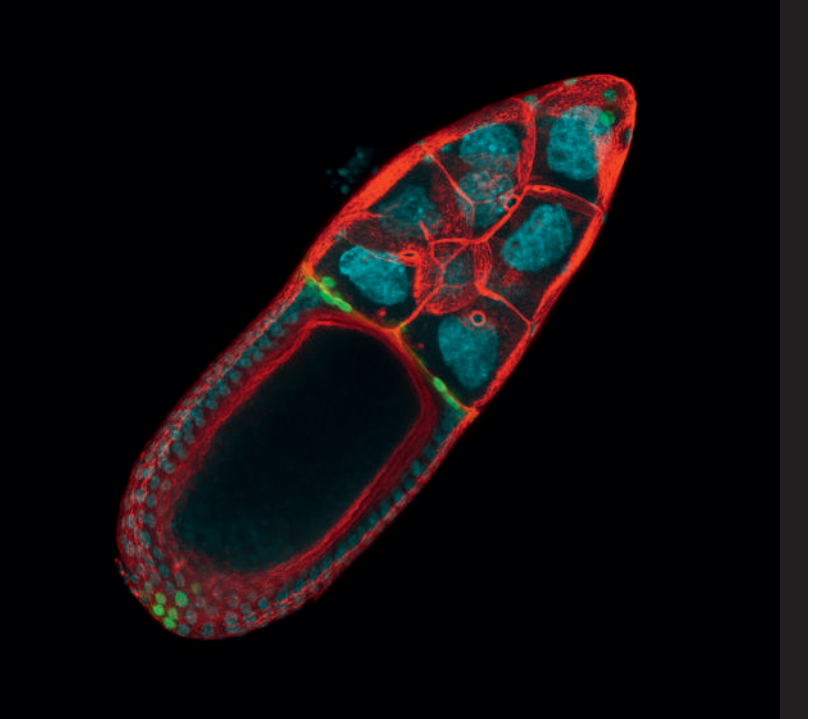


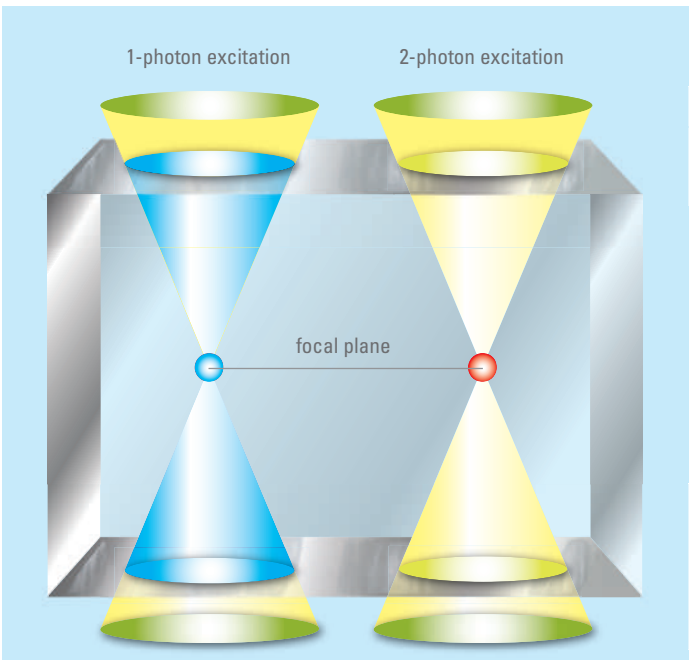
Leica
TCS SP5 MP

Broadband Confocal and
Multiphoton Microscope

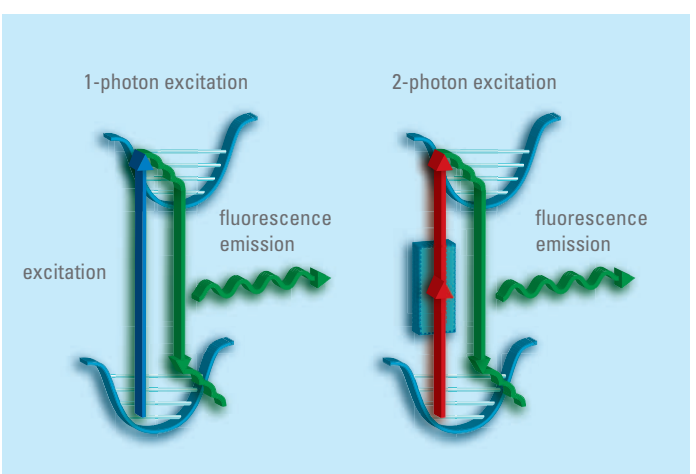
The Solution for Deep Imaging

Leica
MICROSYSTEMS

Deep Imaging



In 2-photon excitation fluorescence emission occurs only on the focal plane.



Energy diagram of fluorescence with 1-photon and 2-photon excitations

Since the advent of confocal microscopy, immense progresses have been made in cellular biology, neurosciences, medical research. Today, it is a major challenge to penetrate deeper into samples for improved studies of cells, organs or tissues. An efficient method to achieve a deep penetration into samples is two-photon and multiphoton excitation with laser scanning microscopes which are equipped with pulsed infrared lasers. Thanks to reduced absorption and scattering of the excitation light, two-photon and multiphoton confocal microscopes reach a penetration depth of about 400 µm.

In the case of two-photon excitation, the dye is excited by the simultaneous absorption of two photons. Due to the non-linearity nature of two-photon absorption, the excitation is limited to the