10.3.4	Crosstalk	94
10.3.5	Sequential Scanning	95
10.3.6	Unmixing	95

10.4	3D Series.....	96
10.4.1	Z-stack.....	96
10.4.2	Section Thicknesses	97
10.4.3	Distances.....	97
10.4.4	Data Volumes.....	97
10.4.5	Depictions.....	98
10.4.5.1	Gallery	98
10.4.5.2	Movie	98
10.4.5.3	Orthogonal Projections	99
10.4.5.4	Rotated Projections	100
10.5	Time Series	100
10.5.1	Scan Speed.....	100
10.5.2	Points	101
10.5.3	Lines.....	101
10.5.4	Planes	101
10.5.5	Spaces (Time-Space).....	101
10.5.6	FRAP Measurements.....	102
10.6	Spectral Series.....	102
10.6.1	Data Acquisition and Utilization.....	102
10.6.2	About Spectral Resolution.....	102
10.7	Combinatorial Analysis.....	102
11.	Care and Maintenance.....	105
11.1	General.....	105
11.2	Cleaning the Optical System.....	105
11.3	Cleaning the Microscope Surface	106
11.4	Maintaining the Scanner Cooling System	106
12.	Transport and Disposal.....	107
12.1	Changing the Installation Location	107
12.2	Disposal.....	107
13.	Contact.....	109
14.	Glossary.....	111
15.	Appendix.....	117
15.1	Safety Data Sheets from Third-party Manufacturers.....	117
15.2	Declaration of Conformity.....	122

1. General

1.1 Copyright

The instructions contained in the following documentation reflect state-of-the-art technology and knowledge standards. We have compiled the texts and illustrations as accurately as possible. Nevertheless, no liability may be assumed for the accuracy of this manual's contents. If you have any comments on this operating manual or on any of our other documentation, we would be pleased to hear from you. The information in this manual is subject to modification at any time and without notification.

All rights to this documentation are held by Leica Microsystems CMS GmbH. Adaptation, translation and reproduction of text or illustrations (in whole or in part) by print, photocopy, microfilm or other method (including electronic systems) is not allowed without express written permission from Leica Microsystems CMS GmbH.

Programs such as LAS and LAS AF are protected by copyright laws. All rights reserved. Reproduction, adaptation or translation of these programs is prohibited without prior written permission from Leica Microsystems CMS GmbH.

The term "Windows" may be used in the following text without further identification. It is a registered trademark of the Microsoft Corporation. Otherwise, no inference with regard to the free usability of product names may be drawn from the use of those names. All other brand names and product names in this document are brands, service marks, trademarks or registered trademarks of the respective manufacturers.

Made in Germany.

© Copyright Leica Microsystems CMS GmbH.
All rights reserved.

1.2 About this Operating Manual

The main area of concentration of this Operating Manual is the safety instructions, which must be strictly observed while working with the laser scanning microscope.

In addition, this Operating Manual provides a rough overview of the operating principle of laser scanning microscopes. It presents you with the first steps for activating and commissioning the system and provides important information about the Leica Application Suite Advanced Fluorescence (LAS AF) software.

The Leica TCS SP5 is supplied with the latest version of the licensed LAS AF software. To maintain information on the most current level, the description of software functions was intentionally omitted from this Operating Manual. Instead, please refer to the online help of the LAS AF, which presents the most up-to-date explanations and instructions about the corresponding software functions.

First, read the chapter "Introduction to the Leica Application Suite Advanced Fluorescence" in this Operating Manual to familiarize yourself with its design and operation. Please refer to the online help for further information on particular functions.

2. The Leica TCS SP5

2.1 Important Notes

2.1.1 System Overview

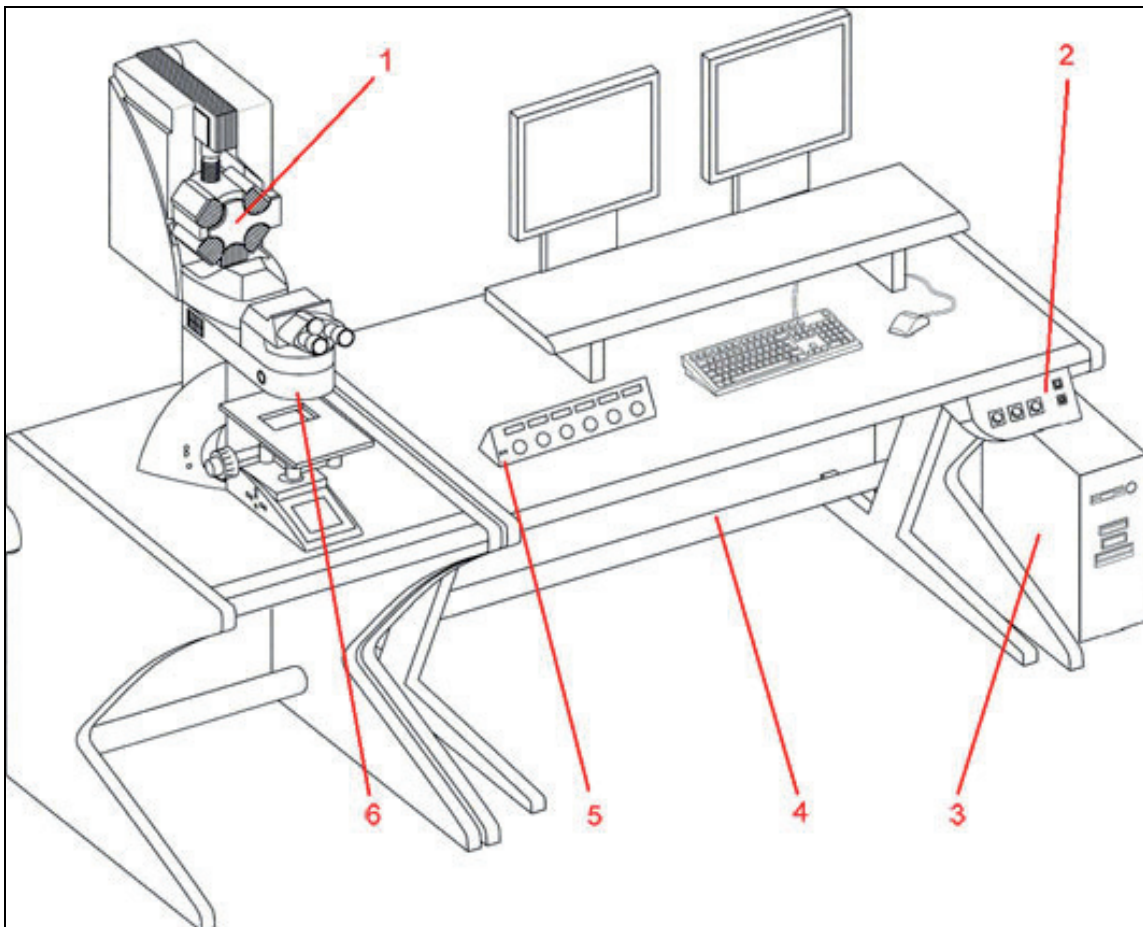


Figure 1: System components (overview)

- 1 TCS SP5 Scanner
- 2 Main switch board
- 3 TCS workstation
- 4 Supply unit
- 5 Control panel
- 6 Microscope stand

2.1.2 Intended Use

The system was designed for confocal scanning (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 in accordance with MPG (German Medical Devices Act) are excluded from proper intended use.

The manufacturer assumes no liability for damage caused by, or any risks arising from, use of the microscopes for purposes other than those for which they are intended, or not using the microscopes within the specifications of Leica Microsystems CMS GmbH. In such cases, the Declaration of Conformity shall be invalid.

2.1.3 Conformity

This device has been 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 instrument for use in buildings that do not include domestic premises and buildings not directly connected to a low-voltage power supply network that supplies buildings used for domestic purposes.
IEC/EN 61000-3-2	"Electromagnetic Compatibility (EMC)" Part 3-2: Limits — Limits for harmonic currents
IEC/EN 61000-3-3	"Electromagnetic Compatibility (EMC)" Part 3-3: Limits — Limitation of voltage fluctuations and flicker in low-voltage supply systems.

Refer to the Appendix of this Operating Manual for the Declaration of Conformity for the Leica TCS SP5.

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 USA (area of validity of the CDRH/FDA), the designations of the laser class are to be changed in the text from 3B to IIIb and Class 4 to IV.

2.1.4 Patents

The product is protected under 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.

2.1.5 Serial Number

For the serial number of your Leica TCS SP5, refer to the rear side of the scanner:

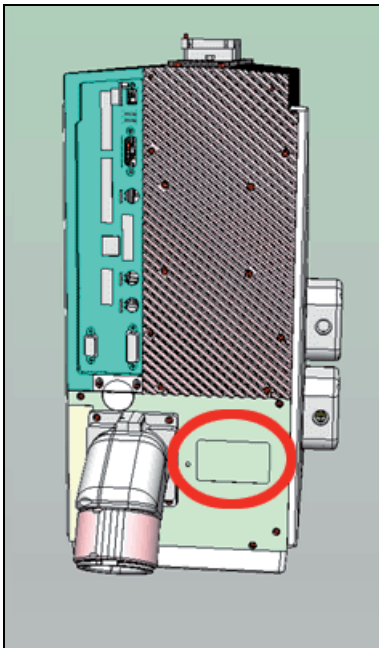


Figure 2: Rear side of the scanner — label with serial number

2.2 Specifications

2.2.1 Dimensions

2.2.1.1 System with Inverted Stand

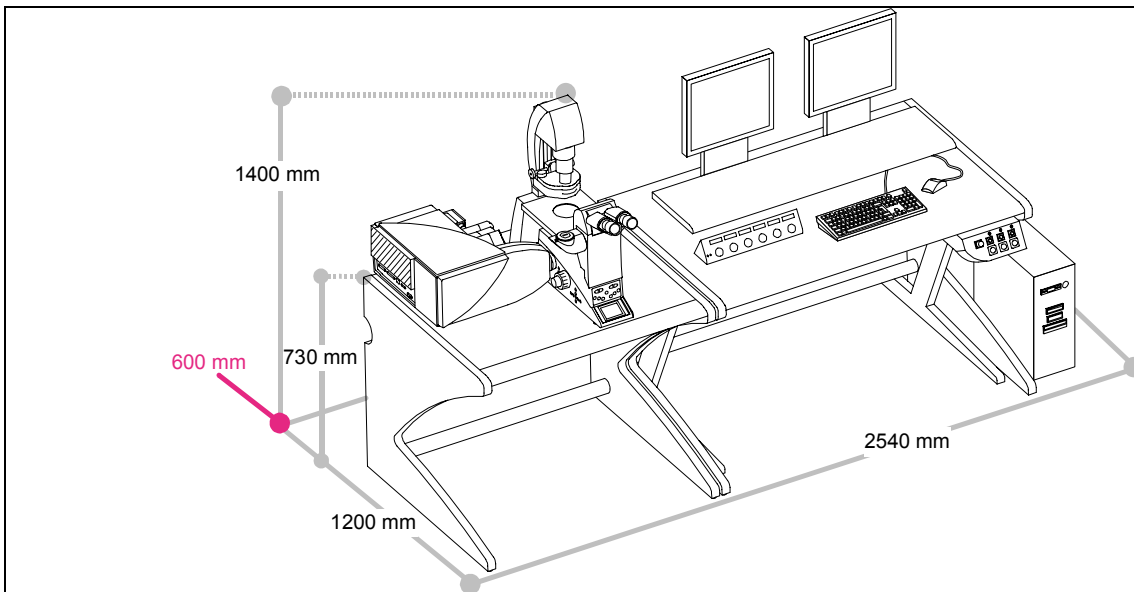


Figure 3: Dimensions of the TCS SP5 with inverted stand

2.2.1.2 System with Upright Stand

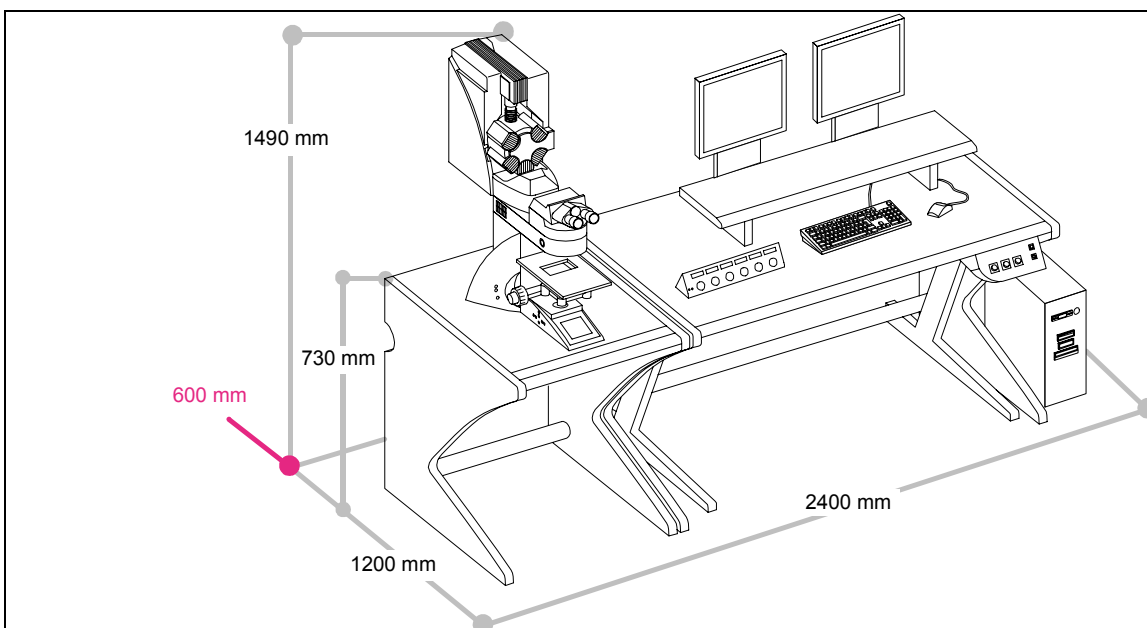


Figure 4: Dimensions of the TCS SP5 with upright stand

2.2.2 Electrical Connection Requirements

Supply voltage:	100 - 240 V AC \pm 10 %
Frequency:	50/60 Hz
Power consumption:	3200 VA
Overtoltage category:	II

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

- 3 x 100 V - 120 V power supply at 20 A
or
- 3 x 200 V - 240 V power supply at 12 - 16A

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

2.2.3 Requirements Regarding Ambient Conditions



Do not expose the system to drafts.

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



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

Also read the notes on protection against dust in Chapter 11 Care and Maintenance
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, note that the system will apply a static load of 200 kg/m².



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

2.2.4 Permitted Ambient Conditions

Permissible temperature range for operation:	+18 to +25 °C
Temperature range for optimum optical behavior:	+22 °C ±1 °C
Permitted relative humidity:	20 - 80% (non-condensing)
Permitted vibration speeds: Frequency range [5 Hz–30 Hz]: Frequency range [> 30 Hz]:	< 30 μm/s (RMS) < 60 μm/s (RMS)
Pollution degree:	Class 2

2.2.5 Waste Heat/Required Cooling Performance

The TCS SP5 system features a maximum power consumption of 3.2 kW (VIS system) or 6.2 kW (MP system), respectively.

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

2.3 Features

2.3.1 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
Diode 405	405	< 60	< 6	Continuous wave (cw)
Diode 405 p	405	< 5 (mean power)	< 0.3 (mean power)	pulsed, 60 ps
DPSS 442	442	< 50	< 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)
HeNe	543	< 1.5	< 0.5	Continuous wave (cw)
DPSS 561	561	< 25	< 8	Continuous wave (cw)
HeNe	594	< 4	< 1	Continuous wave (cw)
HeNe	633	< 15	< 4	Continuous wave (cw)
Ar-UV, external	351, 364	< 500	< 10	Continuous wave (cw)

Table 1: Table of usable lasers (without MP)

2.3.2 Overview of Usable MP Lasers (IR Lasers)

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

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

Laser type	Wavelength [nm]	Luminous power at laser output [W]	Luminous power in focal plane [W]	Pulse duration
MaiTai ps	780 - 920	< 1.2	< 0.6	pulsed, 1.0 - 1.5 ps
MaiTai ps wideband	710 - 950	< 2.5	< 1.2	pulsed, 1.0 - 1.5 ps
MaiTai ps broadband	710 - 990	< 2.5	< 1.2	pulsed, 1.0 - 1.5 ps
MaiTai ps HP	690 - 1020	< 3.0	< 1.5	pulsed, 1.0 - 1.5 ps
Chameleon ps	720 - 950	< 2.0	< 1.0	pulsed, 1.0 - 1.5 ps
Chameleon ps XR	705 - 980	< 2.5	< 1.2	pulsed, 1.0 - 1.5 ps
Chameleon ps Ultra	690 - 1040	< 3	< 1.5	pulsed, 1.0 - 1.5 ps

Table 2: Table of usable MP lasers

2.3.3 Which Laser Class Does the Product Have?

Laser variant	Wavelength range	Configuration	Laser class
VIS	400 - 700 nm, (visible laser radiation)	Combination of lasers from Chapter 2.3.1 (without lasers having wavelengths of 350 - 400 nm)	3B / IIIb
UV	350 - 700 nm, (visible and invisible laser radiation)	Combination of lasers from Chapter 2.3.1 (VIS and UV lasers)	3B / IIIb
MP	350 - 1050 nm, (visible and invisible laser radiation)	Combination of lasers from Chapter 2.3.1 (VIS/UV lasers) and Chapter 2.3.2 (IR lasers)	4 / IV

2.3.4 Required Laser Safety Measures

Please observe the laser safety measures for laser class 3B / IIIb (VIS and UV systems) or laser class 4 / IV (MP systems) in accordance with applicable national and federal regulations. The owner/operator is responsible for observing the laser safety regulations.

3. Safety Instructions and their Meanings



DANGER

This kind of warning alerts you of an operating procedure, practice, condition, or instruction in the operating manual that must be strictly observed and followed, as otherwise you expose yourself to the risk of fatal injury.



WARNING! LASER RADIATION

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.



WARNING! ELECTRICAL VOLTAGE

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.



WARNING! HARMFUL SUBSTANCES

A harmful substances warning points out a substance that can be harmful to your health.



CAUTION

A safety instruction 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.



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

4. General Safety Instructions

4.1 Laser Class for VIS and UV Systems

In accordance with IEC/EN 60825-1, this laser scanning microscope is a laser product of Class 3B / IIIb.



Never expose eyes or skin to direct radiation! The laser light can cause permanent eye damage!

4.2 Laser Class for MP Systems

In accordance with IEC/EN 60825-1, this laser scanning microscope is a laser product of Class 4 / IV.



Never expose eyes or skin to direct or indirect radiation! Laser light can cause permanent eye damage and skin injuries!

4.3 What Should the Owner/Operator of the Laser Scanning Microscope Observe?

The owner/operator of this product is responsible for proper and safe operation and safe maintenance of the system and for following all applicable safety regulations. The owner/operator is fully liable for all consequences resulting from the use of the system for any purposes other than those listed in the operating manual or the online help.



This laser product may be operated only by persons who have been instructed in the use of the system and the potential hazards of laser radiation.



The owner/operator is obligated to perform and monitor suitable safety measures in accordance with IEC / EN 60825-1 and the corresponding national and federal regulations.



To assure classification as a 3B/IIIb or 4/IV laser product in accordance with IEC/EN60825-1 and electrical safety compliance, all safety devices, interlocks, and safety systems of the laser product 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 damage. In these cases, Leica Microsystems CMS GmbH shall not assume any liability.



In accordance with IEC/EN 60825-1: "Safety of laser products, Part 1: Equipment classification, requirements and user's guide," the owner/operator 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 owner/operator is fully liable for all consequences resulting from the use of the system if it is opened, improperly serviced or repaired by persons other than authorized Leica 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.



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

Leica Microsystems CMS GmbH shall 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.

4.4 Safety Instructions for the User

Read and observe the safety instructions in the operating manual and the safety labels located on the system. Failure to observe the safety instructions may lead to serious injuries and to significant damages to the system and loss of data.



The instrument is a Class 3B or 4 laser product (depending on the laser used) in accordance with IEC/EN 60825-1. This laser product may be operated only by persons who have been instructed in the use of the system and the potential hazards of laser radiation.



Before carry out operating steps with the system for the first time, first read the corresponding description of the function in the online help. For an overview of the individual functions, refer to the table of contents of the online help.

As it is impossible to anticipate every potential hazard, please be careful and apply common sense when operating this product. Observe all safety precautions relevant to Class 3B/IIIb lasers and Class 4/IV lasers for MP systems.

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

4.5 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.

4.6 Maximum Current Load of the Multiple Socket Outlet at the Supply Unit

The total power consumption of all loads connected to the multiple socket outlet (Figure 5) must not exceed 800 VA.

The terminals are intended for:

- TCS workstation
- Monitor 1
- Monitor 2
- Microscope

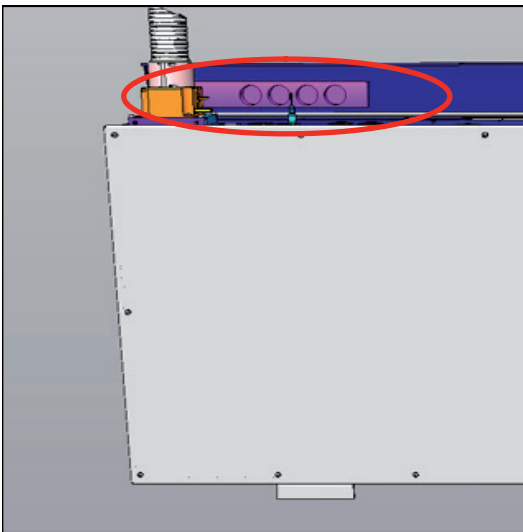


Figure 5: Multiple socket outlet, rear side of supply unit

5. Safety Devices

5.1 Disconnecting the Power Supply

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 (Figure 6).

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.

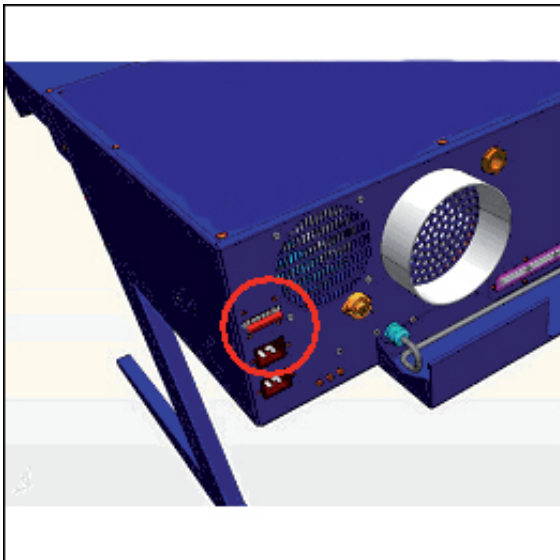


Figure 6: Supply unit with main circuit breaker

5.2 Detachable-key Switch

The detachable-key switch for protection against unauthorized use of the laser products is located on the main switch board (see Figure 7).

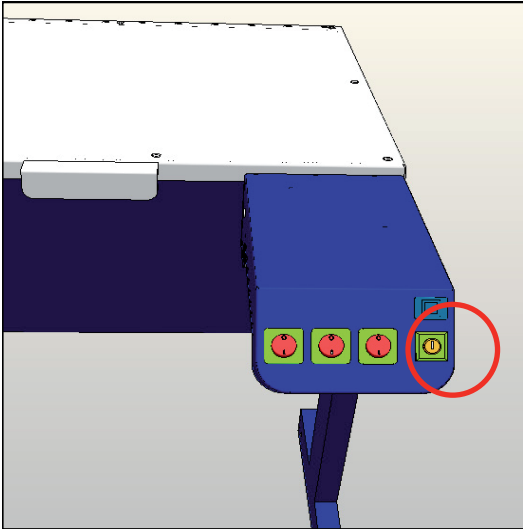


Figure 7: Detachable-key switch for the internal lasers



For external lasers, please refer to the manual supplied by the laser manufacturer for the position of the detachable-key switches.

5.3 Emissions Warning Indicators

The operational readiness of lasers located in the supply unit is signaled by an emission warning indicator (Figure 8). 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.

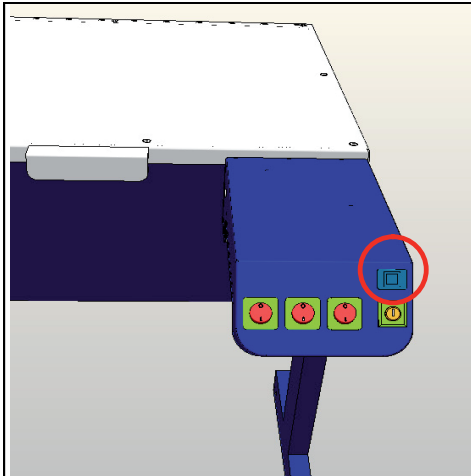


Figure 8: Emission warning indicators on the main switch board



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 detachable-key switch.
- 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 external lasers, please refer to the manual supplied by the laser manufacturer for the position of the emission warning indicator.

5.4 Remote Interlock Connector on the Supply Unit

The remote interlock jack is located on the rear side of the supply unit (12 V DC operating voltage, Figure 9).

The remote interlock plug, which contains a shorting bridge, is connected to this jack.

Remote interlock devices such as those connected to the room, the door or other onsite safety interlock systems can also be connected to the remote interlock connector. The laser beam path is interrupted if the contact is open.



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

The overall length of the cable between the two connecting pins of the remote interlock connector must not exceed 10 m.



Figure 9: Position of the remote interlock connector

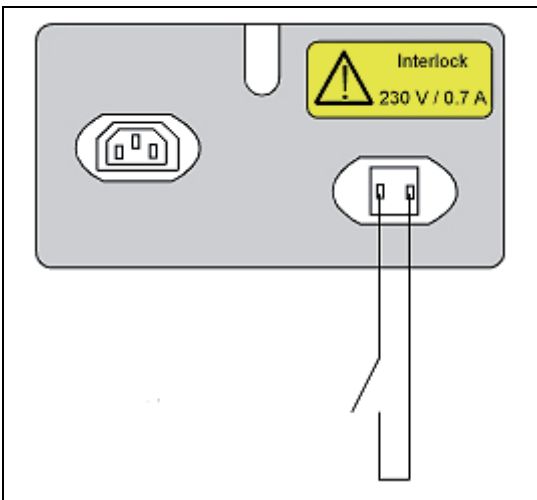


Figure 10: Example of a remote interlock

5.5 Remote Interlock Connectors on External Lasers



For external lasers, please refer to the manual supplied by the laser manufacturer for the position of the remote interlock connector.

5.6 Remote Interlock Connector on the Scanner

The interlock jack is located on the rear side of the scanner (operating voltage: 12 V DC, Figure 11).

The inverted microscope, or the mirror housing if an upright microscope is used, is connected to this jack. This ensures that the safety switch of the microscope is integrated in the interlock circuit.

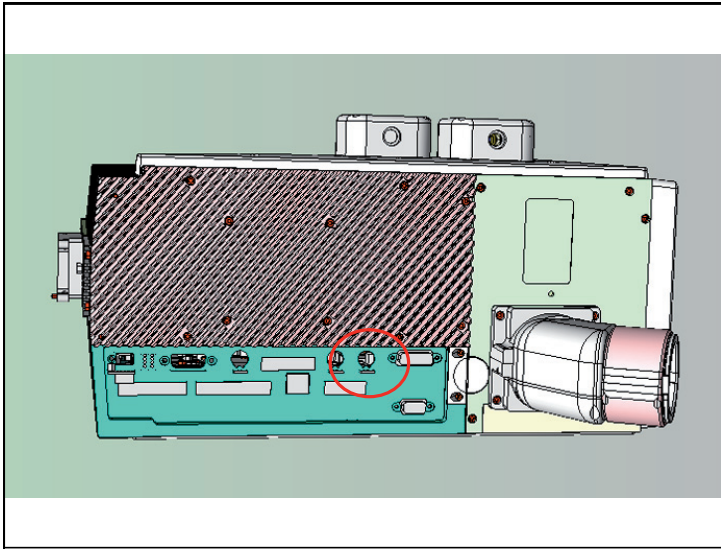


Figure 11: Location of the interlock jack

5.7 Function and Position of Safety Switches

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

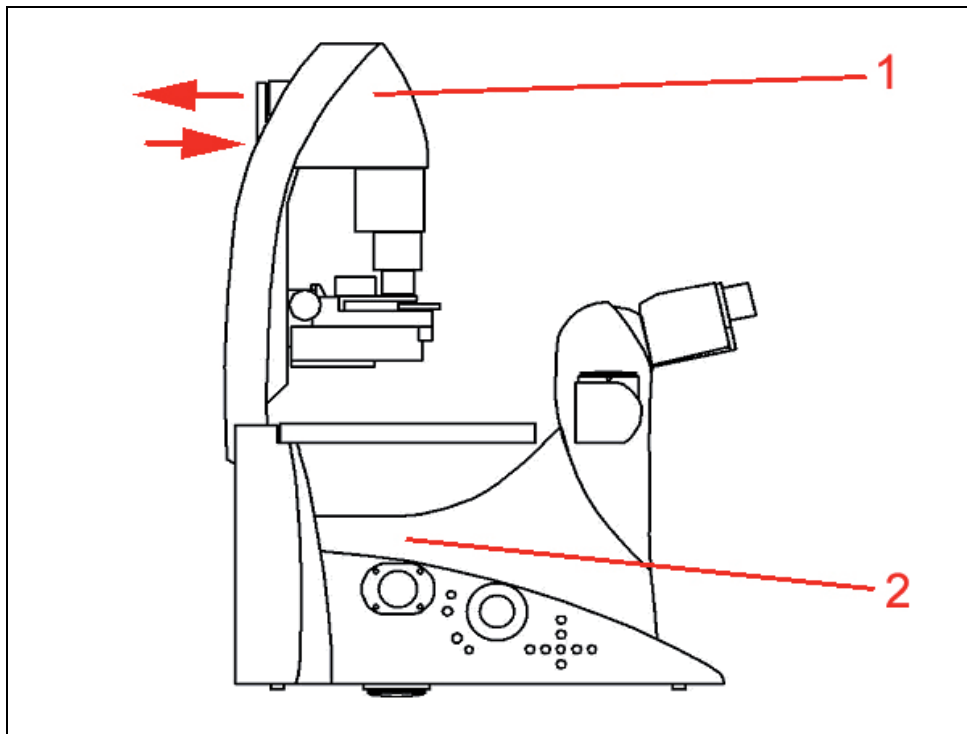


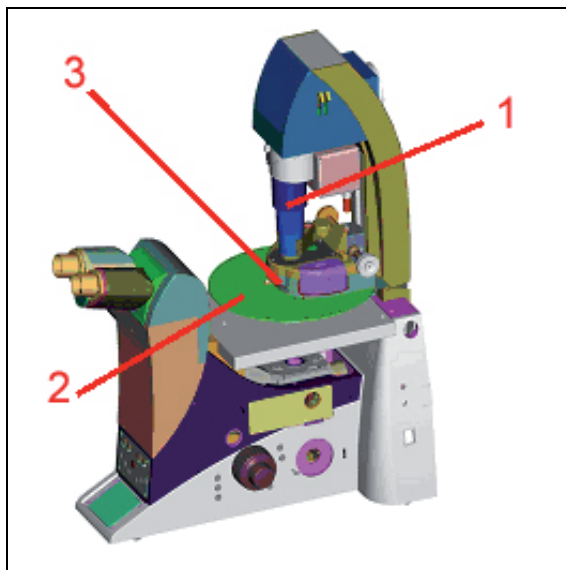
Figure 12: Position of the transmitted-light illumination arm (1) and switching from scan mode to eyepiece (2).

Position	Activated by:	Type of microscope	Activated if:	Function
1	Transmitted-light illuminator arm	Inverted stand DMI 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	The deflection mirror for the scanner is swung out by motor.	Prevents stray light if the user switches from confocal observation to eyepiece observation.

5.8 Special Laser Safety Equipment

5.8.1 Safety Beam Guide and Beam Stop

On inverted microscopes, the safety beam guide and the beam stop serve as protection against laser radiation emission and are located between the condenser base and the transmitted-light detector (see Figure 13).



1 Safety beam guide

2 Beam stop

(illustrated is the version of the beam stop for MP systems)

3 Condenser base

Figure 13: Inverted stand



If you reorder a condenser base (Figure 13, item 3) be aware that the condenser base is delivered without the beam stop (Figure 13, item 2). The existing beam stop (Figure 13, item 2) must always be reinstalled. Please consult the microscope stand's operating instructions provided.



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 operation.

5.8.2 Shielding in MP Systems (IR Lasers)

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 a fiber optic cable 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 a fiber optic cable (Figure 14). This shields the laser beam until it leaves the microscope objective and reaches the specimen.

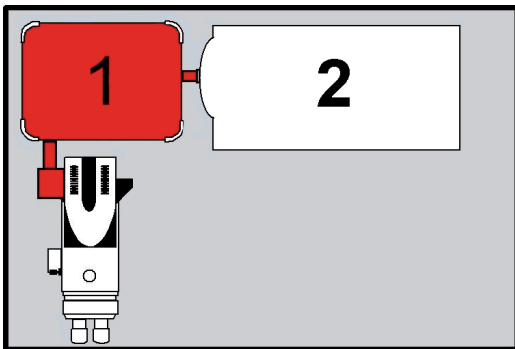


Figure 14: Safety beam guide (1) and IR laser (2)

5.9 Safety Labels on the TCS SP5

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

5.9.1 Inverted Stand DMI 6000 CS

Angled rear view of right side of stand

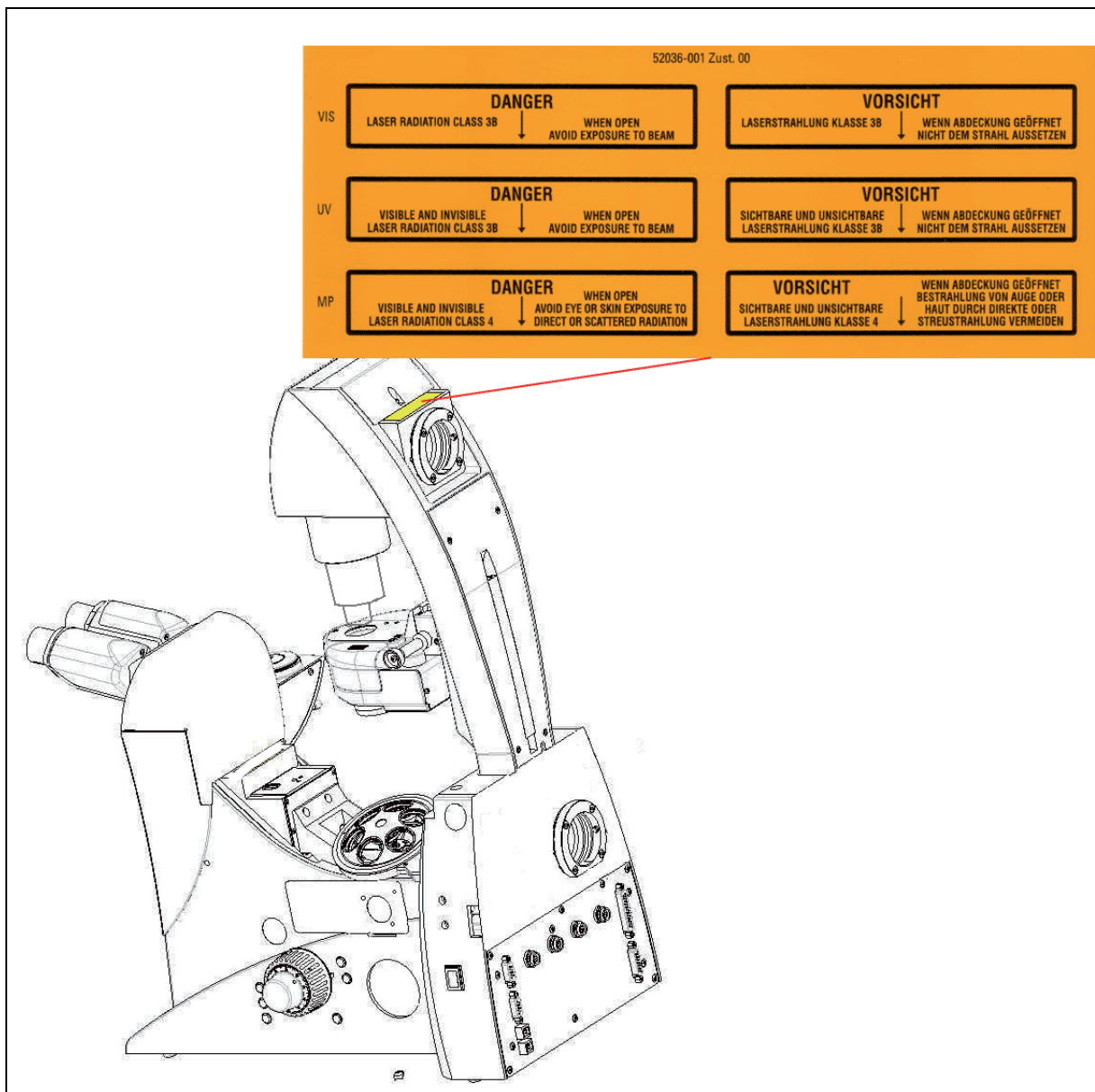


Figure 15: Safety label for DMI 6000 CS inverted stand

Angled rear view of right side of stand

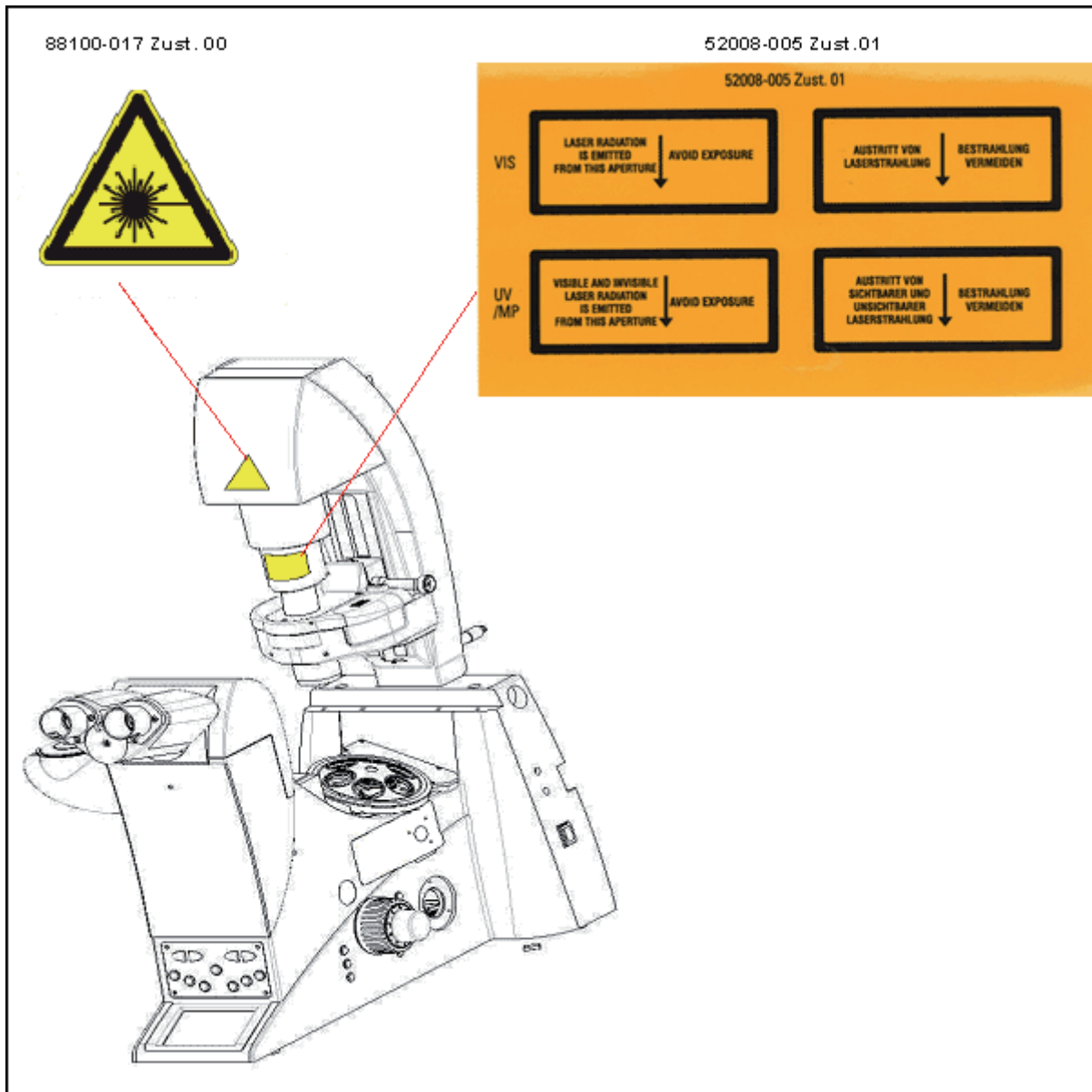


Figure 16: Safety label for DMI 6000 CS inverted stand

5.9.2 Upright Stand DM 5000/6000 CS

Angled rear view of right side of stand:

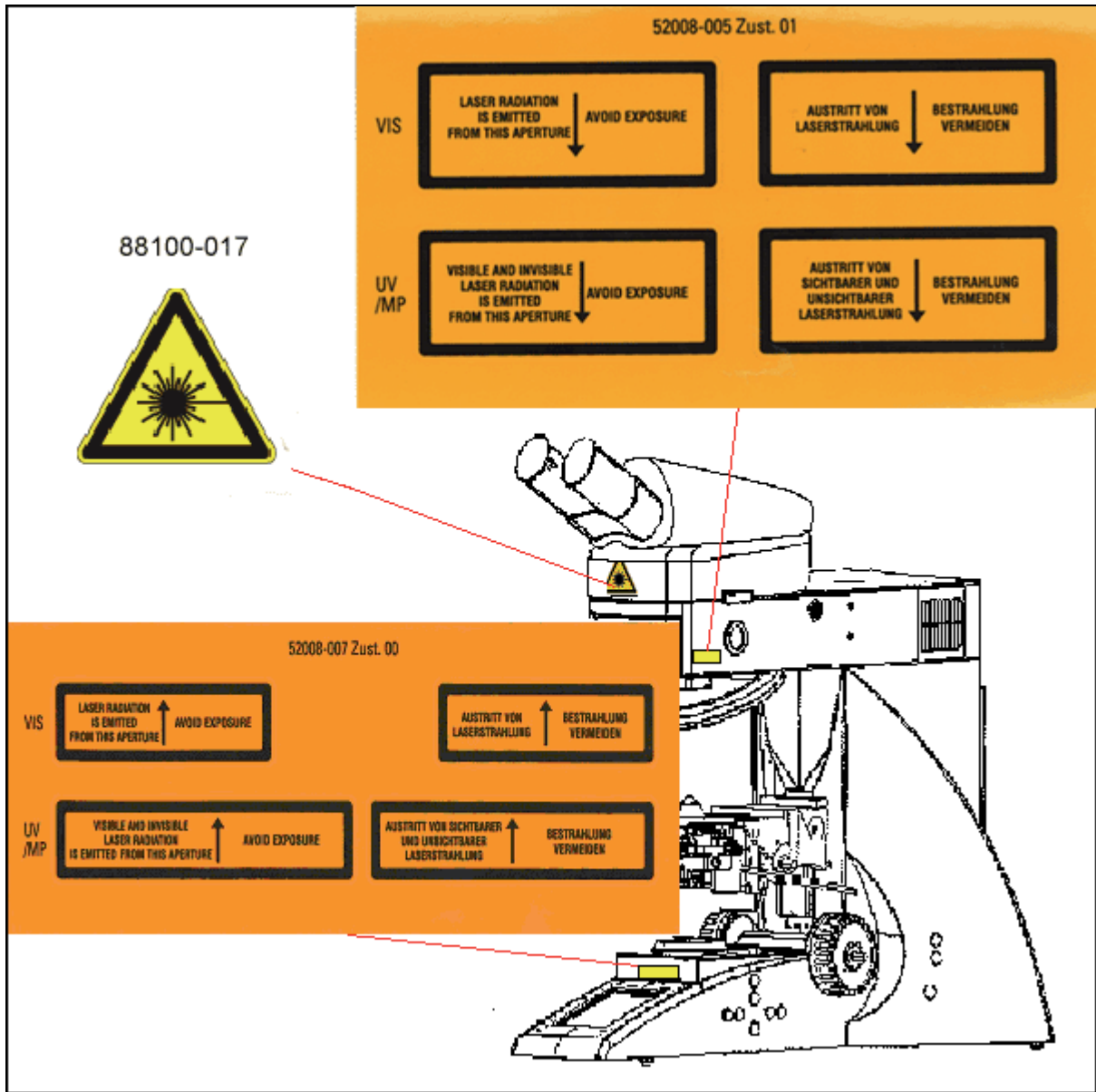


Figure 17: Safety label for DM 5000/6000 CS upright stand

Rear view of stand:

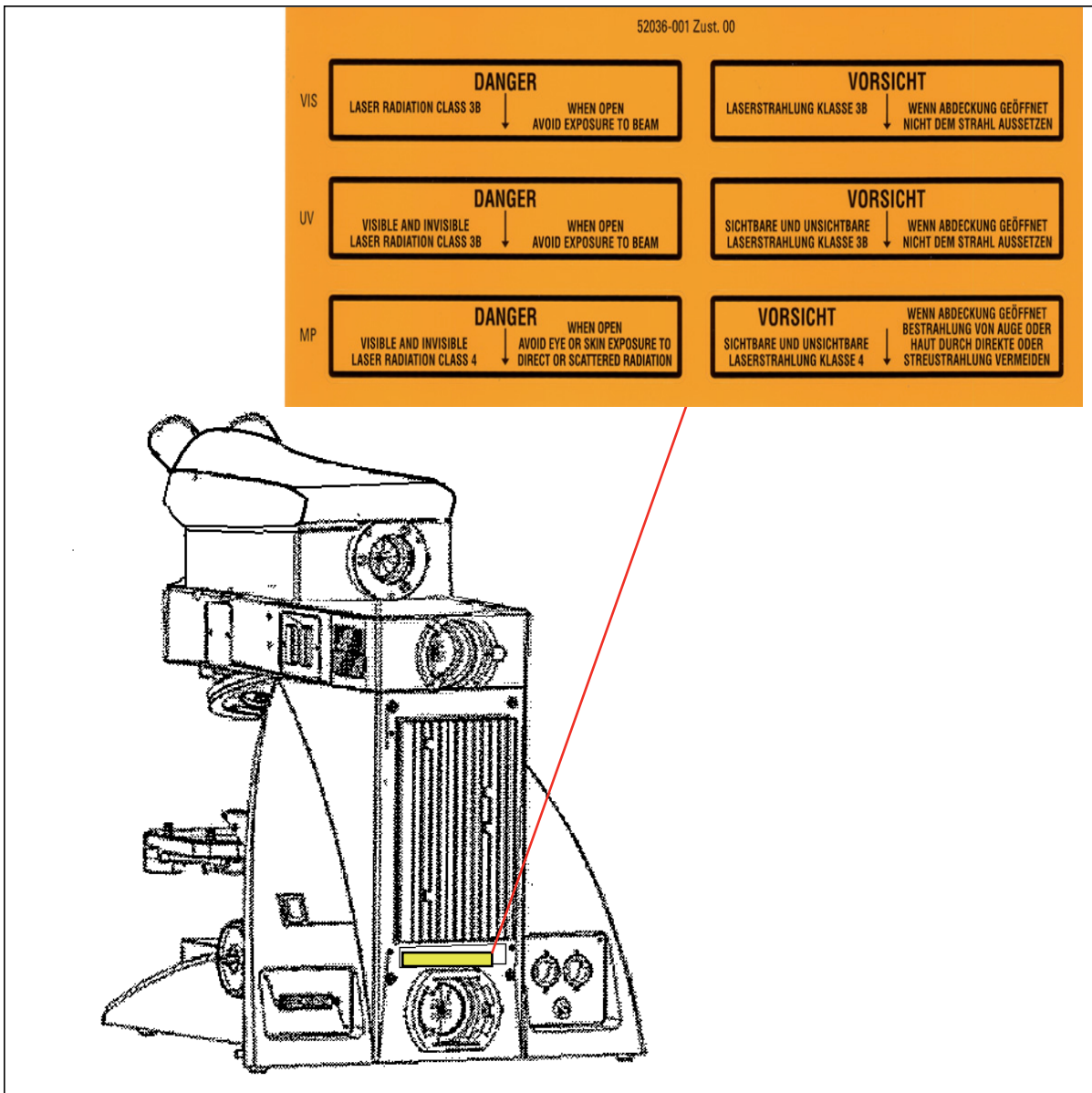


Figure 18: Safety label for DM 5000/6000 CS upright stand

5.9.3 Scan Head

Angled front view of left side of scan head:

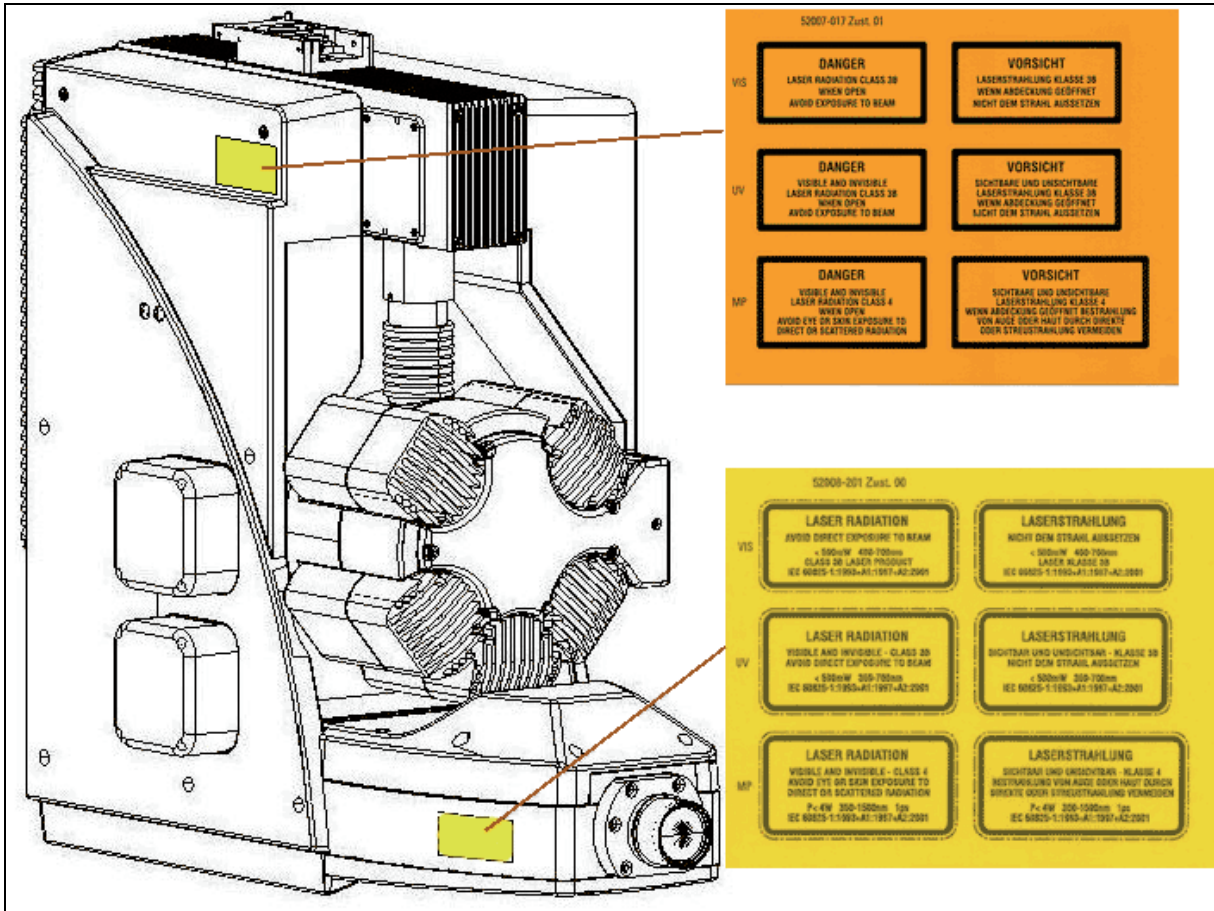


Figure 19: Safety label for the scanner

5.9.4 Supply Unit

View of TCS SP5 supply unit:

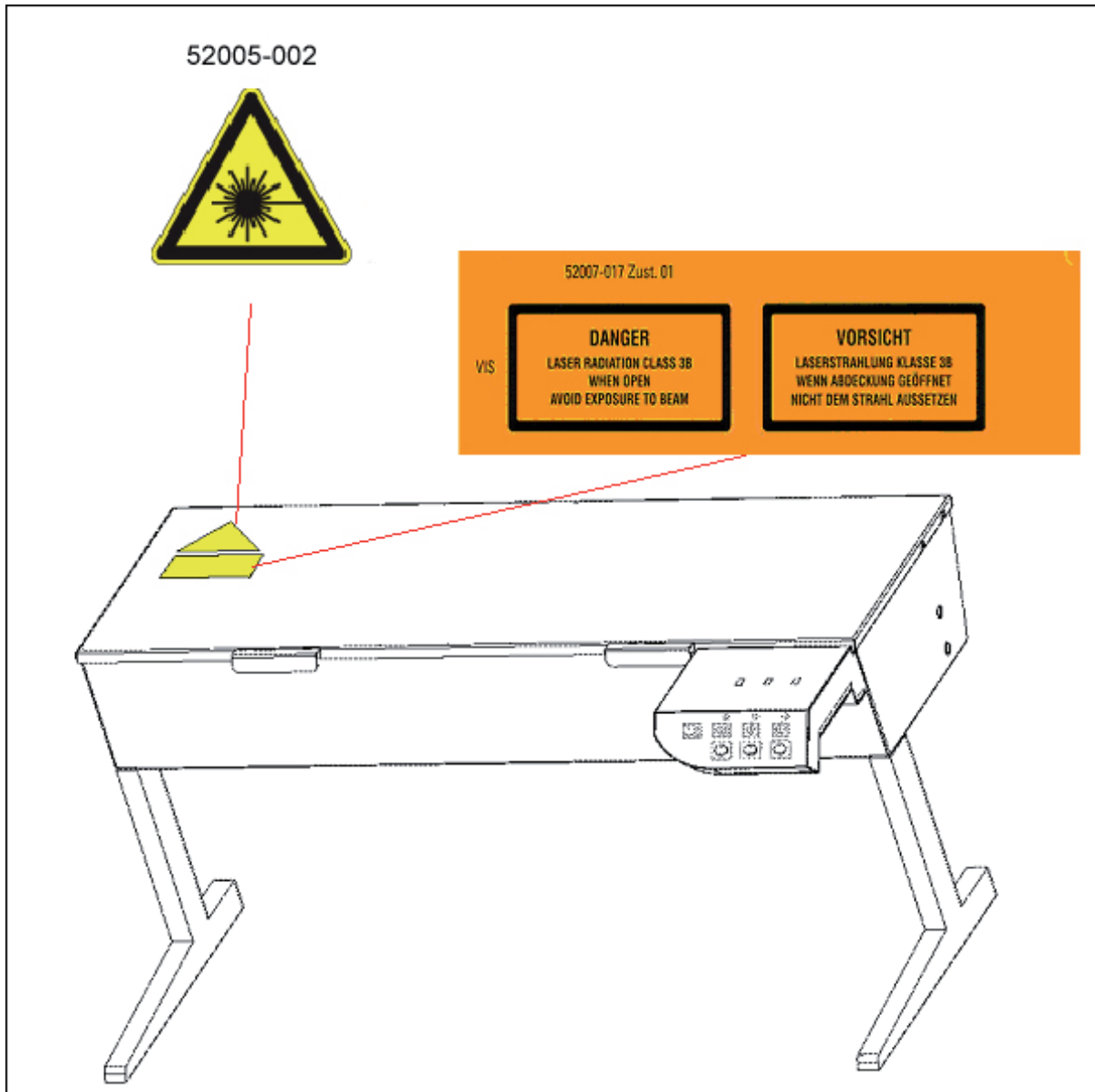


Figure 20: Safety label for the TCS SP 5 supply unit (front side)

5.9.5 MP Beam Coupling Unit

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

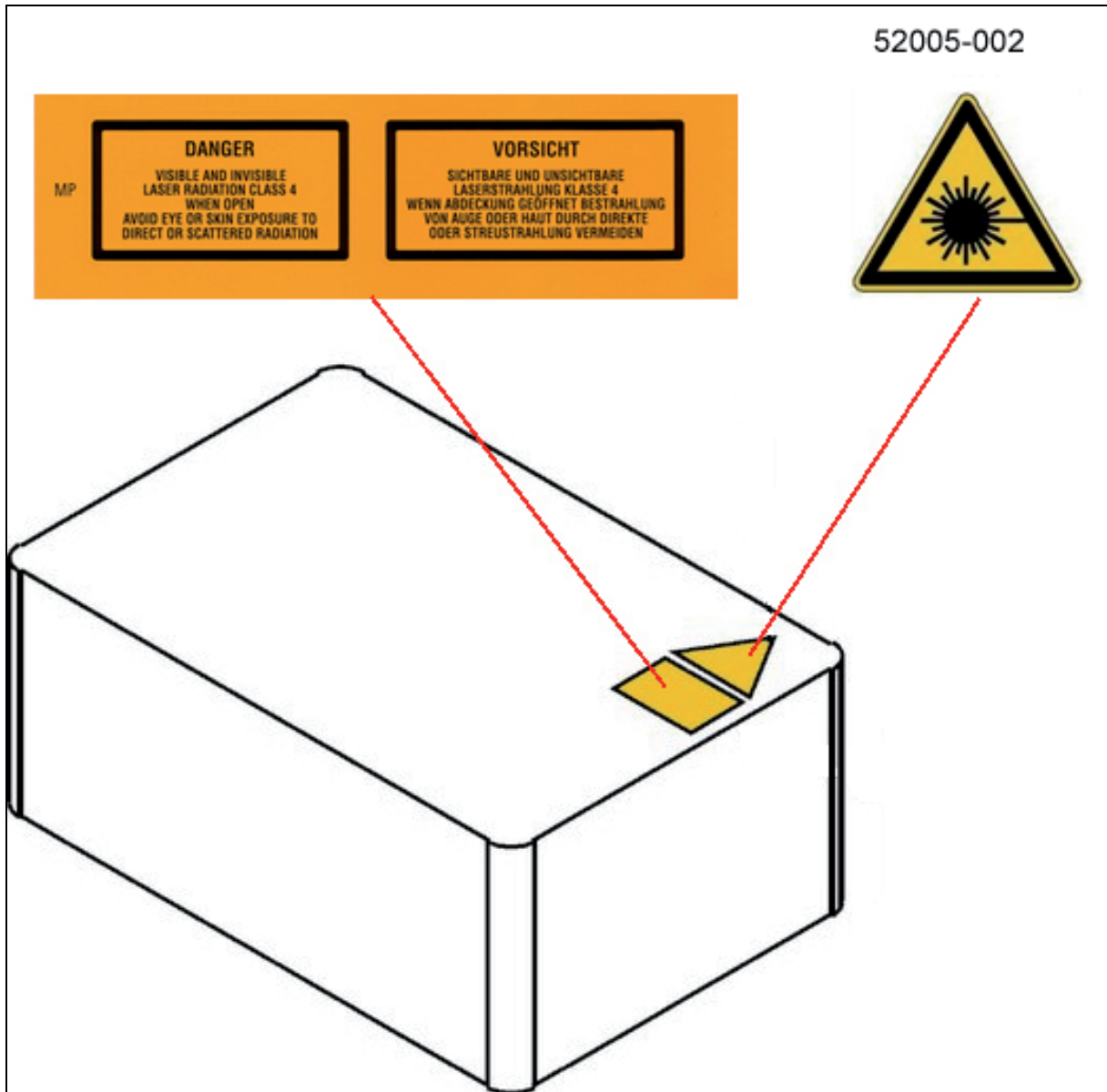


Figure 21: Safety label for the MP beam coupling unit (top side)

5.9.6 Cover (for Replacement Flange)

Front view of cover:

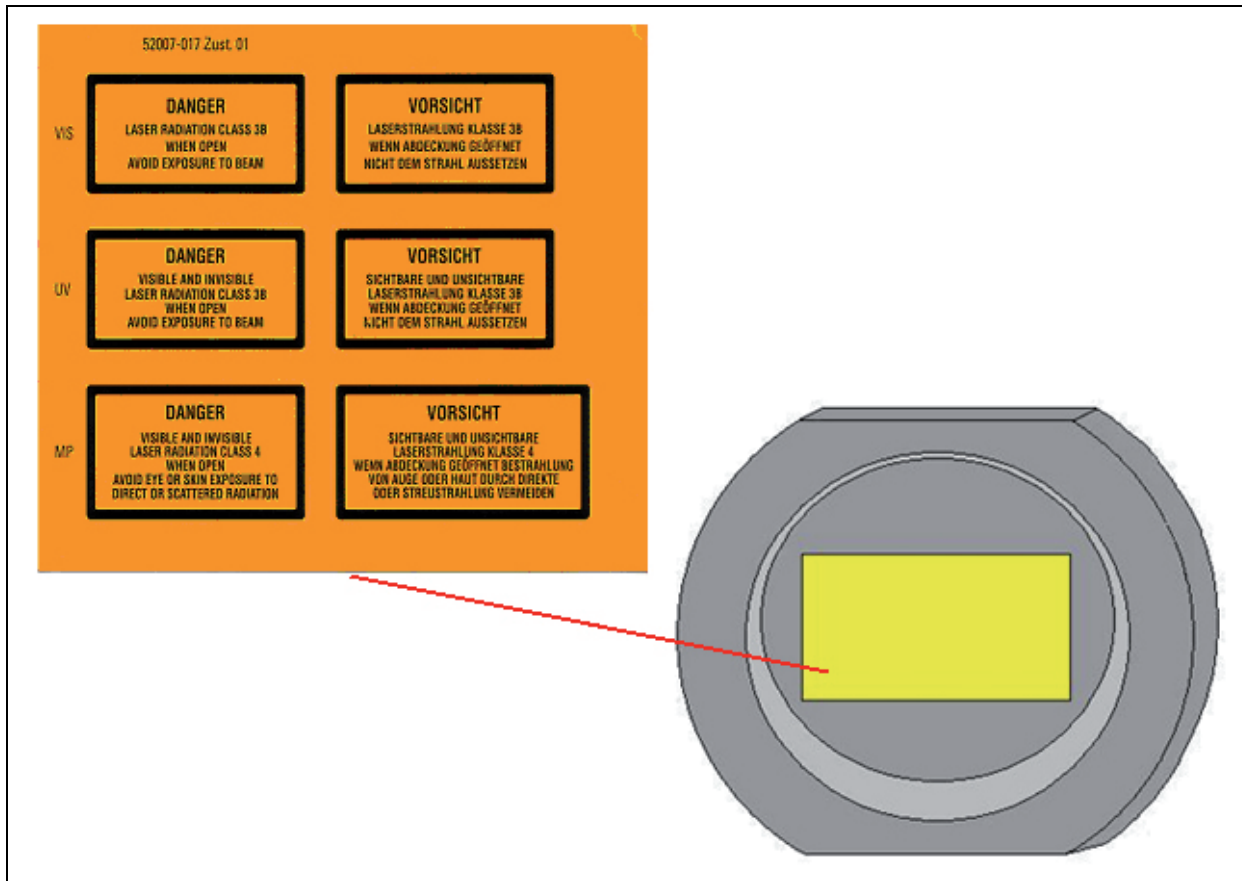


Figure22: Cover for replacement flange

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

5.9.7 Mirror Housing

Front view of the mirror housing:

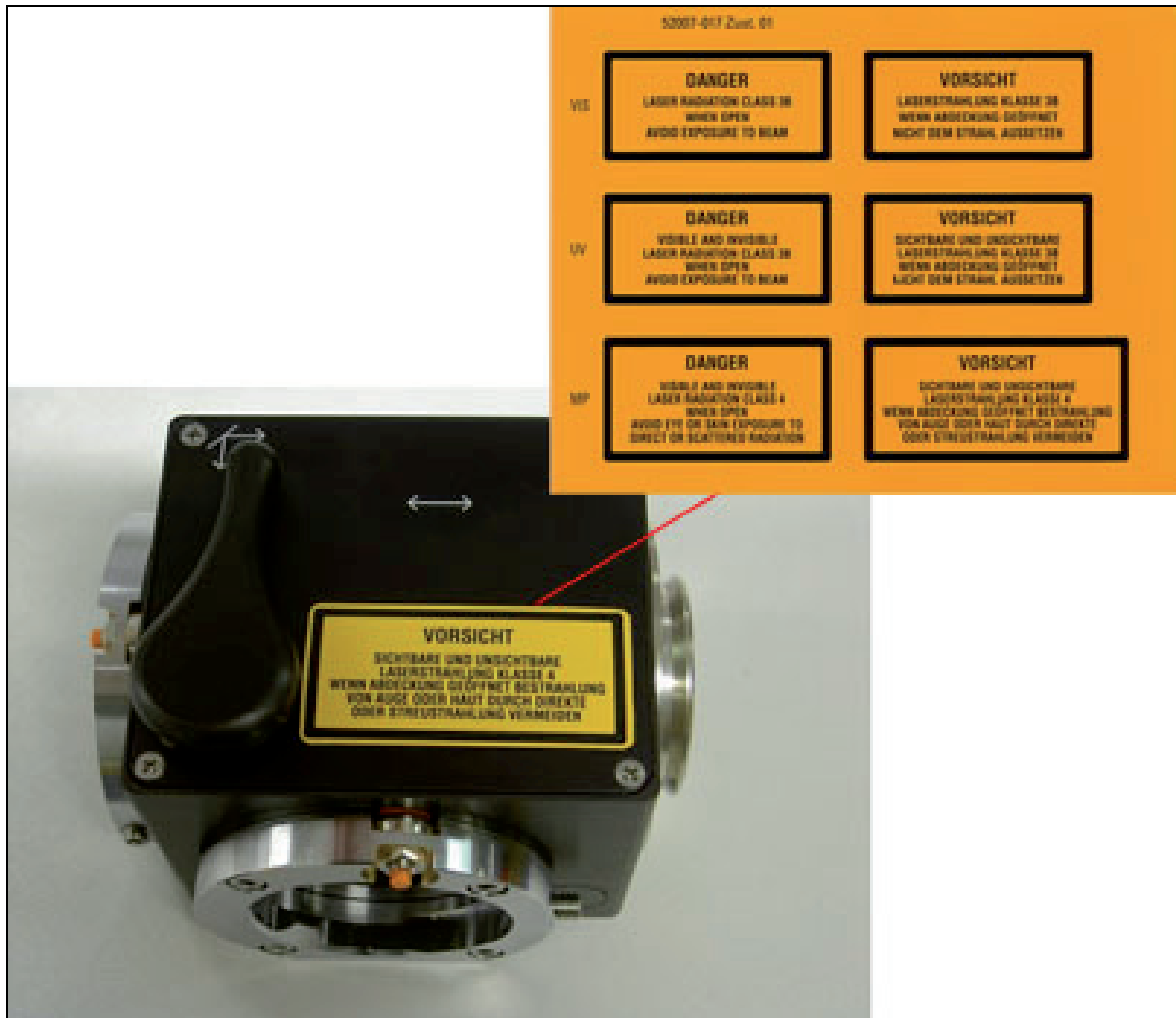


Figure 23: Safety label for mirror housing (top)

6. Safety Instructions for Operating the System

6.1 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 completely dry before connecting it to the power supply or turning it on.