focal volume and the photobleaching outside the focal plane is reduced. Only inside the confocal volume the photon density is sufficiently high to allow two photon absorption by the fluorophore.

Multiphoton excitation performance improves with pulsed laser excitation in the NIR spectra. Longer excitation wavelengths are scattered less in biological tissue allowing a deeper penetration in very thick specimen. Emission/Fluorescence signal is not degraded either by scattering from within the sample.

Advantages of multiphoton exitation:

- **Greater penetration depth due to lower scattering**
- **Intrinsical optical sectioning properties – no need for a detection pinhole**
- **Bleaching restricted to focal plane – no volume bleaching**
- **Reduced phototoxicity due to spatial confinement, which is ideal for living cells.**
- **Uncaging, photoactivation or photobleaching in a diffraction-limited volume**

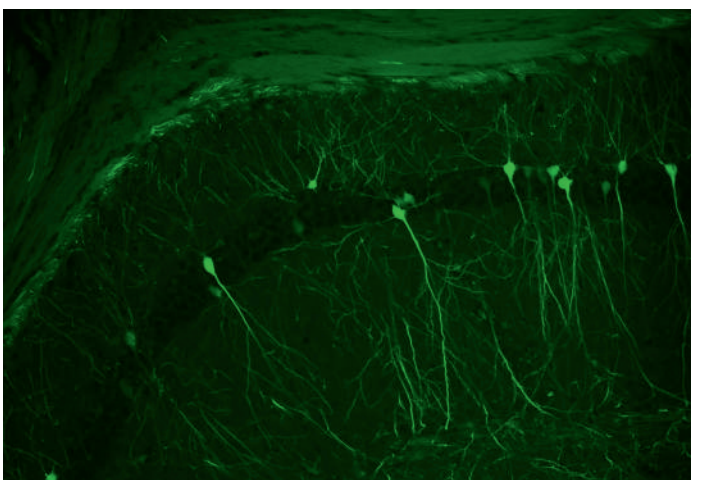
The Leica TCS SP5 MP covers a wide range of imaging applications (multiphoton and one photon) by combining two technologies in one system: a conventional scanner for maximum resolution and a resonant scanner for high time resolution.