6.2 General Safety Instructions for Operation



Do not look into the eyepieces during the scanning operation.



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.



Be sure to follow the included operating instructions for the microscope.

6.3 Eye Protection

6.3.1 MP System with Upright Microscope Stand



Using protective eyewear (order number: 15000010000032) is essential. Appropriate safety goggles for IR laser radiation are provided with the system when delivered.



During the scanning operation, all persons present in the room must wear safety goggles.

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.

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

6.3.2 MP System with Inverted Microscope Stand and VIS or UV Systems

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

6.3.3 VIS and UV Systems with Inverted or Upright Stand

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

6.4 Specimen Area

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

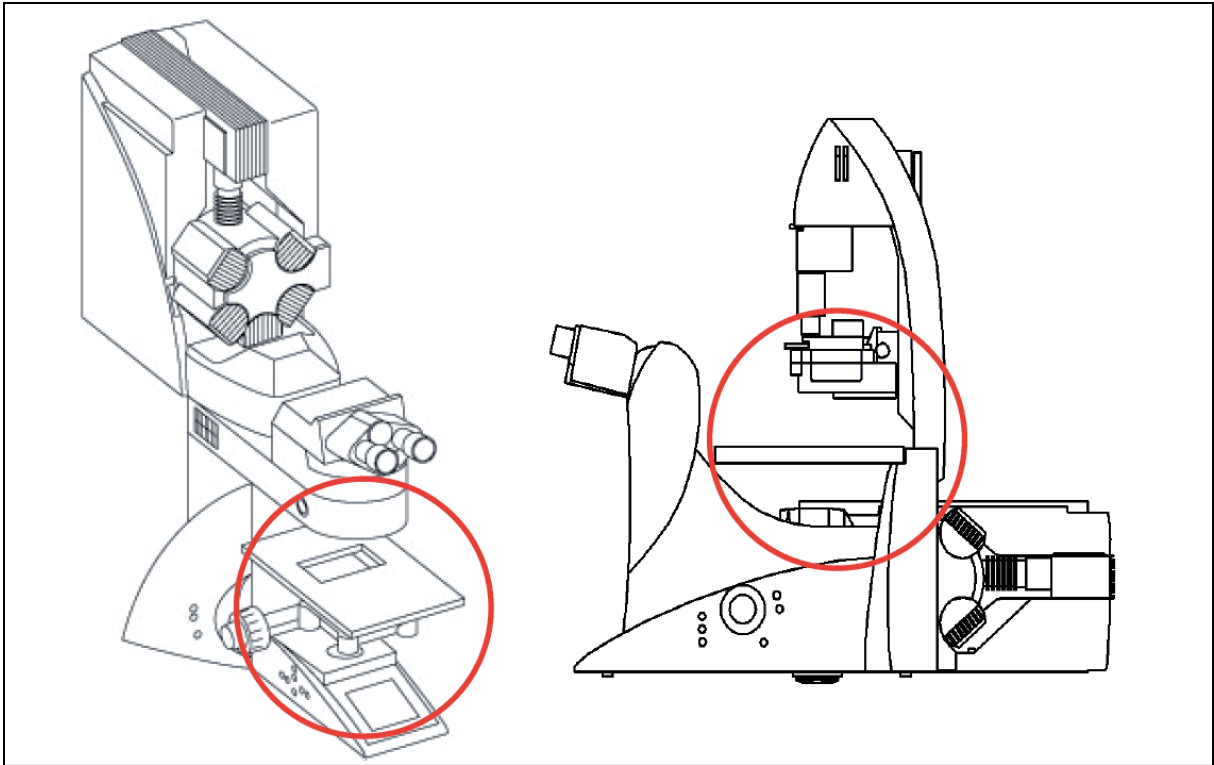


Figure 24: Specimen area of upright and inverted stand



During the scanning operation, the laser radiation is 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, special caution is absolutely necessary as soon as one or more of the laser emission warning indicators are lit.

If the laser scanning microscope is used as prescribed and the safety instructions are observed during operation, there are no dangers to the operator. Always keep your eyes at a safe distance of at least 20 cm from the opening of the objective.

6.5 Changing Specimens



Never change specimens during a scanning operation.

To change specimens, proceed as follows:

Upright microscope	Inverted microscope
Finish the scanning operation.	Finish the scanning operation.
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.

6.6 Changing Objectives



Do not change objectives during a scanning operation.

To change objectives, proceed as follows:

1. Finish the scanning operation.
2. Rotate the objective nosepiece 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 nosepiece must be closed using the supplied caps.



For MP systems, dry objectives (air objectives) may not be used with a numerical aperture (NA) larger than 0.85. This does not apply to immersion objectives (oil, water).



If a piezo focus is installed on your TCS SP5, please also observe the safety notes related to changing objectives with a piezo focus in 6.10.1.

6.7 Changing the Transmitted-light Lamp Housing

If no transmitted-light lamp housing is connected, to protect from the potential escape of laser radiation, the opening (Figure 26 or Figure 27) must be securely sealed with the cover (Figure 25) that accompanies the system.

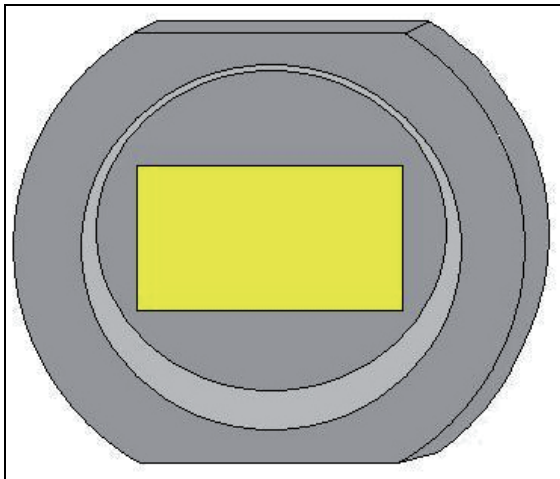


Figure 25: Cover



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

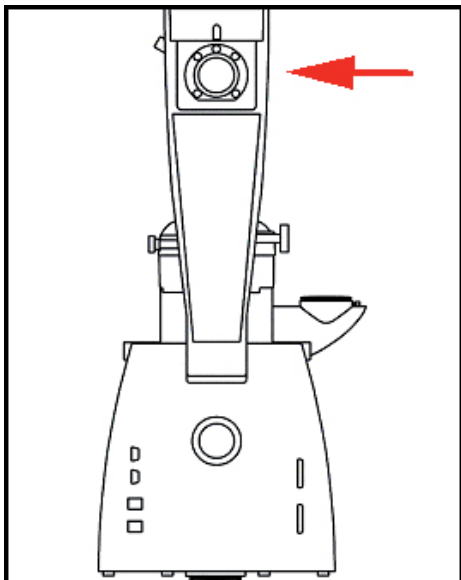


Figure 26: Port for connecting the transmitted-light lamp housing on the inverted stand

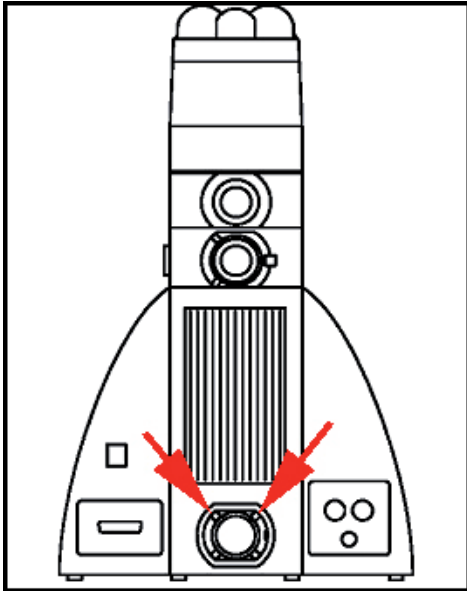


Figure 27: Port for connecting the transmitted-light lamp housing or mirror housing on the upright stand

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

1. Switch off the lasers.
2. Disconnect the lamp housing from the power supply.
3. Remove the lamp housing.
4. Modify the lamp housing as needed.
5. After finishing the tasks, screw the new lamp housing back onto the microscope stand.

6.8 Mirror Housing on Upright Stand

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 (Figure 28).

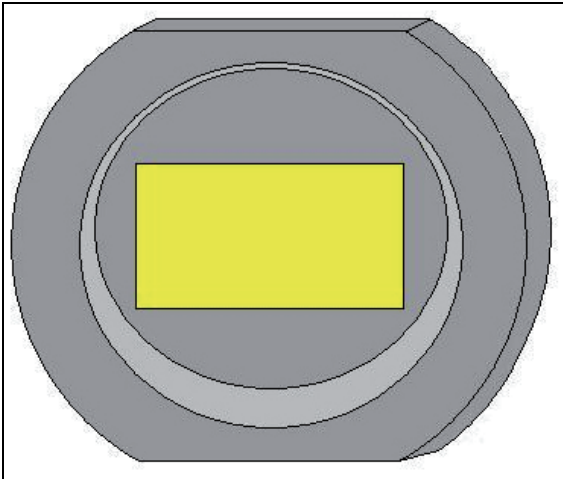


Figure 28: Cover



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

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

- If the mirror housing is removed, you must close off the port on the stand (Figure 27) using the cover (Figure 28).
- The interlock jack on the mirror housing (see Figure 29, item 1) must be connected to the scan head at all times.
- The unused output on the mirror housing must be covered with the cover provided (see Figure 29, item 3).



When installing the cover (Figure 29, item 3), ensure that the button (Figure 29, item 2) is pressed by the cover.



Figure 29: Mirror housing on upright stand

6.9 Changing Filter Cubes, Beam Splitters or Condenser



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

To change filter cubes or beam splitters proceed as follows:

Upright microscope	Inverted microscope
Finish the scanning operation.	Finish the scanning operation.
Remove the cover of the fluorescence module (see operating manual for microscope stand).	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 a fiber optic cable.



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 numerical aperture of the S70 microscope condenser could pose a hazard due to laser radiation. Therefore, only S1 and S28 Leica microscope condensers should be used.

6.10 Piezo Focus with Upright Microscope Stand

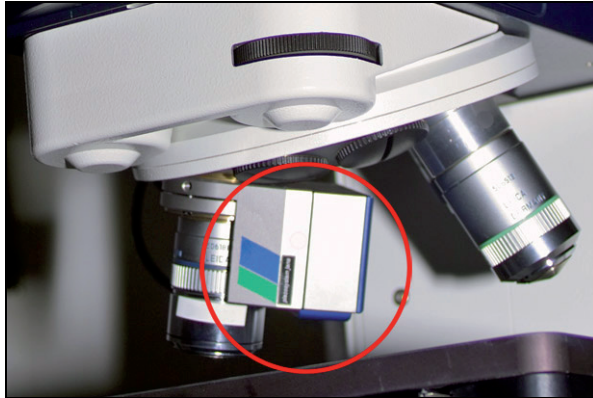


Figure 30: Piezo focus on objective nosepiece

If a piezo focus is installed on your TCS SP5, please also observe the following safety notes:



Before switching the system on or launching the LAS AF software, ensure that there is no slide or specimen on the stage and that the stage is in its lowest possible position.

The slide or objective may otherwise be damaged or destroyed by the initialization of the piezo focus when starting the system/software.

The objective can be moved by 150 μm in either direction. The total travel is 300 μm .



Figure 31: Piezo focus controller

Piezo focus controller display:

Upper position:	350 μm
Middle position:	200 μm
Lowest position:	50 μm

xz-Scanning range:	250 μm
--------------------	-------------------



Do not make any adjustments to the piezo focus controller, as it has already been optimally set up by Leica Service.

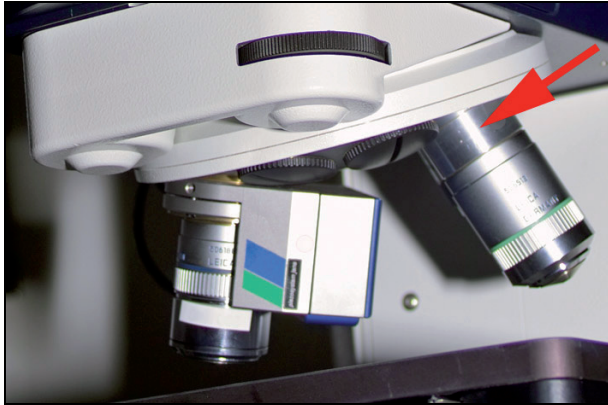


Figure 32: Spacer on objective



Please note that the focus position of an objective with piezo focus is 13 mm lower than those without piezo focus. A spacer (Figure 32) is installed on all other objectives to ensure the same focal plane.

6.10.1 Objective Change with Piezo Focus Configuration



Do not change objectives automatically! The automatic motion may damage the cable of the piezo focus.



Before changing the objective on the piezo focus, finish the scan process, move the objective to be changed out of the beam path, move the stage as far down as possible and remove the slide or specimen from the stage. The slide or objective may otherwise be damaged or destroyed by the initialization of the piezo focus when starting the system/software.



When replacing the objective on the piezo focus, you must perform a teach-in for the new objective in LAS. Please see the instructions on this topic in the microscope manual.

7. Starting Up the System

7.1 Switching On the System



With the motorized stage (156504145) for DMI 6000 (inverted):

Before the system start or start of the LAS AF, the illuminator arm of the inverted microscope must be swung back, because the motorized stage can be initialized and damage the condenser.

With the motorized stage (156504155) for DM 6000 (upright):

Before the system start or start of the LAS AF, the stage must be moved downwards, because during initialization, it can come into contact with the objective nosepiece and damage the objectives.

1. Switch on the workstation (PC switch) at the main switch board.

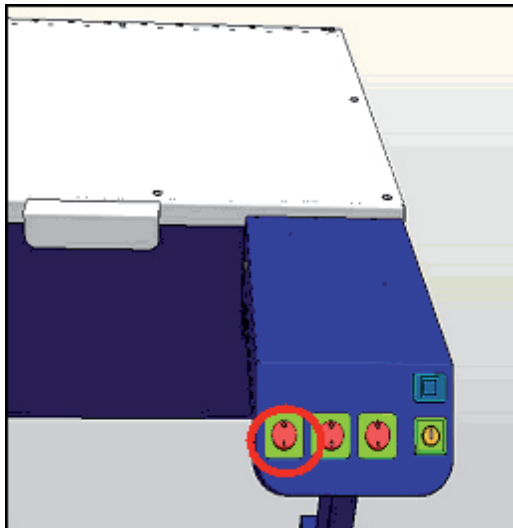


Figure 33: Switching on the workstation



You do not have to start the operating system—it starts automatically when you switch on the computer. Wait until the boot process is completed.

2. Log on to the computer. After you simultaneously press the **Ctrl**, **Alt**, and **Del** keys, the logon information dialog box appears.



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



After logging on with your own user ID, you may change your password by pressing the keys **Ctrl**, **Alt**, and **Del** at the same time. Then, click **Change password**. The **Change password** dialog box opens.

3. Check whether the microscope stand is switched on. If the readiness indicator (Figure 34, item 1) on the electronic box is lit, the microscope stand is operating. If the readiness indicator is not lit, activate the toggle switch (Figure 342) of the electronic box.



Figure 34: Switching on the microscope

4. Switch on the scanner on the main switch board.

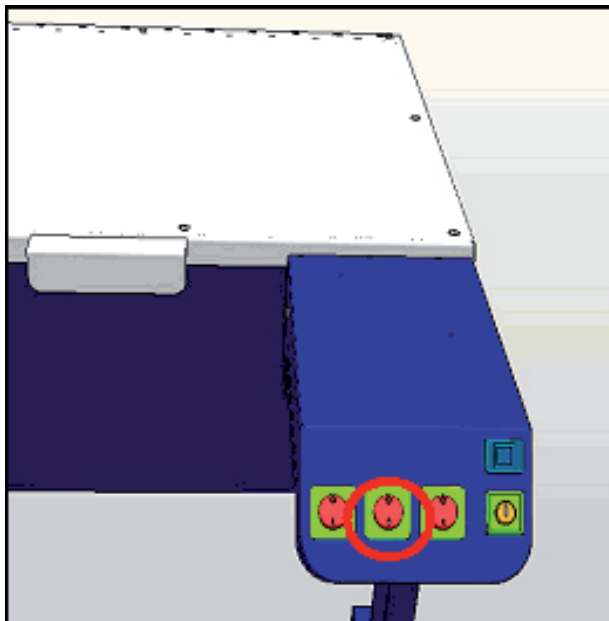


Figure 35: Turning on the scanner

5. Switch on the lasers on the main switch board.

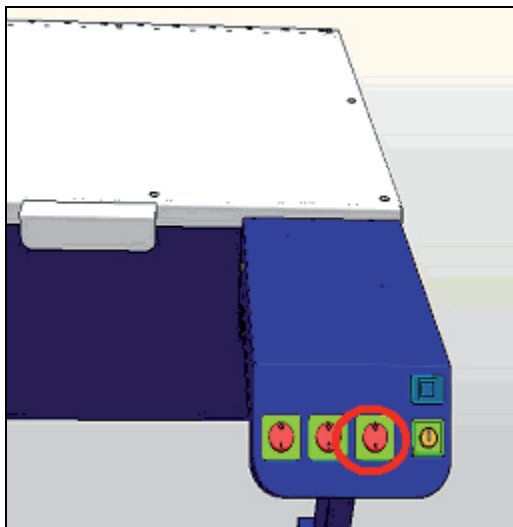


Figure 36: Switching on the lasers



The power supplies and fan of the system have been started.

- Engage the detachable-key switch on the main switch board.

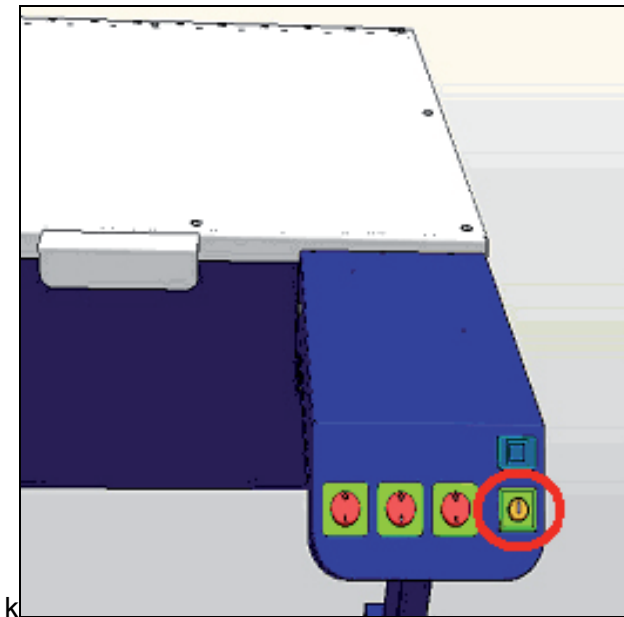


Figure 37: Activating the detachable-key switch



From this time on, laser radiation may be present in the specimen area of the laser scanning microscope. Follow the safety instructions provided in Chapter 6 Safety Instructions for Operating the System.

For switching off the system, refer to Chapter 8 Switching Off the System.

7.2 Starting the LAS AF



With the motorized stage (156504145) for DMI 6000 (inverted):

Before the system start or start of the LAS AF, the illuminator arm of the inverted microscope must be swung back, because the motorized stage can be initialized and damage the condenser.

With the motorized stage (156504155) for DM 6000 (upright):

Before the system start or start of the LAS AF, the stage must be moved downwards, because during initialization, it can come into contact with the objective nosepiece and damage the objectives.

1. Click the LAS AF icon on the desktop to start the software:

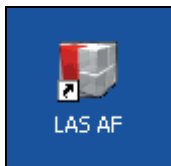


Figure 38: LAS AF icon on the desktop

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

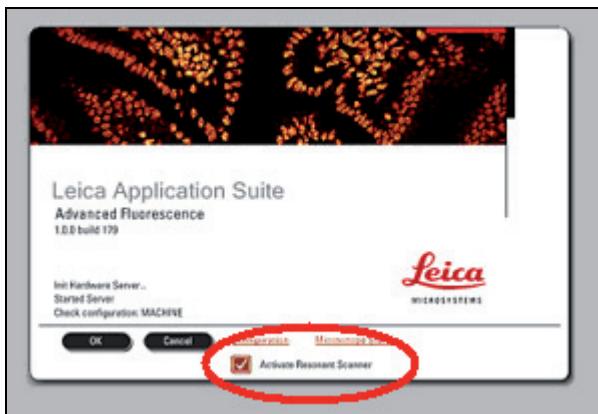


Figure 39: Resonant or non-resonant mode

3. Start the LAS AF by clicking the "OK" button.

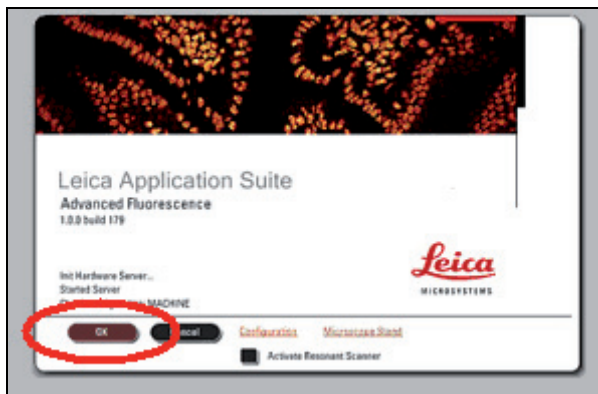


Figure 40: LAS AF start window

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



Figure 41: LAS AF main view

7.3 Setting Up Users



The default user name for the Leica TCS SP5 system is "TCS_User". No default password is set. It is recommended to set up a separate user ID for each user (set up by the system administrator). This will create individual directories that can be viewed by the respective user only. 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.

1. Log on as administrator. To do so, use the username (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:
 - User ID
 - Password (must be re-entered in the next line for confirmation purposes)
4. Select the following two check boxes:
 - **User must change password at next logon** (this allows the new user to define his or her own password at logon)
 - **Password never expires** (this allows a defined password to be valid until either 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 disk drives are provided with two partitions (C:\ and D:\). Set up the user directory on partition D:\.

8. Switching Off the System

1. Save your image data: On the menu bar, select **File** → **Save as** to save the data record.
2. Close the LAS AF: On the menu bar, select **File** → **Exit**. Exit the LAS AF.
3. On the main switch board, switch off the lasers using the detachable-key switch (Figure 43, 2). The emission warning indicator (Figure 43, item 1) goes out.
4. Shut down the computer. On the toolbar, select **Start** → **Shutdown** to shut down the TCS workstation.

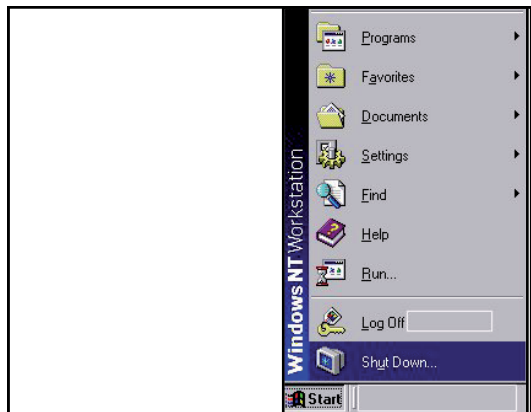


Figure 42: Shutting down the computer

5. Next, turn off the switches on the main switch board for the TCS workstation (Figure 43, 5) and the TCS SP5 scanner (Figure 43, item 4).
6. The external fan of the argon laser will switch off automatically after several minutes. Also set the switch for the lasers (Figure 43, item 3) to "O" at this point.

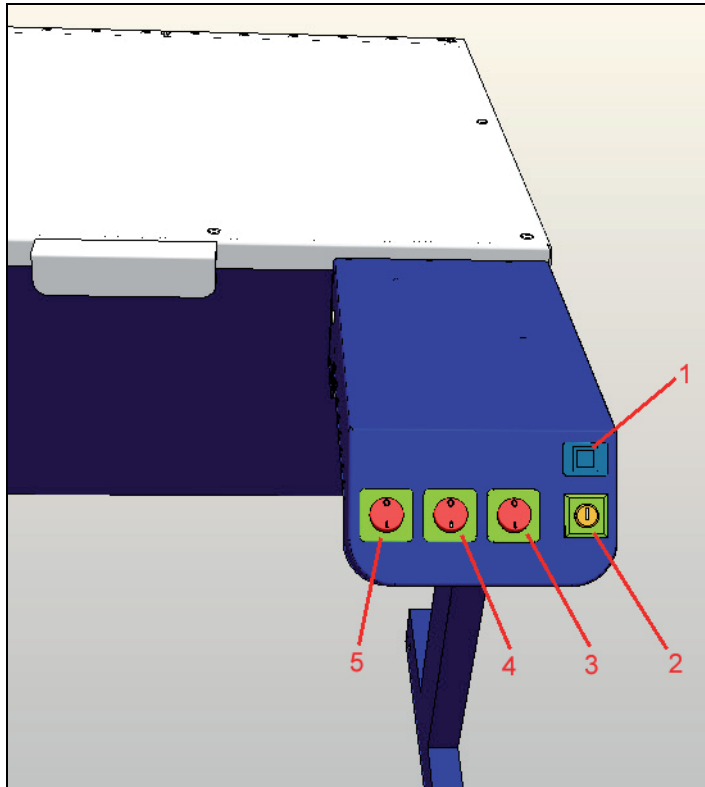


Figure 43: Main switch board: 1 = emission warning indicator, 2 = detachable-key switch, 3 = switch for laser, 4 = switch for scanner, 5 = switch for workstation



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

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 the respective manual from the manufacturer.

9. Introduction to LAS AF

9.1 General

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

The "experiment 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.

9.2 Online Help

9.2.1 Structure of the Online Help

The online help is divided into 6 different books:

Books	Contents
General	This book contains a guide for using the online help and a legal notice.
Structure of the graphical user interface	This book describes the structure of the LAS AF user interface and features topics about the various menus, tabs, operating steps and symbols.
Dialog descriptions	This book describes each individual dialog window of the graphical user interface.
Learning the basics	This book describes the fundamental steps for an experiment.
Instructions	This book contains step-by-step instructions for 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, filters, etc.

9.2.2 Accessing the Online Help

The online help can be accessed in three ways:

In the respective context (**context-sensitive**)

Via the **Help** menu

With the key combination **CTRL + F1**

In the respective context (**context-sensitive**)

Click the small question mark located in the top right corner of every dialog window. Online help opens directly to the description for the corresponding function.

Via the **Help** menu

Click the Help menu on the menu bar. The menu drops down and reveals search-related options, including the following:

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 in the table of contents to display the corresponding information.
Index	Enter the term to be searched for. The online help displays the keyword that is the closest match to the specified term. 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 the LIST TOPICS button. A hierarchically structured list of topics is displayed.
About	Opens the User Configuration dialog box, where you can, for example, select the language in which the online help is shown.

9.2.3 Full-text Search with Logically Connected Search Terms

Click 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. After the operator, enter the second search term you would like to associate with the first search term:

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", 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", but not containing the word "sections".

9.3 Structure of the Graphical User Interface

9.3.1 General Structure of the Graphical User Interface

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

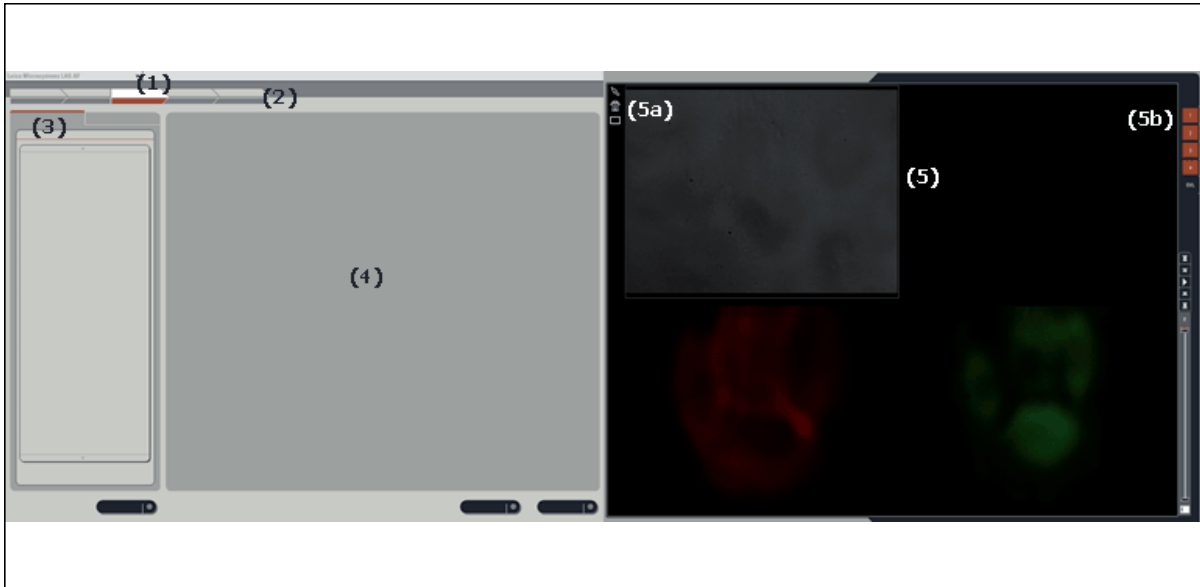


Figure 44: LAS AF user interface

- 1 **Menu bar:** The various menus for calling up functions are available here.
- 2 **Arrow symbols:** Operating steps with the individual functions. These operating steps mirror the typical sequence of scan acquisition and subsequent image processing. The functions are grouped correspondingly into these operating steps.
 - Configuration
 - Acquire
 - Process
 - Quantify
 - Application

- 3 **Tab area:** Each operating step (arrow symbol) has various tabs in which the settings for the experiment can be configured.

Acquire	Experiments: Directory tree of opened files
	Setup: Hardware settings for the current experiment
	Acquisition: Parameter settings for the scan acquisition
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 plotted regions of interest (ROI)

- 4 **Working area:** This area provides the "Beam Path Settings" dialog window in which the control elements for setting the scanning parameters are located.
- 5 **Viewer display window:** Displays the scanned images. In the standard setting, the Viewer display window consists of the image window in the center and the buttons for image editing (5a) and channel display (5b).

9.4 Key Combinations

To speed up recurring software functions, special key combinations have been defined:

CTRL + N	Opens a new experiment
CTRL + O	Starts the "Open dialog window" for opening an existing file.

10. Introduction to Confocal Work

10.1 Preparation

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

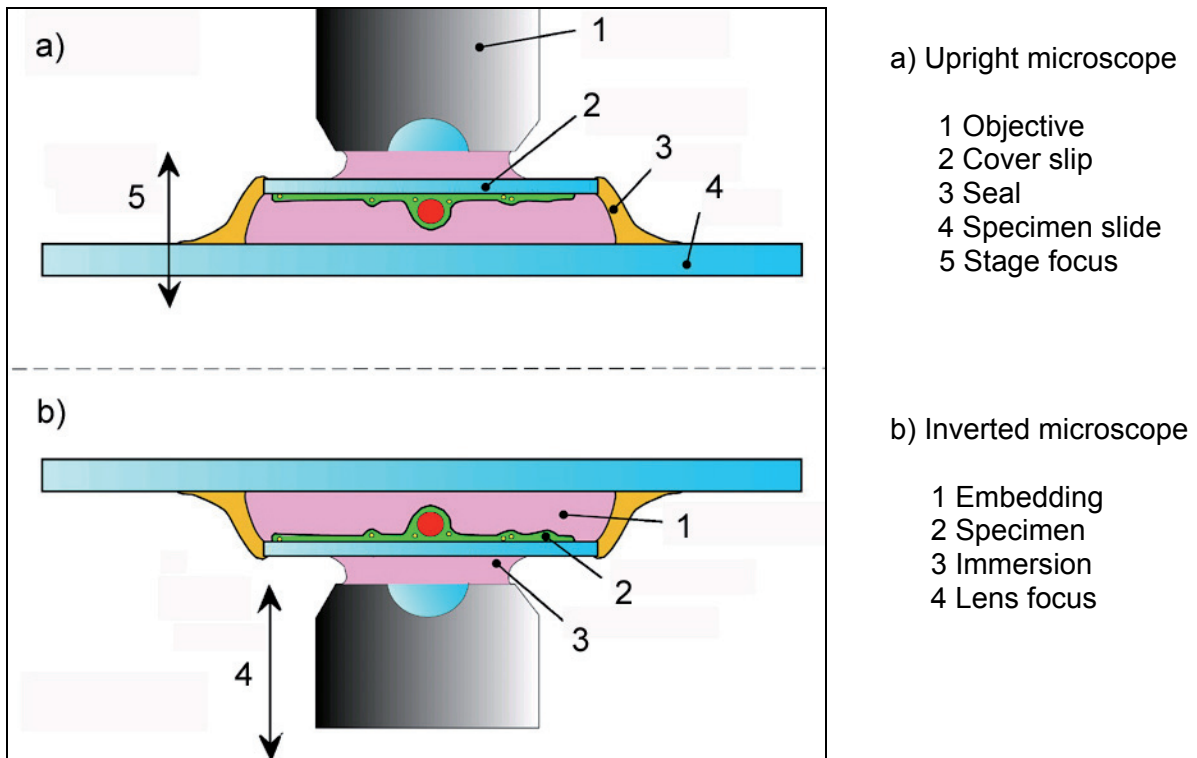


Figure 45: Arrangement of cover slip and specimen on an upright microscope (a) and inverted microscope (b). When using objectives with cover slip correction, ensure that the cover slip (i.e. the top side of embedded specimens) is facing down.

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, of course, is to place a specimen in the microscope. When placing specimens in an inverted microscope, ensure that fixed specimens on slides are inserted with the cover slip facing down (Figure 45). Failing to do so is a frequent reason for not being able to find the specimen or focus on it in the beginning.

10.1.1 The Objective

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

Medium		Refractive Index
Water	Imm	1.333
PBS	Emb	1.335
Glycerol 80 % (H ₂ O)	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 Table of various immersion media

When using immersion objectives, ensure that an adequate quantity of immersion medium is applied between the front lens of the objective and the specimen. Immersion oil, glycerol 80% or 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.

10.1.2 Conventional Microscopy

To view the specimen conventionally through the eyepieces, ensure that "VIS" operating mode is selected. "SCAN" is for use with the laser scanning operation image process. Select a suitable position and focus on the specimen.

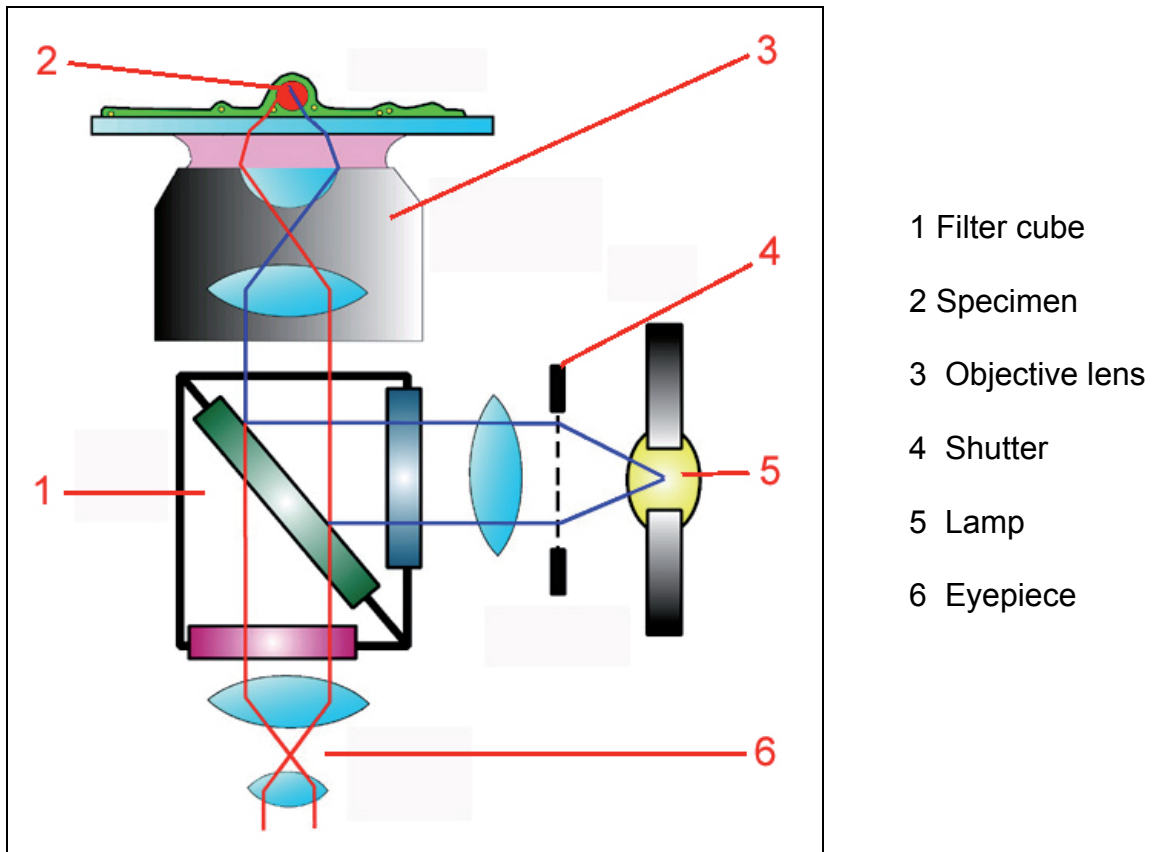


Figure 46: Incident light fluorescence scheme: light from a mercury lamp is collimated, selected spectrally via an excitation filter and applied to the specimen via a color splitter mirror. A shutter permits the specimen to be darkened. The emission (longer wavelength than the excitation) is visible through the color splitter mirror and emission filter via the eyepiece. The excitation filter, color splitter mirror and emission filter are grouped in a filter cube.

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

The filter cubes (Figure 46) that correspond to the fluorescence must be positioned within the beam path 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 keys 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	BP 340-380	400	LP 425
B/G/R	BP 420/30	415	BP 465/20
B/R	420/20;530/45	435;565	465/30;615/70
BFP/GFP	BP 385/15	420	BP 460/20
CFP	BP 436/20	455	BP 480/40
D	BP 335-425	455	LP 470
E4	BP 436/7	455	LP 470
FI/RH	BP 490/15	500	BP 525/20
G/R	BP 490/20	505	BP 525/20
GFP	BP 470/40	500	BP 525/50
H3	BP 420-490	510	LP 515
I3	BP 450-490	510	LP 515
K3	BP 470-490	510	LP 515
L5	BP 480/40	505	BP 527/30
M2	BP 546/14	580	LP 590
N2.1	BP 515-560	580	LP 590
N3	BP 546/12	565	BP 600/40
Y3	BP 545/30	565	BP 610/75
Y5	BP 620/60	660	BP 700/75
YFP	BP 500/20	515	BP 535/30

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

10.1.3 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. This 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 $d_B = 1.22 \cdot \lambda / NA$, with λ representing the excitation wavelength and NA the numerical aperture of the objective used (Figure 48).

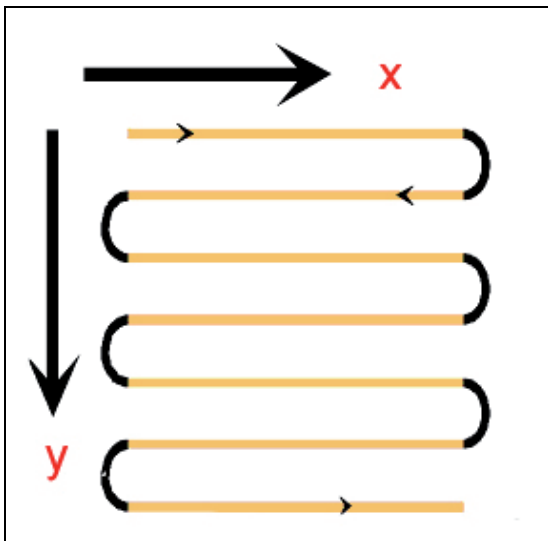


Figure 47: Illustration of the raster scan. Two mirrors move the illumination spot in x and y directions across the specimen in rows so that the entire image can be reconstructed in parallel.

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 (Figure 47). 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 rotates the axis; the rotation ceases at 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.