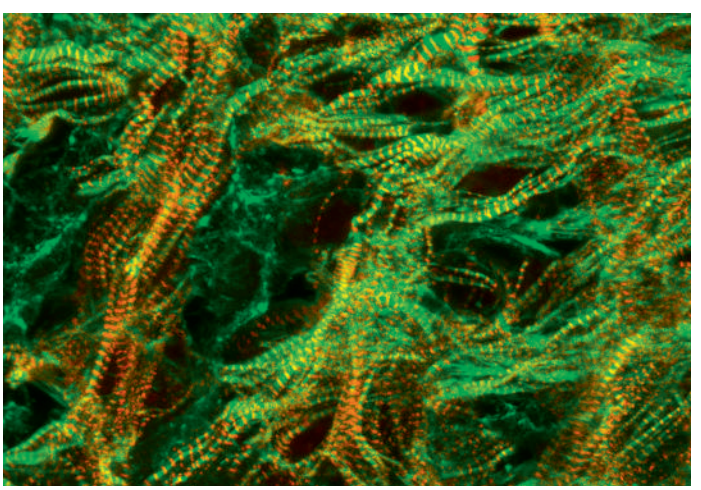
Applications

The invention of multiphoton microscopy in the 1990's raised a tremendous interest and has become a widespread imaging method in the biological sciences since then. Meanwhile there is plethora of applications and publications involving multiphoton microscopy.

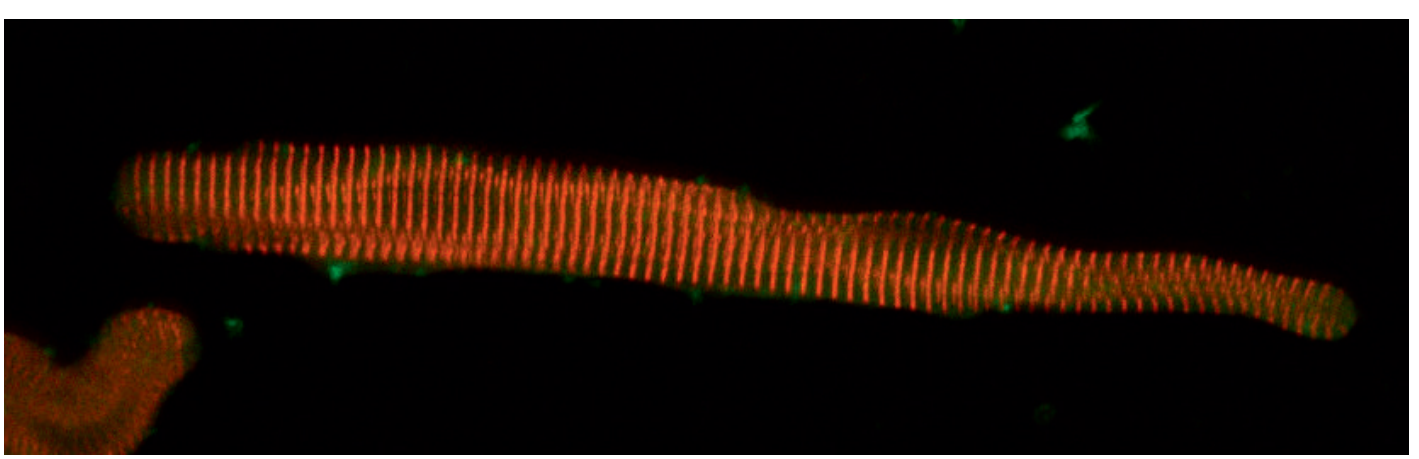
It is now established as the method of choice for non-invasive deep-penetration fluorescence microscopy of thick highly scattering samples and has been used for a diversity of specimen, e.g. lymphatic organs, kidney, heart, skin and brain (slices as well as intact organs).

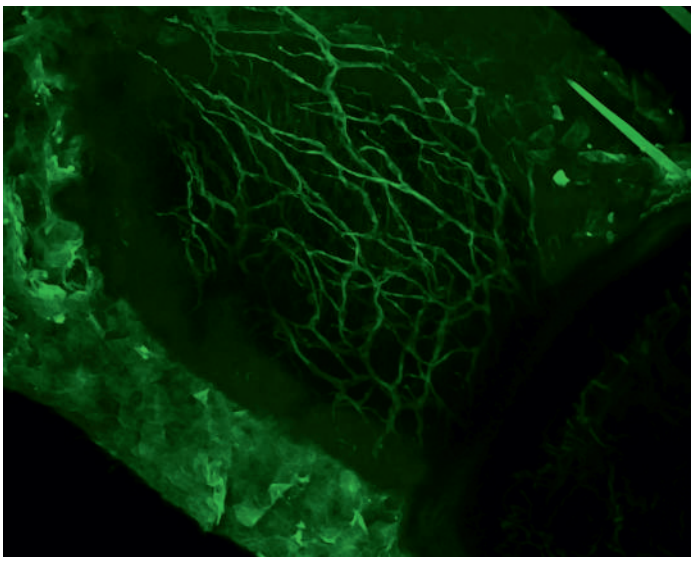
Various research fields, e.g. immunology (lymphocyte tracking, embryology, cancer research and particularly neuroscience (e.g. for the study of calcium dynamics and neuronal plasticity) take the advantage of the deep *in vivo* imaging with multiphoton.