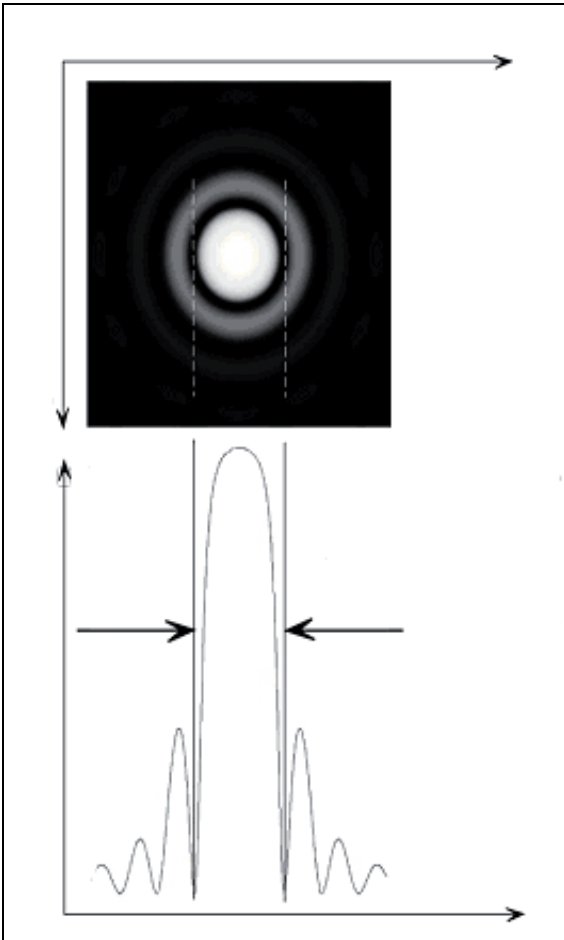


Figure 48: Smallest possible, diffraction-limited illumination spot (Airy disk). Below: an intensity profile.

To scan a line, the x mirror must travel once 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 in synchronization and can be displayed on the monitor.

10.1.4 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 10.1.3, the illumination of the specimen is focused on the smallest possible spot—hence the term "focal." 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, known as the pinhole. This pinhole cuts off all information not coming from the focal plane (Figure 49).

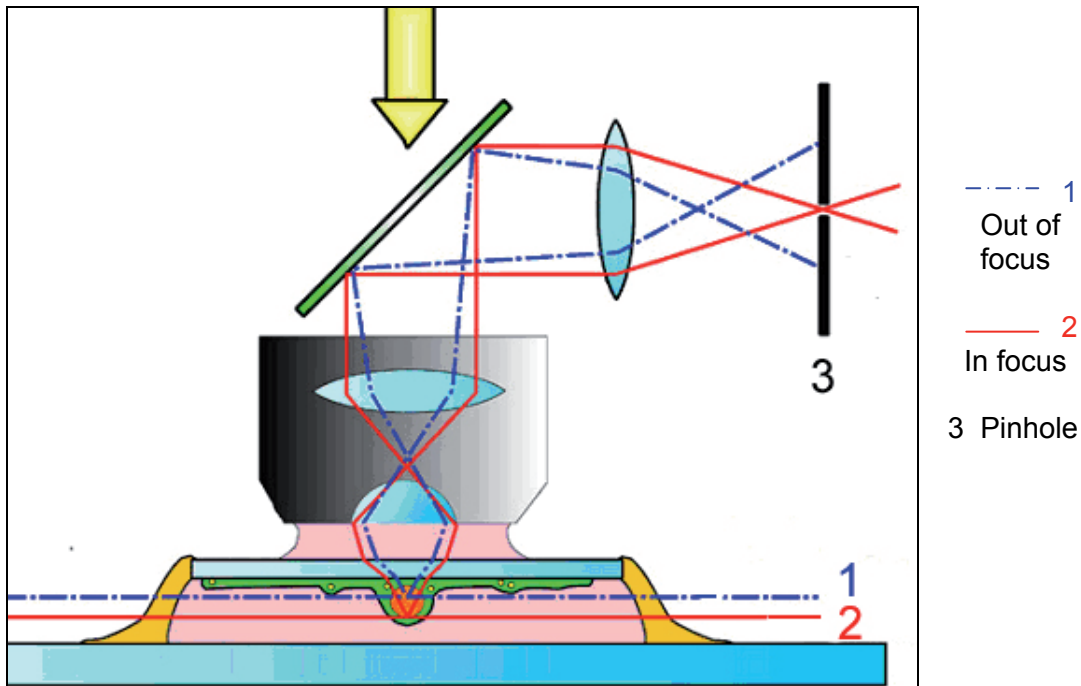


Figure 49: Creating an optical section using an incident-light process. Light not originating from the focal plane is cut off by a spatial filter (here, a pinhole). Only information from the focal plane can reach the detector.

The diaphragm thus acts as a spatial filter, but 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 dialogue 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.

10.2 Acquiring Optical Sections

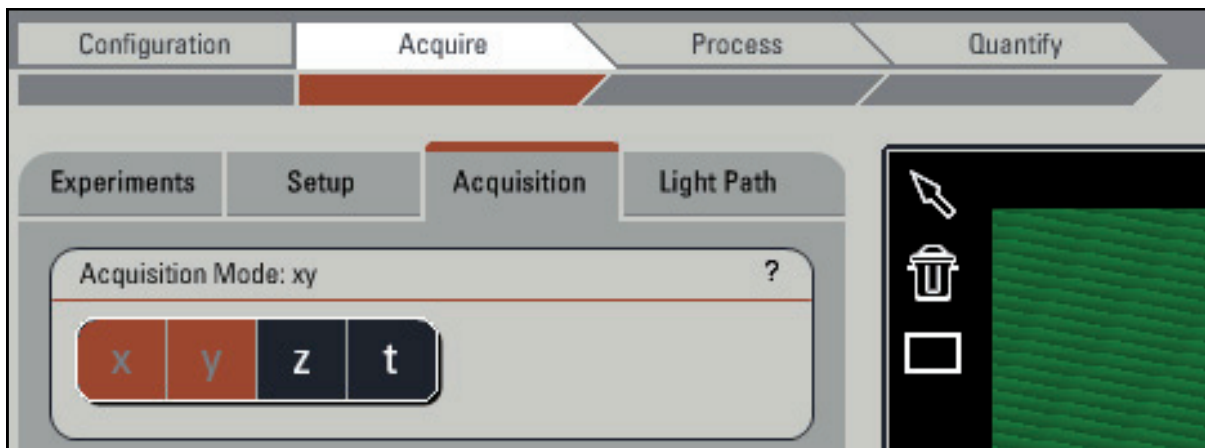
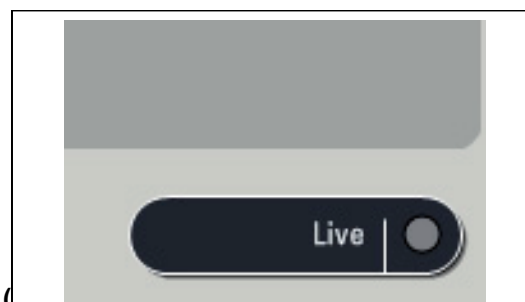


Figure 50: Use the "Acquire" arrow key to acquire data in all Leica LAS AF applications.

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 keys at the top. The functions required for data acquisition (the sole focus of these chapters) are grouped under "Acquire" (Figure 50). For descriptions of the individual functions, please see the online help.

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

10.2.1 Data Acquisition



Press the "Live" button to begin data acquisition (

Figure 51). 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.

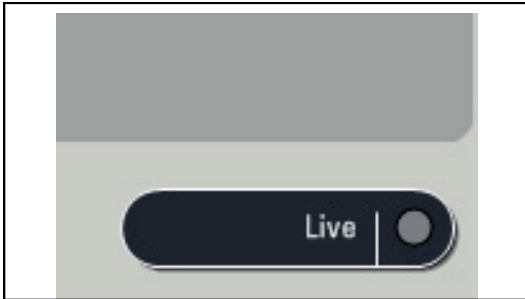


Figure 51: The "Live" button starts data acquisition in all Leica LAS AF applications.

This is a preview mode suitable for setting up the instrument. Stopping data acquisition will also immediately stop the scanning operation, 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 scanning has the advantage of exposing the specimen only 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 scanning the individual images. Time series, lambda series and z-stacks can be created in this manner (Figure 52).

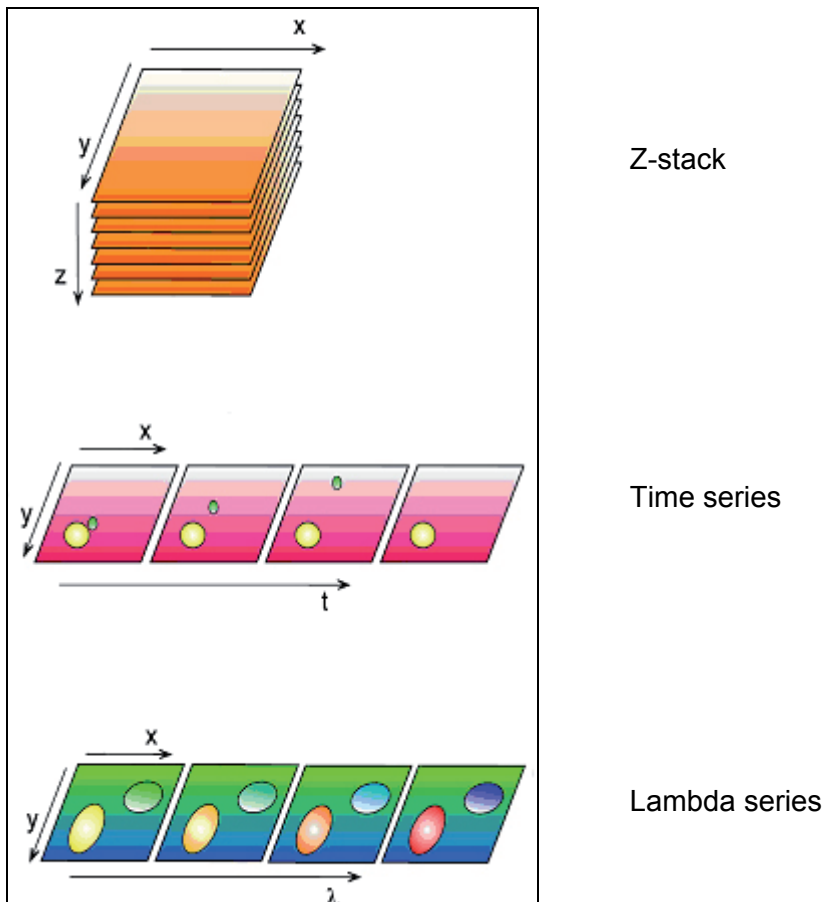


Figure 52: Stack acquisition for 3D, time and lambda series

When using the instrument in "LiveDataMode", all captured images are automatically stored with the time of capture. A preview mode is not available in that case (Figure 53).

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.

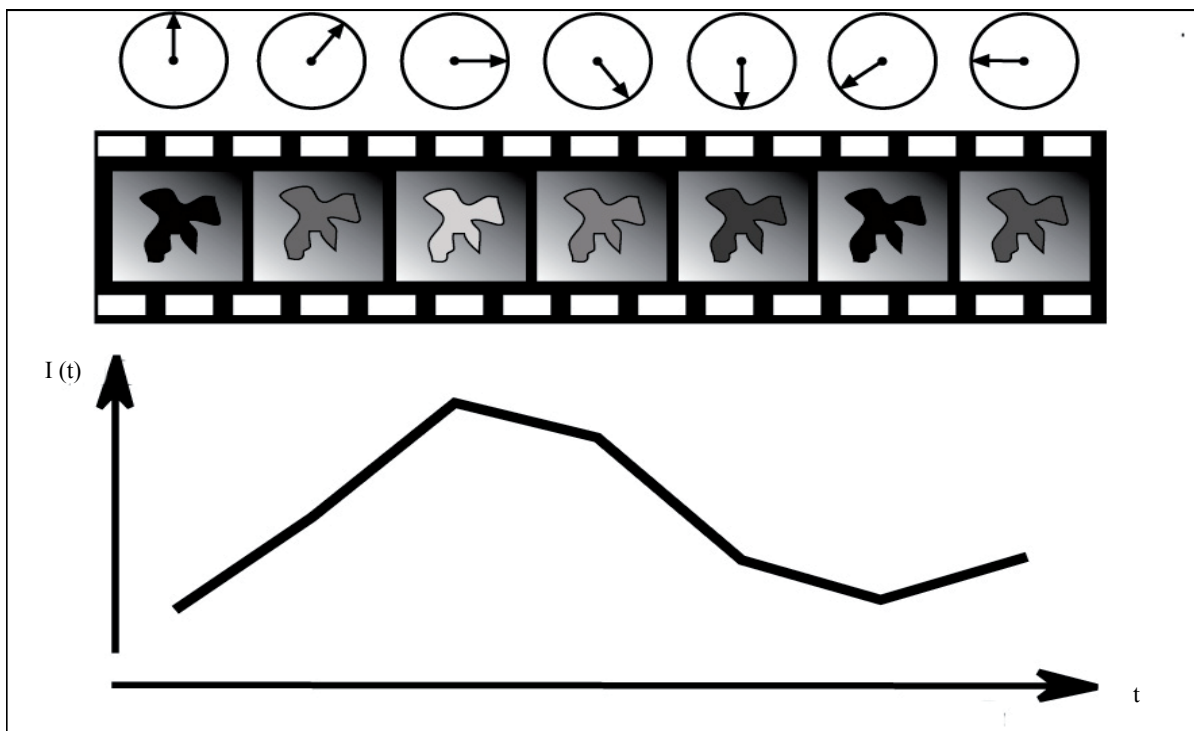


Figure 53: LiveDataMode supports the continuous acquisition of data while changing setting parameters, manipulating the specimen or performing bleaching sequences between the individual scans. The clock continues running throughout the experiment and intensity changes in regions of interest can be rendered graphically online.

The setting parameters for scanning 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 standard specimen is a *Convallaria majalis* rhizome section with a histological fluorescent dye. The specimen can be used for a wide range of fundamental problems and has the advantage that it practically does not bleach.

10.2.2 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. Using this slider, the intensity is adjusted continuously via an acousto-optical 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 fluorescent dye, the line used, the density of the dye in the specimen, the location and width of the selected emission band, the scanning speed and the diameter of the emission pinhole.

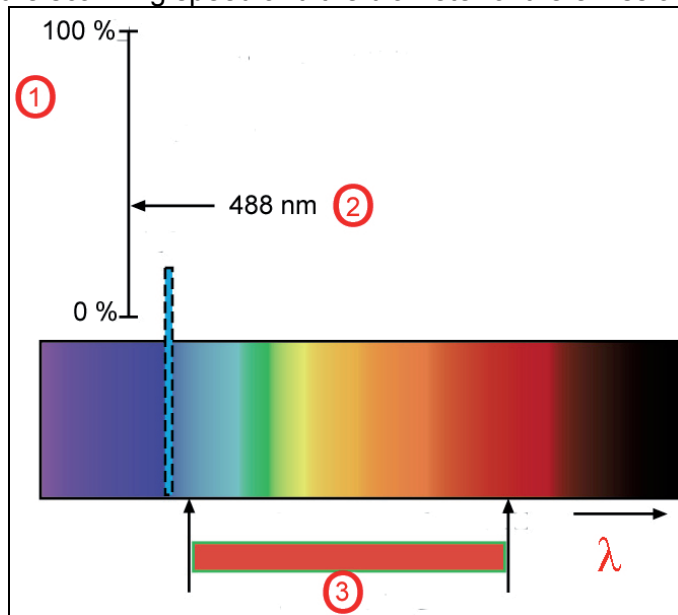


Figure 54: Selecting the illumination intensity (1) via acousto-optical tunable filters (AOTF, 2) and selecting the emission band in the SP detector (3).

If you select the "FITC" parameter set, the 488 nm argon line and a suitable band between 490 nm and 550 nm is set.

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. If the laser scanning microscope is used as prescribed and the safety instructions are observed, there are no dangers to the user's eyes. Always keep your eyes at a safe distance of at least 20 cm from the opening of the objective. Read the safety instructions in this user manual carefully.

If all of the other settings are in order, darker and lighter images will be visible on the monitor when moving the slider for the illumination.

10.2.3 Beam Splitting

The simplest case would involve the selection of a laser line roughly at the maximum of the excitation spectrum of a given fluorescent dye. 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 dye, of course). One can thus also excite the fluorescence on the blue side of the excitation maximum, which has the advantage of providing a wider band for the collection of the emission (Figure 55).

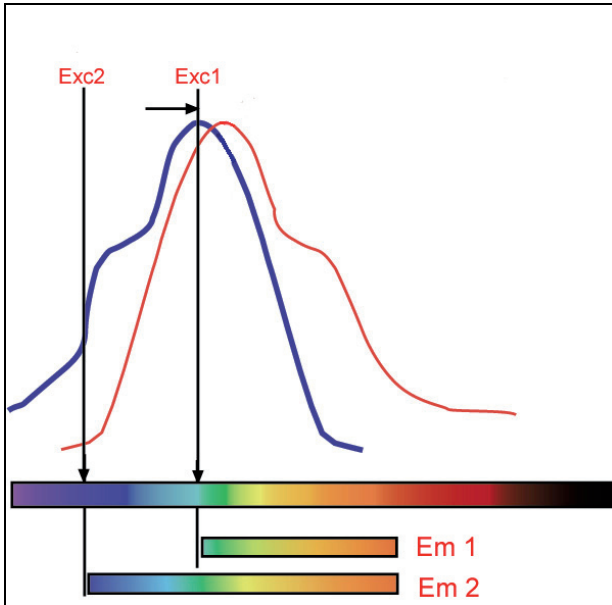


Figure 55: Excitation spectrum of a fluorescent dye (blue) and emission spectrum (red). An excitation in the maximum (Exc1) would result in only a narrow band to be collected on the emission side (Em1). A significantly wider emission band (Em2) is available from an excitation in the blue range, at which point the intensity of the laser can be increased without detrimental effects.

A little experimentation 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.

10.2.4 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 of the fluorescent dyes 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 dyes have a Stokes shift between 10 nm and 30 nm. However, it is sometimes even more than 100 nm, for example with natural chlorophyll, an excellent dye for curious experimenters.

The emission characteristics of dyes 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 using the system.

An adjustable bar below the spectral band has been assigned to each confocal detector. The limits at the left and right of these bars indicate the limits for the selected emission band.

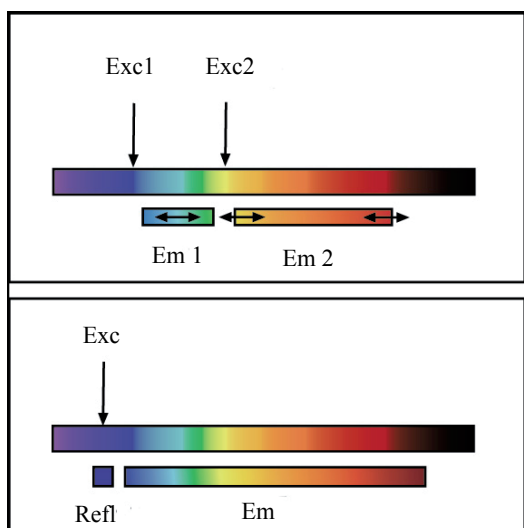


Figure 56: SP detector setting options for two fluorescences with different excitations (above) or for fluorescence and reflection with one excitation (below).

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 possible during live acquisition of images. The effects of settings on the images are immediately apparent and suitable values can thus be selected empirically (Figure 56).

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 narrowest 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.

10.2.5 The Pinhole and Its Effects

The reason for deploying a confocal microscope 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 10.1.4. Ideally, the diameter of this pinhole would be infinitely small, but this would no longer allow light to pass, making it impossible to create an image. However, the effect would be lost if the pinhole were too wide, as the image would contain excessive blurred portions 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 (Figure 57). 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.

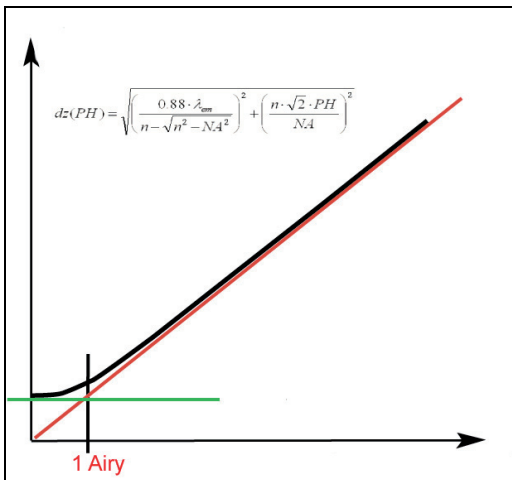


Figure 57: Relation of optical section thickness (y-axis) to pinhole diameter (x-axis).

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) (Figure 58).

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.

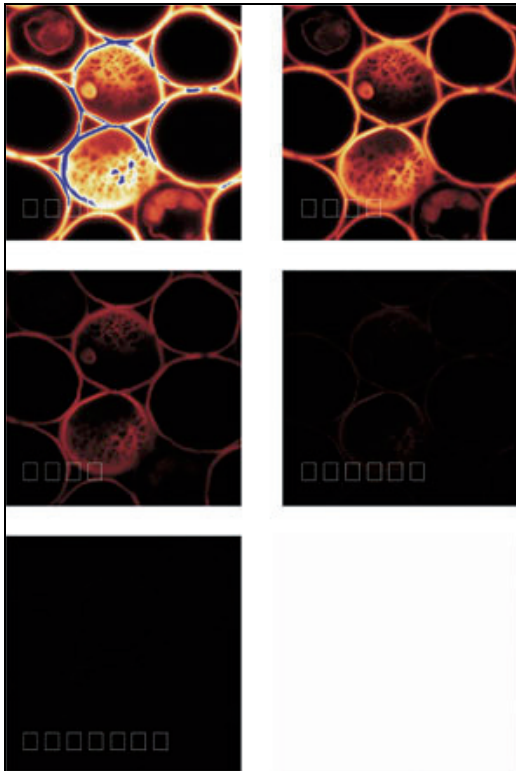


Figure 58: Optical sections with a variety of pinhole diameters (63x/1.4 objective). Pinhole diameters from top left to bottom right: 4 AE; 2 AE; 1 AE; 0.5 AE; 0.25 AE. The strong loss of light can be seen clearly with the small diameters, as can the pronounced background in the images with very large diameters.