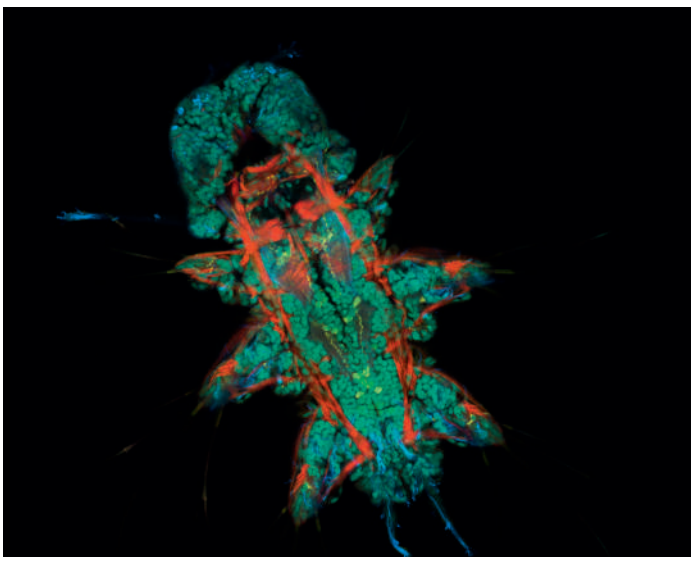


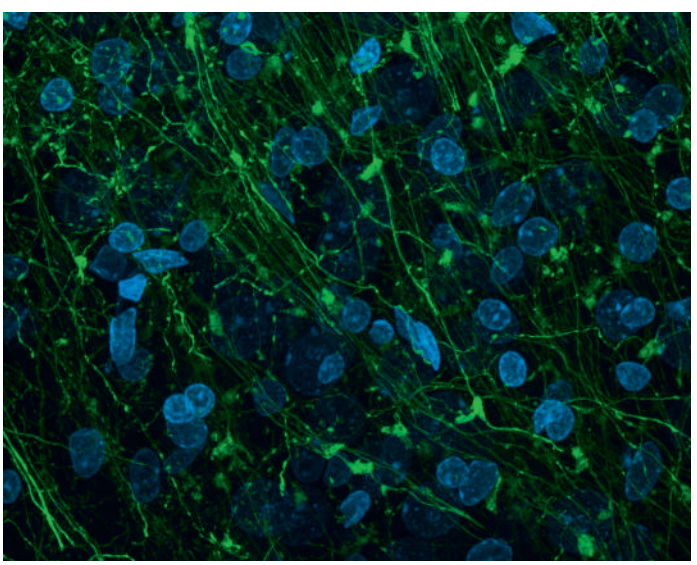


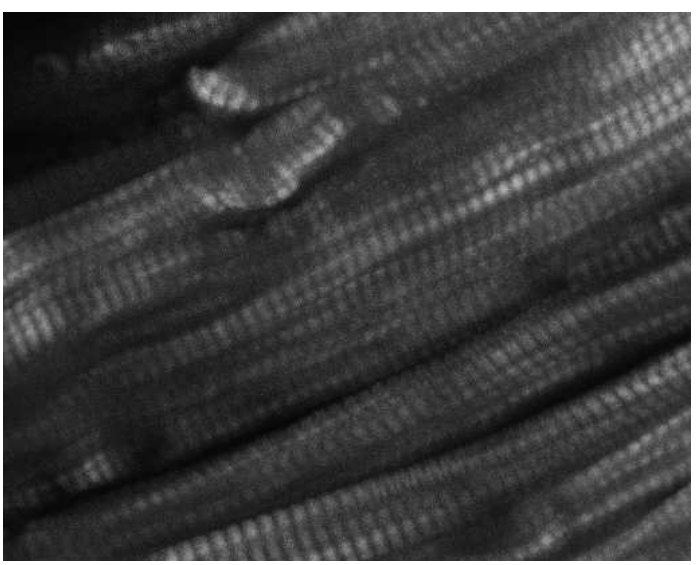
Top: hyppocampal region in mouse brain slice.
Courtesy of Dr. Michael E. Calhun, Hertie Institute, Tübingen, Germany.
Middle: mouse embryo, detail of the heart.
Courtesy of Dr. Elisabeth Ehler, King's College, London, UK.
Bottom: adult rat cardiomyocytes

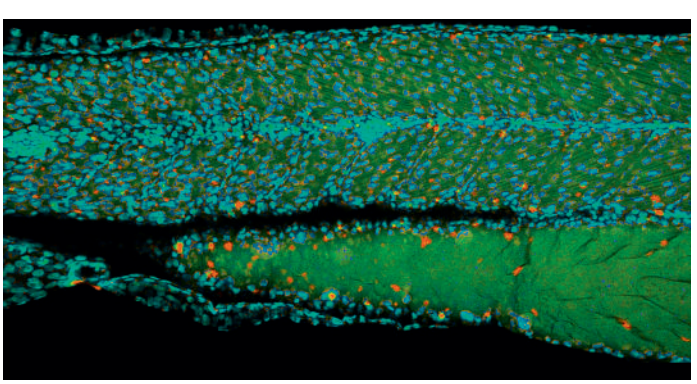












With a multiphoton-setup one can also take advantage of another non-linear optical effect, second-harmonic generation (SHG). SHG-signal is generated from highly ordered structures and has been used to image collagen fibres, microtubules, the striation pattern of muscles and starch granules, but also to measure membrane potential with SHG-suitable dyes.

Top left: peripheral nozizeptive nerves in the paw of a SNS-EGFP mouse. Courtesy of Dr. Rohini Kuner, Medical Faculty, University Heidelberg, Germany
Top right: plathyneris spec. Courtesy of Dr. Leonid Nezlin, RSA, Moscow, Russia)
Middle left: nuclei and pyramidal neurons in mouse brain slice.
Middle right: SHG-signal in mouse heart muscle
Bottom left: dorsal musculature of zebrafish

FLIM–FCS–FCCS

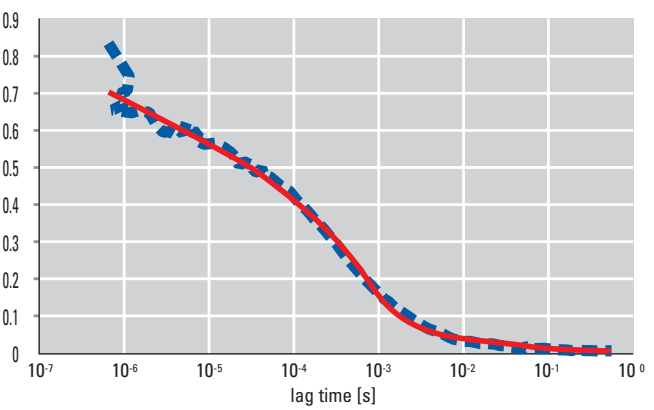
With FLIM (Fluorescence Lifetime Imaging Microscopy) a variety of local parameters within cells or other structures can be measured, such as ion concentrations, molecule interaction, FRET and membrane potential. The researcher thereby acquires information about processes occurring on a molecular scale.

Time-correlated single photon counting technology guarantees perfect exploitation of photons. The Spectral FLIM detectors which are located within the SP5 spectral scanner allow even to combine spectral and lifetime information adding a new dimension to your image data.