A pinhole diameter of 1 Airy is therefore the default setting. Switching objectives also automatically 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. Of course, smaller pinhole diameters may also be selected 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.

10.2.6 Image Detail and Raster Settings

Depending on the objective used, conventional microscopes show a circular cutout 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, by reversing the operation, 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 or rectangle is exactly circumscribed by the field of vision, then 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, the magnification can be increased up to around 40x simply by moving a slider. As always in microscopy, the total magnification must be appropriate, i.e. within a suitable range, in order to obtain good images. Other scales are important for overviews and bleaching experiments.

As errors can easily be made when interpreting scanned 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.

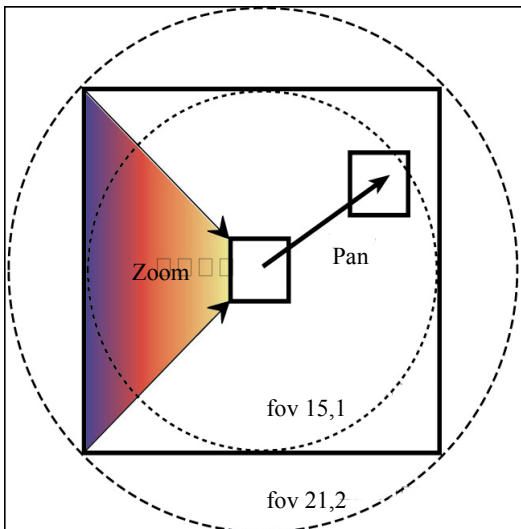


Figure 59: Fields for the conventional scanner (formerly: 21.2 mm, now: 22 mm) and the resonant scanner (15.1 mm). A smaller scanning angle increases the magnification (zoom), while a scan offset shifts the image detail (pan) within the field of view.

The edge length of the displayed field of a conventional scanner corresponds to 15 mm without magnification by the objective (1x scale). Field numbers of 21.2 and 22, respectively, are thus also fully utilized (Figure 59).

That is suitable for most good research microscopes. How many elements 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 $d = \lambda / 2 \cdot NA$. A line can thus contain a maximum of $15 \text{ mm} / d$ resolution elements (also known as "resels"). When using an actual objective such as a plane apochromat 10x/0.4, the edge length corresponds to 1.5 mm ($15 \text{ mm} / 10$) and $d = 0.625 \mu\text{m}$ when using blue-green light with a wavelength of 500 nm. Such an image would thus contain $1500 / 0.625 = 2400$ optical resolution elements along each edge (x and y direction).

Rendering this resolution in a digital pixel image would require working with twice the resolution to prevent losses (Nyquist theorem). That would be an image with 4800 x 4800 pixels. Some purists require 3x oversampling, i.e. 7200 x 7200 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 image capture sizes of up to 64 megapixels (8000 x 8000 pixels) (Table 5).

Magnification		63	40	10
Numerical Aperture		1.4	1.25	0.4
Optical Resolution (400 nm)	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 resolution elements at 400 nm 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 truly capture 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 512 x 512 format, this means limiting the image to a field 10 - 15 times smaller to avoid 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 a cutout in the center of the scan field. That is not always 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".

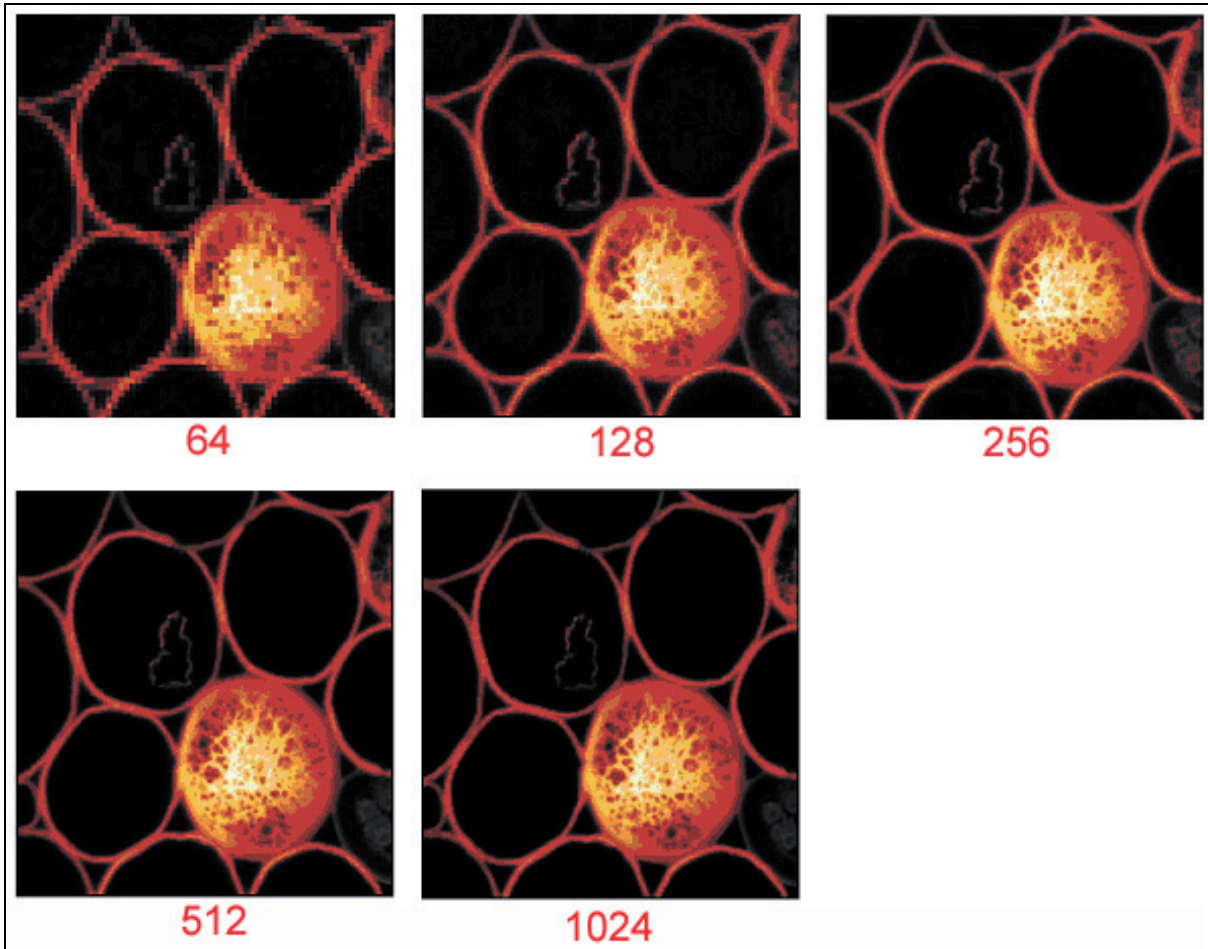


Figure 60: The same image detail in a variety of pixel resolutions. Please note that the printing medium may not be capable of reproducing the full detail of high resolutions. You may therefore have difficulty detecting the differences between the top two images, despite the enormous differences in the 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 pan and zoom 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 elements in x, y and z can be found under "Voxel Size". At Zoom 1, the images calculated above would have a grid spacing between 200 nm and 300 nm. Larger gaps would lead to a loss of resolution when using an objective with an aperture of 0.4 (Figure 60).

Rectangular formats are important for higher image scanning 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 (Figure 61).

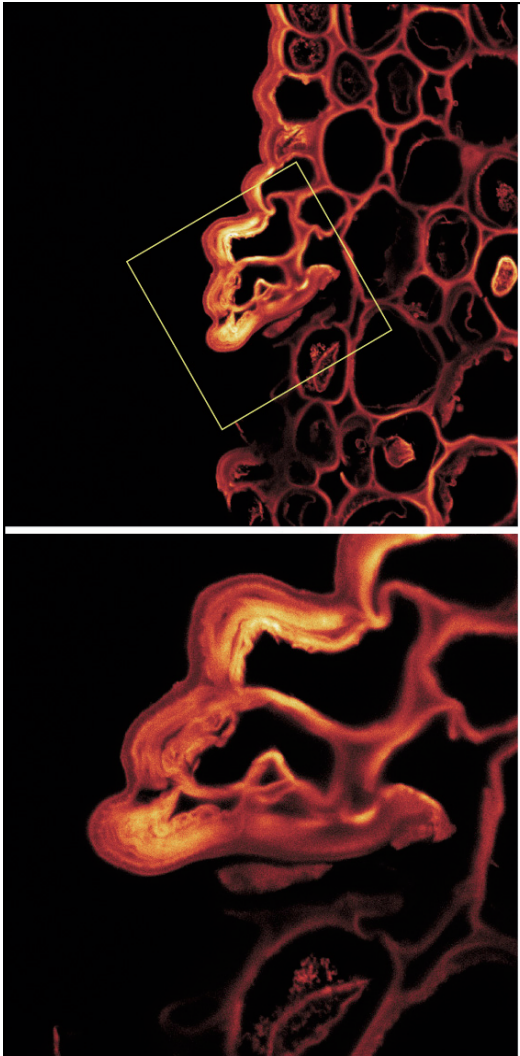


Figure 61: Zoom, Pan and Rotation combined in one example.

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 shape, color and size as required. Scales were not added to the images in this document for the sake of clarity.

10.2.7 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 scan. 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 grayscale value of 255, even though differences (information) were originally present in the signal. This image information is then lost (Figure 62).

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".

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—with the light switched off. 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).

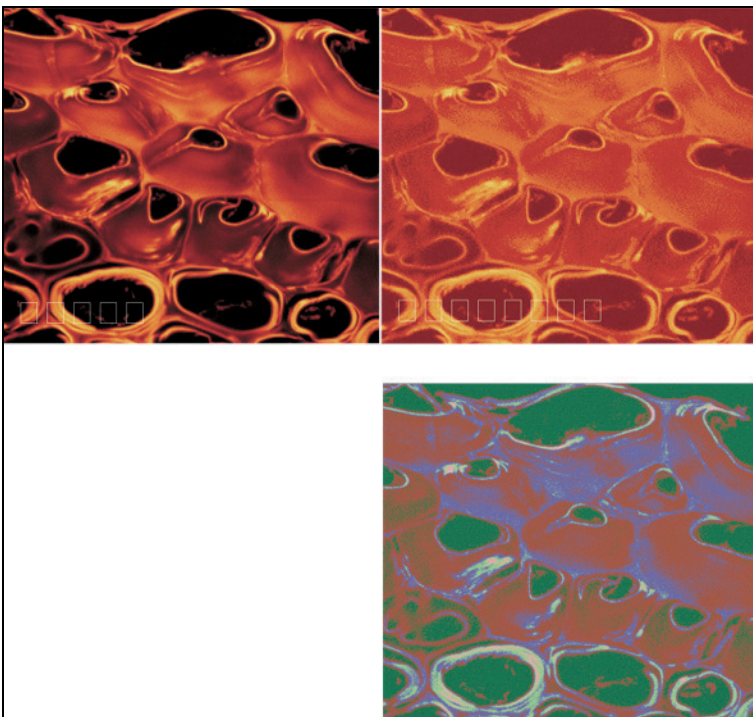


Figure 62: At the top left you see an 8-bit image (256 gray scales). At the right, the same specimen with a considerably smaller dynamic range. Around 6 gray scales can be made out in the false-color image at the bottom. That corresponds to less than 3 bits.

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 nonspecific or self fluorescence in the specimen at the time of the scan. 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.

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 voltage at the PMT until no more blue pixels are visible. We recommend focusing to ensure that the brightest signals in the field of view are really 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. However, this compromise depends on the specimen and the application.

The signal-to-noise ratio may be influenced by a number of other factors in addition to illumination intensity: the speed at which data are 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 method offer additional options for enhancing the signal. The change of the scan speed itself leads to averaging in the pixels, as data are 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 scanned and then averaged with the subsequent image scanned 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 call 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. Thus there is no general rule of thumb for all application situations. Choosing the best method is a matter of experimentation and experience.

10.2.8 Profile Cuts

So far, we have always assumed that images are scanned parallel to the focal plane. That is both correct and appropriate for conventional microscopy. However, a confocal point scanning system offers interesting new options for capturing data. For example, profile cuts through the specimen can be made 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). This is similar to slicing through a cake, permitting impressions to be gained online about the contents of the specimen. Camera-based systems (including "confocal" systems) can only compute such profiles out of complete stacks (Figure 63).

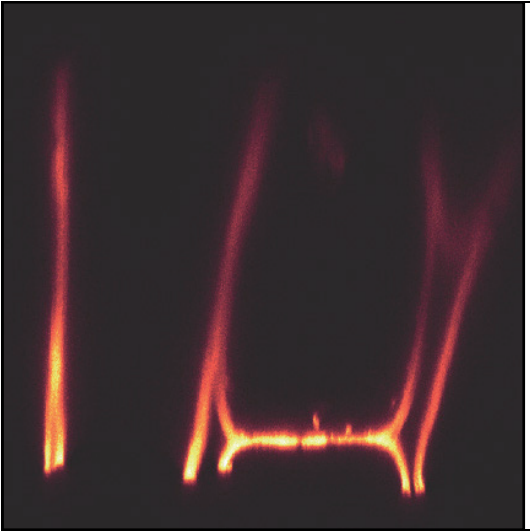


Figure 63: Profile cut through the *Convallaria majalis* specimen, indicating a thickness of approx. 30 μm

10.3 Multiparameter Fluorescence

In many cases today, specimens are used that contain more than one fluorescent dye. Multiple dyes 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 dyes and autofluorescence are also usable parameters (Figure 64).

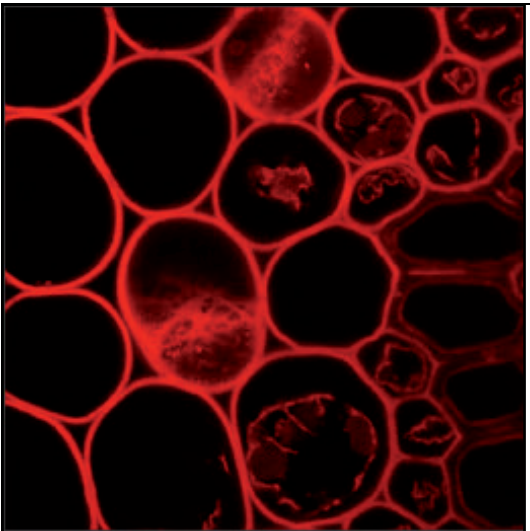


Figure 64: Simultaneous scan of two fluorescences, in this case excited by a single laser line. The depiction in the colors green and red is arbitrary.

10.3.1 Illumination

Specimens with multiple dyes generally require illumination with multiple colors (in this case: laser lines) simultaneously. That is not always the case, however: there are naturally also dyes with differing emissions that can be excited by the same wavelengths. A distinctive example would be a botanical specimen with a FITC dye 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.

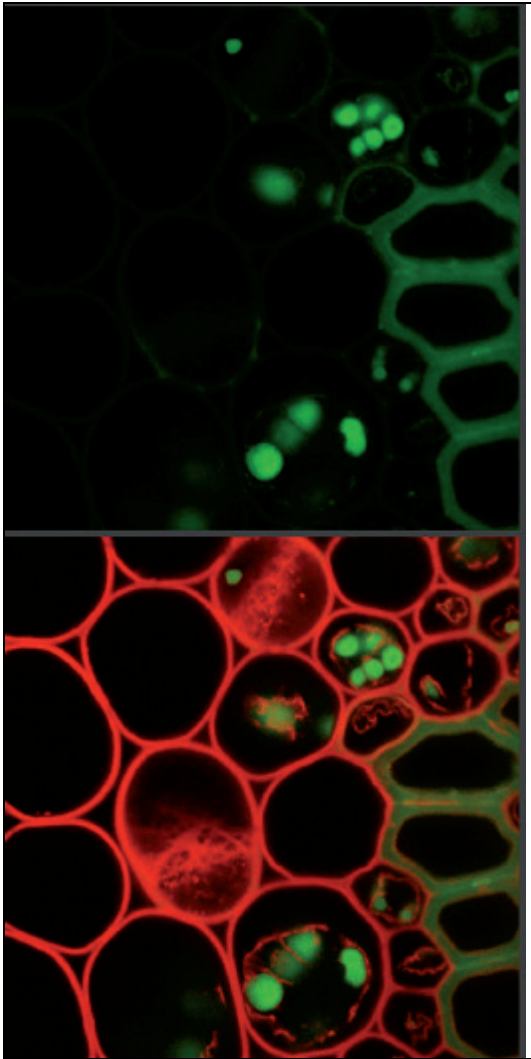


Figure 65: Simultaneous scan of two fluorescences, in this case excited by a single laser line. The depiction in the colors green and red is arbitrary.

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

Under normal circumstances, however, dyes will be used that require different excitation wavelengths. 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 10.2.2 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 dyes, 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 dye combinations.

10.3.2 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 2 nm. 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 single, 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 594 nm and 633 nm HeNe lines. In these cases, an AOBS is a significant advantage: thanks to the very small bands (approx. 1 to 2 nm), both lines can be used for excitation, while capturing an emission band of 35 nm in between with the SP detector.

10.3.3 Emission Bands

Naturally, the same boundary conditions apply for the emission bands as described in 10.2.4—with the difference that two laser lines limit the band for all dyes 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 10.2.

10.3.4 Crosstalk

The emission spectra of dyes (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 on both sides, but with very low amplitude. The red side, in particular, can be a problem. Crosstalk or bleed-through refers to the fact that the emission of a dye not only contributes to the signal in one channel, but in other detection channels as well. This should, of course, 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 can 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 dye, 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 dyes 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 room to adjust the emission bands. This parameter can also be used for balancing: if a dye is very dominant, the selection of a different excitation line can reduce the intensity of the dye (and thus improve the separation against the other dye) 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 reducing crosstalk is selecting suitable emission bands. The emission characteristics of the dyes 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, at least in the graphic on the monitor. Naturally, the stored characteristics are not necessarily identical to the actual emissions, as many factors (e.g. pH value, polarity, metabolic products) can affect the spectrum. However, in this case it is also possible to change and optimize the settings during data acquisition.

10.3.5 Sequential Scanning

Another way to reduce crosstalk is to scan the information for the various dyes sequentially instead of simultaneously. This has two advantages: Whenever different laser lines are used for excitation (and this is generally the case), sequential scanning provides significantly improved separation, as only one dye is excited at a time and the emissions are thus solely from that dye, regardless of the spectral range in which the signals are captured. This is, of course, the ideal state—in practice, other dyes may also be excited slightly; nevertheless, the separation is clearly better than that achieved using simultaneous scanning. Generally, crosstalk can be almost completely eliminated this way.

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

An obvious disadvantage is that the scan takes twice as long with two dyes; however, the advantages listed above compensate for this.

10.3.6 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 10.3.4 and 10.2.5) 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 dye's emissions distributed across the various scanning channels. This process is applied to each of the dyes. The result is a distribution matrix that can be used to redistribute the signal strengths so that they correspond to the dyes. This is described for two dyes in the following figures, but it is equally valid for any number of dyes. The precondition is that the number of channels used is at least the same as the number of dyes. 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. Since 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 scanned 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 dyes in the various channels is determined directly using individual dye reference data. When using this method, it is important to ensure that the parameter settings of the instrument are not altered, as the laser intensity and gain at the PMT naturally affect these coefficients.

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

10.4 3D Series

Altering the position of the focus between two scans permits a whole series of optical sections to be captured that represent the structure in 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 dyes—their local connections.

10.4.1 Z-stack

To capture such a 3D series ("z-stack"), set the upper and lower limits simply by moving to the top of the specimen, marking the location, and then moving to the bottom and marking it. Next, determine the number of sections to be scanned between the two positions; the rest will be handled automatically by the instrument.

10.4.2 Section Thicknesses

As described in sections 10.1.4 and 10.2.5, the thickness of the optical section depends on the wavelength, the numerical aperture of the objective, and, of course, 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. Choosing the pinhole diameter 1 Airy will result in section thicknesses between 0.5 μ m and 2.5 μ m for apertures from 0.7 to 1.4. These are typical values in practice. In product documentation—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.

10.4.3 Distances

The thickness of the optical sections is important when capturing z-stacks. If the spacing between the scans 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 the other 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 scan result with neither gaps nor superfluous oversampling, set the spacing between the scans to around one-half to one-third of the optical section thickness. Practically speaking, this is between 0.7 - 0.2 μ m. Therefore, between 1 and 5 sections are scanned per micrometer in z, largely depending on the aperture of the objective used.

10.4.4 Data Volumes

Another factor that must be considered when scanning 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 512 x 512 pixels and one channel, with a standard 8-bit grayscale, is 0.25 MB. One hundred of these images (i.e. a specimen about 20 μ m thick at high resolution) take up some 25 MB—which, just a couple of years ago, was an unwieldy amount of data. If images in 5 channels are captured simultaneously and the image size is 1000 x 1000 pixels, this stack will already be 500 MB—almost enough to fill a common CD. At 16-bit grayscale and 8000 x 8000 pixels, this results in 64 GB of data, too much for even most of today's computers to digest. A critical assessment of the data capture parameters to be used is definitely called for here.