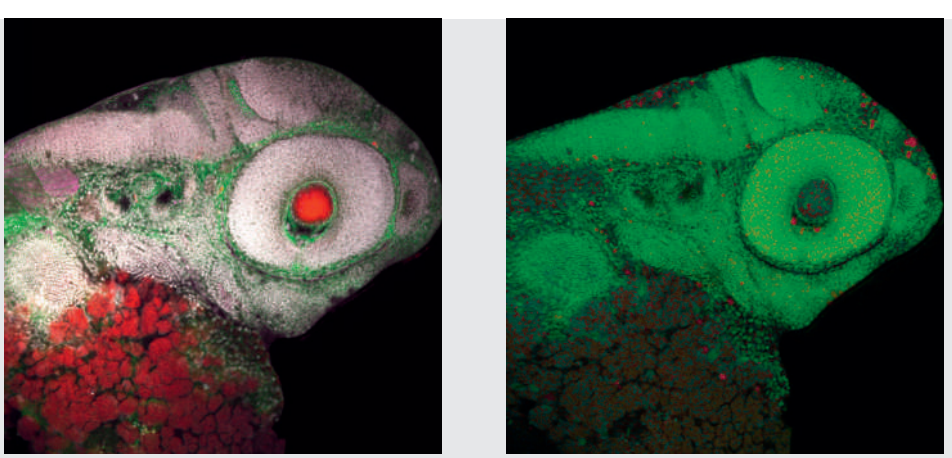
FLIM measurements can be performed with pulsed visible or MP lasers. Advantages of MP excitation are the deep tissue penetration, and less photobleaching outside the focus. Tuneable excitation wavelength allows the usage of a wide range of fluorochromes and fluorescent proteins

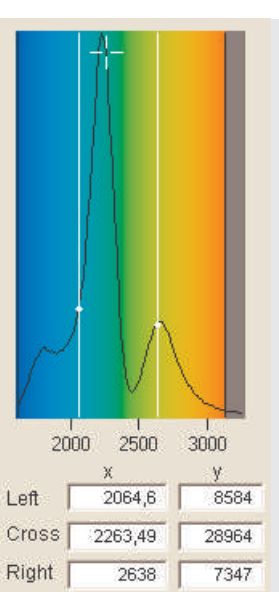
FCS (Fluorescence Correlation Spectroscopy) is a method to measure concentration and diffusion rates quantitatively down to the single molecule level. The data can be used to analyze molecule interactions and transport processes within living cells as well as in vitro. This allows evaluating the dynamics of molecular systems and cellular structures.

FCCS (Fluorescence Cross-Correlation Spectroscopy) is mainly used to measure molecular interactions between molecules of arbitrary size which are labelled with spectrally distinct fluorescent labels. In this context, MP excitation is of special interest because it often allows exciting both labels at one wavelength. This guarantees the same excitation volumes for both interaction partners leading to a better cross-correlation signal.



FCS: Autocorrelation from living cells expressing an EGFP fusion of a nuclear protein. The measurement lasted 50 sec and was acquired in photon mode with a time resolution of 1 MHz.





FLIM: Zebrafish FLIM image (left) and corresponding intensity image (right) with nuclei, muscle tissue and yolk. The histogram contains the color legend for lifetimes.

Flexibility

Leica objective HCX APO L 20x1.0

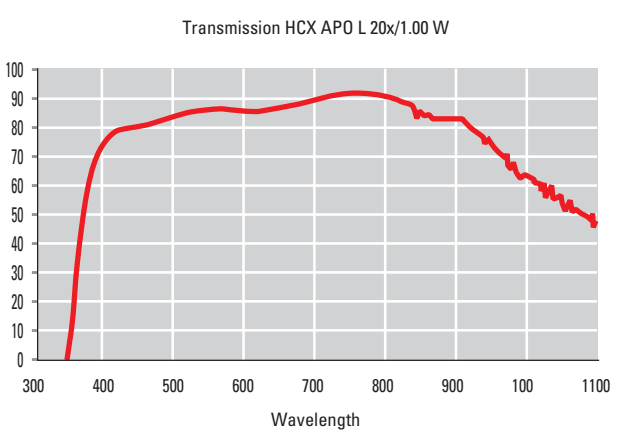
External detectors NDD on DM6000 CFS microscope

Dedicated objectives and microscope stands

Due to the different applications in confocal microscopy, different motorized stands have been developed: the upright microscope DM6000, the inverted microscope DMI6000 for living cell experiments and the new fixed stage DM6000 CFS for electrophysiology. These stands are fully integrated in the LAS AF software.