10.4.5 Depictions

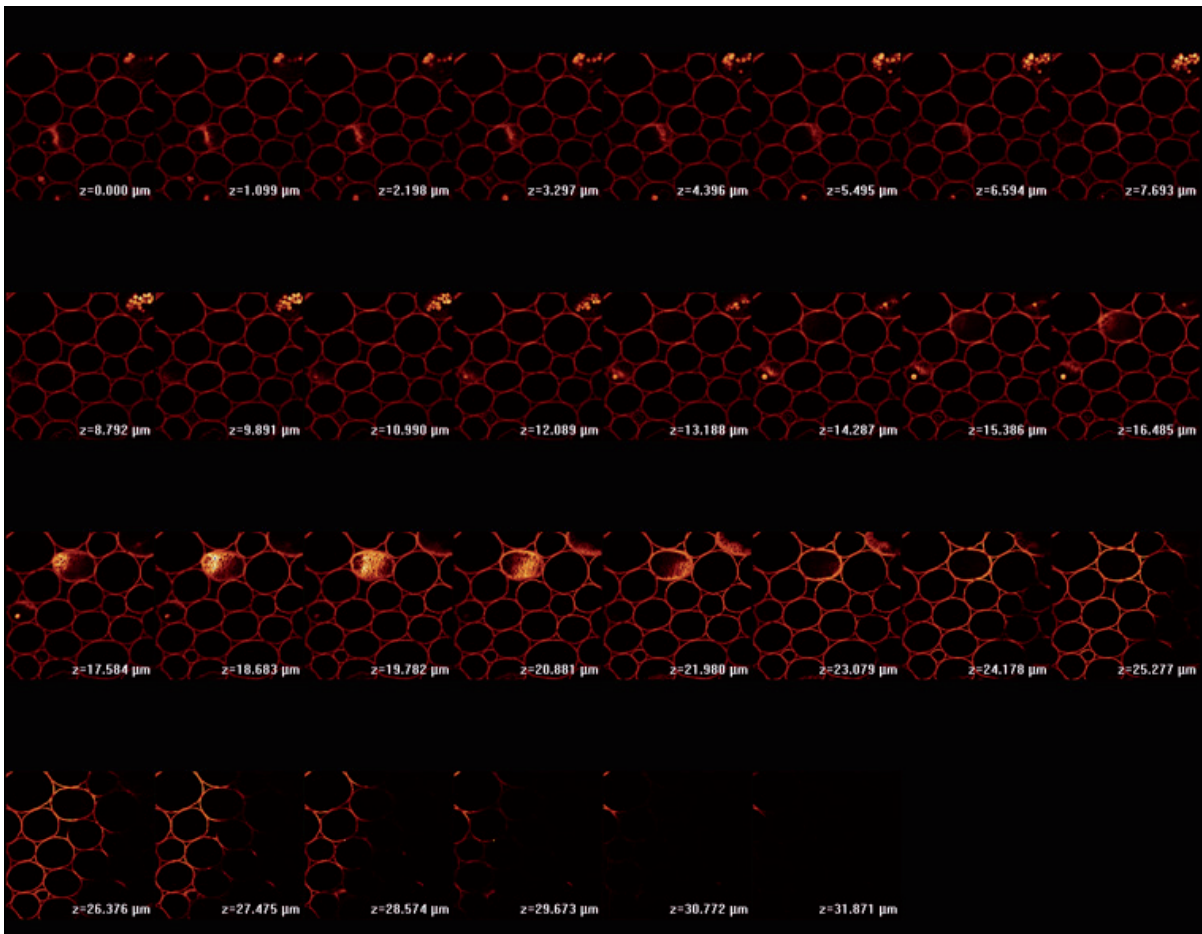


Figure 66: Gallery of a z-stack. This thumbnail gallery is well suited for monochrome publications.

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

10.4.5.1 Gallery

The simplest of these is to display all of the sections of a series in a thumbnail gallery (Figure 66). Changes from section to section can thus be analyzed and the images printed in periodicals.

10.4.5.2 Movie

Many publications today are available on the Internet, making it possible to include movies in which these sequences can be viewed 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 multichannel scans.

10.4.5.3 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.

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 scans.

Coloring each section 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" (Figure 67).

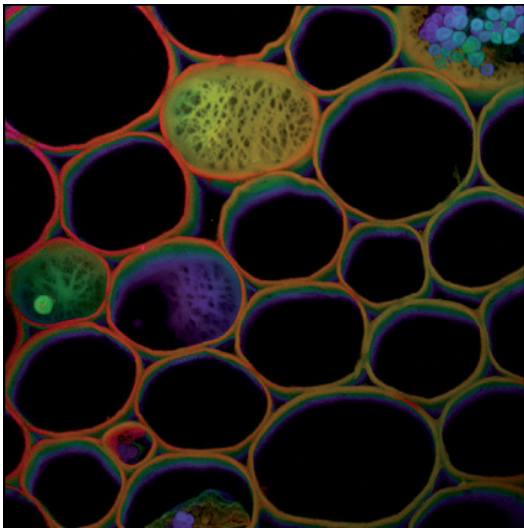


Figure 67: Color-coded relief of the series shown above

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.

10.4.5.4 Rotated Projections

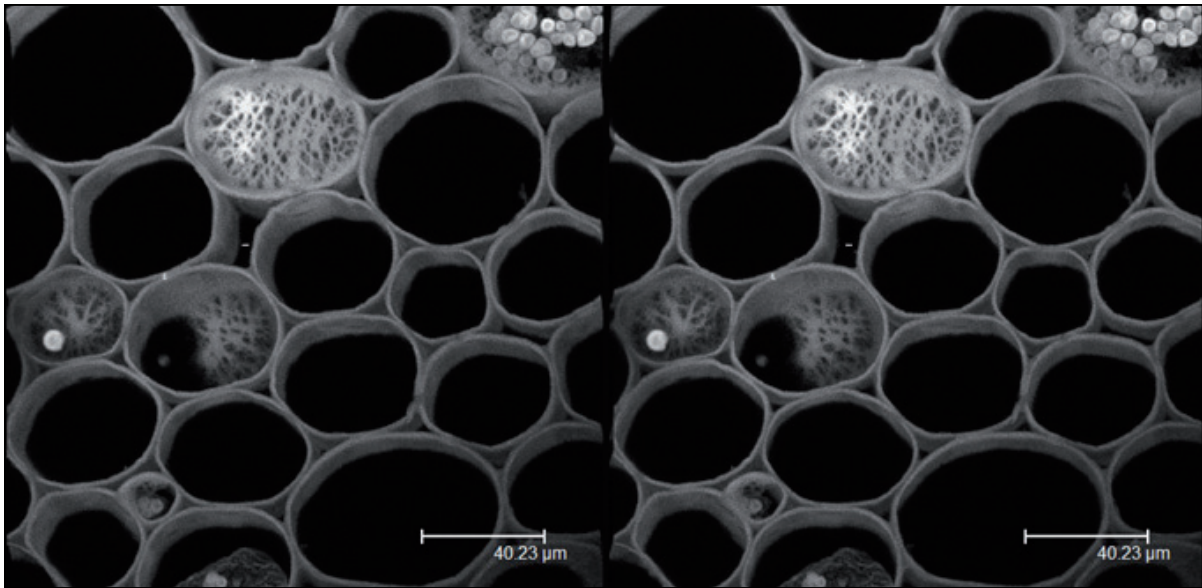


Figure 68: Stereo image of the same 3D data. Although some practice is required, this is nevertheless a worthwhile exercise for any confocal microscope user.

The methods described in 10.4.5.3 initially assume that the projection will be performed along the visual axis. Because the data in the computer exist in a spatially homogeneous state, however, projections from any direction are possible.

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 object viewed with both eyes (Figure 68).

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.

10.5 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.

10.5.1 Scan Speed

Temporal resolution is an important parameter in dynamic processes, especially those related to kinetic studies of cellular biophysical processes. Unfortunately, restrictions 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 resolution 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.

10.5.2 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.

10.5.3 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 (63 μ s) in bidirectional mode.

10.5.4 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 scan to a band-shaped image of 16 lines, a resonant scanner can scan up to 200 images per second (5ms).

This standard scan process (generally at 512 x 512 pixels) will also be used for long-term experiments in which the image of the specimen is scanned 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; however, the system must be extremely stable, free of drift and climate controlled.

10.5.5 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.

10.5.6 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. Here, a part of the specimen is photobleached using strong illumination in order then to measure the recovered fluorescence from the area.

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.

10.6 Spectral Series

Section 10.2.4 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 scanned 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.

10.6.1 Data Acquisition and Utilization

The scanning 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.

10.6.2 About Spectral Resolution

A recent debate has developed about which technology offers the best spectral resolution in conjunction with spectral series, 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.

10.7 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 data graveyards and incorrect conclusions.

11. Care and Maintenance

11.1 General

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

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 use of acids, lyes or other aggressive chemicals. Make sure to keep such substances away from optical or mechanical components.

11.2 Cleaning the Optical System

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

Remove dust by using a fine, dry hair pencil. If this method fails, use a piece of lint-free cloth, moistened with distilled water.

Stubborn 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 which solvents to use 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.

The immersion oil should be removed from oil immersion lenses immediately after it is applied.

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. Apply a drop of the recommended solvent and gently draw the tissue across the lens surface. Repeat this procedure until the lens is completely clean. Use a clean piece of lens tissue each time.

11.3 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.



Whenever the confocal system is moved, it must first be thoroughly cleaned. This applies in particular to systems that are located in biomedical research labs.

This is necessary to remove any existing contamination so as to prevent the risk of putting others in danger. In addition to surfaces, pay particular attention to fans and cooling devices, as dust is particularly likely to accumulate at these locations.

11.4 Maintaining the Scanner Cooling System

The scanner of the TCS SP5 is liquid-cooled. Observe the safety data sheet (reprinted in the Appendix) provided by the manufacturer, Innovatek, regarding the coolant used.



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



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.

12. Transport and Disposal

12.1 Changing the Installation Location



Clean the laser scanning microscope thoroughly before moving it to another place. Whenever any system parts are removed, these also have to be cleaned thoroughly. This applies in particular to systems that are located in biomedical research labs.

This is necessary to remove any possible contamination, thereby preventing the transfer of dangerous substances and pathogens and avoiding hazards and dangers.



In addition to surfaces, pay particular attention to fans and cooling devices, as dust is particularly likely to accumulate at these locations.

12.2 Disposal



If you have any questions related to disposal, please contact the Leica branch office in your country (see Chapter 13).

13. Contact

If you have any further questions related to your TCS SP5, please contact your country's Leica branch office directly. 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

14. 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 curvature of image field 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 image of a point light source. The diffraction discs of two adjacent object points overlap partially or completely, thus limiting the spatial resolution capacity.

Aliasing

An image aberration 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 perpendicular to the ultrasonic wave field as through a grid.

Apochromatic

Describes a correction class for objectives. The chromatic aberration for three wavelengths is corrected for objectives of this type (usually 450 nm, 550 nm and 650 nm) and the sine condition for at least two colors is met. The curvature of image field 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 specimen 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 that 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.

Curvature of Image Field

The curved surface to which a microscopic image is to be clearly and distinctly mapped is described as curvature of image field. It is conditional on the convex shape of the lens and makes itself apparent as an error due to the short focal lengths of microscope objectives. The object image is not in focus both in the center and at the periphery at the same time. Objectives that are corrected for curvature of image field are called flat-field objectives.

Refractive Index

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

Chromatic Aberration

An optical image aberration caused by the varying refraction of light rays of different wavelengths on a lens. Thus light rays of shorter wavelengths have a greater focal length 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 dichroic filters 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; light above this value 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 unwanted 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 Dichroic

Double dichroic filters are interference filters at an angle of incidence of light of 45°. The transmissivity or reflectivity of double dichroic filters depends on two specific wavelengths of light. With a DD 488/568 double dichroic filter, 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 around 80% and the reflection values are between 90% and 95%.

Experiment

A file with Leica-specific data format (*.lei) that consists of one or more individual images or image series. Images recorded with different scan parameters or resulting 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, rhodamine, eosin, DPA.

Fluorescence Microscopy

A light-optical contrast process for displaying fluorescent structures. Auto-fluorescent specimens have what is known as 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 dyes called fluorochromes. Specific dyeing methods allow the precise localization of the dyed structure elements of an object. Fluorescence microscopy provides both the potential for 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 scan 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 laser radiation (infrared).

Confocal Microscopy Techniques

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 focused and unfocused image components, the confocal principle suppresses the structures found outside of the focal plane of the microscope objective. Diaphragms are implemented in optically conjugated locations of the beam path to achieve this. They function as point light source (excitation diaphragm) and point detector (detection diaphragm). 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).

Short-pass Filter

Reflection short-pass filters are interference filters that transmit short-wave light while reflecting long-wave light. An optical 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 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 any additional gain of information. The term "empty magnification" applies whenever 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 specimen; rather, they only diminish the focus and the contrast.

MP Laser

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

Neutral Density Filter

Neutral density filters are semi-reflective glass plates. They are used to distribute the light path independent of wavelength. The incident light is partially reflected and partially transmitted. Neutral density filters are usually placed at angle of less than 45° in the beam path. The ratings of a neutral density filter are based on its reflectivity-to-transmissivity ratio. For example, for a neutral density filter RT 30/70, 30% of the excitation light is reflected and 70% is transmitted.

Numerical Aperture

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

Optical Bleaching

The destruction of fluorescent dyes known as 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 fluorescence 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 displays. The main area of application is measuring ion concentrations in physiology. In contrast with ratiometric procedures, phase visualization obtains more information on the specimen. In addition, this method allows for adapting the display of physiological data to the dynamics of the human eye. 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 and the image points are referred to as pixels.

Flat-field Objective

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, planachromatic, planapochromatic and plan fluorite, are based on the type of additional correction for chromatic aberration.

ROI

Abbreviation for "Region of Interest". A ROI delimits 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 aberration conditional on the varying distance of paraxial light rays of the same wavelength from the optical axis. Light rays that travel through outer lens zones have shorter focal lengths than rays that travel through the lens center (optical 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 45°. The transmissivity or reflectivity of triple dichroic filters depends on three specific wavelengths of light. With a TD 488/568/647 triple dichroic filter, 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 around 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 laser radiation.

VIS Laser

Laser of the wavelength range 400 - 700 nm, visible laser radiation.

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 and the 3D pixels are referred to as voxels.

Z-stack

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

15. Appendix

15.1 Safety Data Sheets from Third-party Manufacturers

The scanner of the TCS SP5 is liquid-cooled.

Following are the safety data sheets from the manufacturer, Innovatek, for the coolant used.



1. Substance/preparation and company name

Trade name: innovatekProtect IP
company: innovatek OS GmbH, Stadtweg 9, 85134 Stammham
Tel: 08405/92590
Fax: 08405/925921
Emergency phone No.: 08405/92590

2. Composition / information on ingredients

Chemical nature:	Ethylene glycol (ethane diol). Corrosion inhibitors.	
Hazardous Compounds:		
Ethanediol	Content (w/w): >90 % EC No: 203-473-3 INDEX No: 603-027-00-1	CAS No: 107-21-1 Hazard symbol: Xn R-phrases: 22
2-ethylhexanoic acid, sodium salt	Content (w/w): 2 % - 3 % EC No: 243-283-8	CAS No: 19766-89-3 Hazard symbol: Xn

R-phrases: 63 The wording of the hazard symbols and R-phrases is specified in Chapter 16 if dangerous ingredients are mentioned.

3. Hazard identification

Harmful if swallowed.

4. First aid measures

General advice:	Remove contaminated clothing.
If inhaled:	If difficulties occur after vapour/aerosol has been inhaled: fresh air, summon physician.
On skin contact:	Wash thoroughly with soap and water.
On contact with eyes:	Wash affected eyes for at least 15 minutes under running water with eyelids held open.
On ingestion:	Rinse mouth immediately and then drink plenty of water, seek medical attention.
Note to physician:	Symptomatic treatment (decontamination, vital functions). Administer 50 ml of pure ethanol in a drinkable concentration.

5. Fire fighting measures

Suitable extinguishing media:	Water spray, alcohol-resistant foam, dry extinguishers, carbon dioxide (CO ₂).
Special protective equipment:	In case of fire, wear a self contained breathing apparatus.
Further information:	The degree of risk is governed by the burning substance and the fire conditions. Contaminated extinguishing water must be disposed of in accordance with local legislation.



6. Accidental release measures

Personal precautions:	Avoid excessive contact with skin and eyes. In case of release of larger amounts remove contaminated clothing and wash body down thoroughly with water. Hand protection. Pick up immediately as product renders floors slippery.
Environmental precautions:	Contain contaminated water/firefighting water. Do not discharge product into natural waters without pretreatment (biological treatment plant).
Methods for cleaning up / taking up:	Bind the liquid by using suitable absorbent material (saw dust, sand, etc.) and dispose of in accordance with the regulations. Wash away spills thoroughly with large quantities of water. In case of release of larger quantities which might flow into the draining system or waters, contact appropriate authorities.

7. Handling and storage

Handling:	Ensure thorough ventilation of stores and work areas.
Protection against fire and explosion:	Take precautionary measures against static discharges. If exposed to fire, keep containers cool by spraying with water.
Storage:	Product is hygroscopic. Containers should be stored tightly sealed in a dry place. Storage in galvanized containers is not recommended.

8. Exposure controls and personal protection

Components with workplace control parameters: 107-21-1: Ethylene glycol	MAK value (D): 26 mg/m ³ = 10 ppm (TRGS 900 (DE)). Top limit category 1. There is no reason to fear a risk of damage to the developing embryo when the MAK value is adhered to. Skin resorption hazard: wear suitable gloves (see below).
Personal protective equipment Respiratory Protection:	Do not inhale gases/vapours/aerosols.
Hand protection:	Chemical resistant protective gloves (EN 374). Recommended: nitrile rubber, protective index 6. Manufacturers directions for use should be observed because of great diversity of types.
Eye protection: General safety and hygiene measures:	Safety glasses with side-shields (frame goggles, EN 166) The usual precautions for the handling of chemicals must be observed.

9. Physical and chemical properties

Form:	Liquid
Colour:	Colourless
Odour:	Product specific
pH value (500 g/l, 20 °C):	7.0 - 9.0
Solidification temperature:	< -18 °C (DIN/ISO 3016)
Boiling point/range:	>= 165 °C (ASTM D 1120)
Flash point:	>120 °C (DIN/ISO 2592)
Lower explosion limit:	3.0 % vol.
Upper explosion limit:	15.0 % vol.



10. Stability and reactivity

Substances to avoid:	Strong oxidizing agents.
Hazardous reactions:	No hazardous reactions when stored and handled according to instructions.
Hazardous decomposition products:	No hazardous decomposition products if stored and handled as prescribed/indicated.

11. Toxicological data

LD50/oral/rat: >2000 mg/kg
LD50/dermal/rabbit: >2000 mg/kg (literature data)
Primary skin irritation/rabbit: non-irritant

Information on Ethylene glycol:
Further information:

Developmental toxicity was observed after oral ingestion of high doses in studies with rats and mice, but this effect was not seen in a study with rabbits.

Experiences in humans:

Lethal dose if swallowed approx. 1.5 g/kg body weight. Lethal dose approx. 90-110 g for adults, and correspondingly less for children. Smaller doses can result in: consciousness is affected, kidney damage, damage to the central nervous system.

Additional information:

The statements are based on the properties of the individual components. There is no reason to fear a risk of damage to the developing embryo or fetus when the MAK value is adhered to. The whole of the information available provides no indication of a carcinogenic effect.

12. Ecological information

Ecotoxicity:

Toxicity to fish: *Leuciscus idus*/LC50 (96 h): >100 mg/l
Aquatic invertebrates: *daphnia magna*/EC50 (48 h): >100 mg/l
Aquatic plants: algae/EC50 (72 h): >100 mg/l
Microorganisms/Effect on activated sludge: Inhibition of degradation activity in activated sludge is not to be anticipated during correct introduction of low concentrations.

Persistence and
Degradability:

Elimination information:
Test method: OECD 301A (new version)
Method of analysis: DOC reduction
Degree of elimination: >70 %
Evaluation: readily biodegradable.

Additional information:

Other ecotoxicological advice: Do not release untreated into natural waters. The product has not been tested. The statement was derived from the properties of the individual components.

13. Disposal considerations

innovatekProtect must be dumped or incinerated in accordance with local regulations.



Stand:
2.12.2004

Contaminated packaging: Uncontaminated packs can be reused. Packs that cannot be cleaned should be disposed of in the same manner as the contents.

14. Transport information

Not classified as hazardous under transport regulations.
(ADR RID ADNR IMDG/GGVSee ICAO/IATA)

15. Regulatory information

Regulations of the European union (Labelling) / National legislation / Regulations:
Directive 1999/45/EEC („Preparation Directive“)

Hazard Symbol:	Xn:	Harmful
R-phrases:	22:	Harmful if swallowed
S-phrases:	2:	Keep out of reach of children
	24/25:	Avoid contact with skin and eyes
	46:	If swallowed, seek medical advice immediately and show this container or label

Hazard determinant component for labelling:	Ethanediol
Other regulations:	none

16. Further Information

Full text of hazard symbols and R-phrases if mentioned as hazardous components in chapter 2:
Xn: Harmful
22: Harmful if swallowed.
63: Possible risk of harm to the unborn child.

Vertical lines in the left hand margin indicate an amendment from the previous version.
This safety data sheet is intended to provide information and recommendations as to: 1. how to handle chemical substances and preparations in accordance with the essential requirements of safety precautions and physical, toxicological, and ecological data. 2. how to handle, store, use, and transport them safely.
No liability for damage occurred in connection with the use of this information or with the use, application, adaption, or processing of the products here described will be accepted. An exception will be made in the case that our legal representatives should come to be held responsible and liable by reason of intent or gross negligence. No liability will be accepted for damage indirectly incurred.
We provide this information and data according to our present level of knowledge and experience. No assurances concerning the characteristics of our product are hereby furnished.

15.2 Declaration of Conformity



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 $\leq 16A$
- 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

A handwritten signature in blue ink, appearing to read "Rafael Storz".

Dr. Rafael Storz

Mannheim, Germany
October 19, 2005

Leica Microsystems CMS GmbH Phone: +49 (0)621 7028 - 0
Am Friedensplatz 3 Fax: +49 (0)621 7028 - 1028
D-68165 Mannheim (Germany) <http://www.leica-microsystems.com>
Copyright © Leica Microsystems CMS GmbH • All rights reserved
Order No.: 156500002 V05