A full range of objectives HCX PL APO with outstanding performance has been developed (quality: class CS). All relevant parameters have been optimized for high resolution, large image field and excellent color correction in the wide spectral range from UV to IR (U-V-I).

The new Leica HCX PL APO L 20 x 1.0 water immersion objective has been specifically developed for the new DM6000 CFS microscope. This objective fits perfectly for electrophysiological studies and developmental biology. It offers high resolution, a large field of view and a high transmission in both visible and infrared regimes. To achieve a 1.0 NA with a 20x objective means that large specimen can be imaged as a whole while still preserving details at confocal resolution.



External/non-descanned detection

For detection, the internal spectral detectors in the scan head can be used. But given the intrinsic confocality of the method, excitation is limited to the focal plane. Higher collection efficiency can be ensured by the extremely short coupling of detectors. Therefore the Leica TCS SP5 allows for using large photo sensor areas, as found in external detectors, which can be coupled in directly behind the objective (RLD: Reflected Light Detector) or directly behind the condenser (TLD: Transmitted Light Detector).

Progress in research depends – last but not least – on the enhancement of the information content of images. To obtain images rich in details the acquisition of four colors simultaneously is fundamental. The Leica TCS SP5 MP provides a four channel solution: the NDDs can be installed as RLDs or as TLDs. To ensure full flexibility, either two channel detectors can be coupled in on both sides (RLD/TLD) or four channel detectors (RLD or TLD) on one side.

IR lasers and attenuation system

The IR lasers for multiphoton microscopy offered by Leica Microsystems are directly coupled to a dedicated port in the scanhead of the confocal microscope and controlled by LAS AF software.

To attenuate the laser power, a continuously adjustable electro-optical modulator (EOM) or a polarizing filter wheel are offered. They are used together in combination with high power IR lasers.

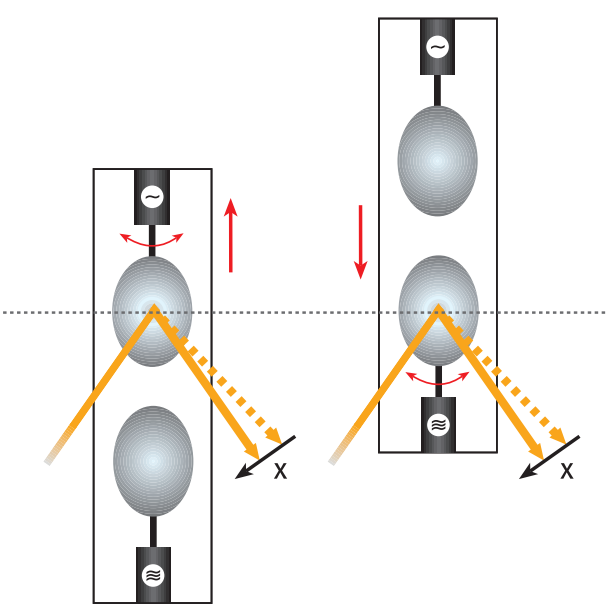
Using multi-photon excitation, the fluorescence is only generated in the diffraction limited focal volume. Photomanipulation experiments (uncaging, photoactivation or photobleaching) will be possible at this focal plane by using the EOM.

Tandem scanner

All multiphoton applications, morphological and high-speed dynamic studies, are covered by the Leica the Leica Tandem Scanner TCS SP5: Two scanners – one conventional and one resonant – are combined in the system and enables to switch between both scanners – fully motorized and computer controlled.

The conventional scanner is optimized for morphological studies – brain, tissues, and cytoskeleton – allowing high spatial resolution. 8196 x 8196 pixel images can be obtained in combination with a large field of view (23 mm – intermediate image plane).

The resonant scanner of the Leica TCS SP5 works at 16000 Hz frequency in bidirectional mode. The system acquires 25 images per second with 512 x 512 pixels and at higher speed up to 250 images with 512 x 16 pixels. Dynamic processes with high time resolution can be imaged and measured, e.g. Ca2+waves.



Tandem Scanner:

By means of a motorized and computer controlled high precision device, a conventional and a resonant galvanotmetric driven scan mirror are exchanged into the proper position for scanning, while the scan electronics is switched simultaneously

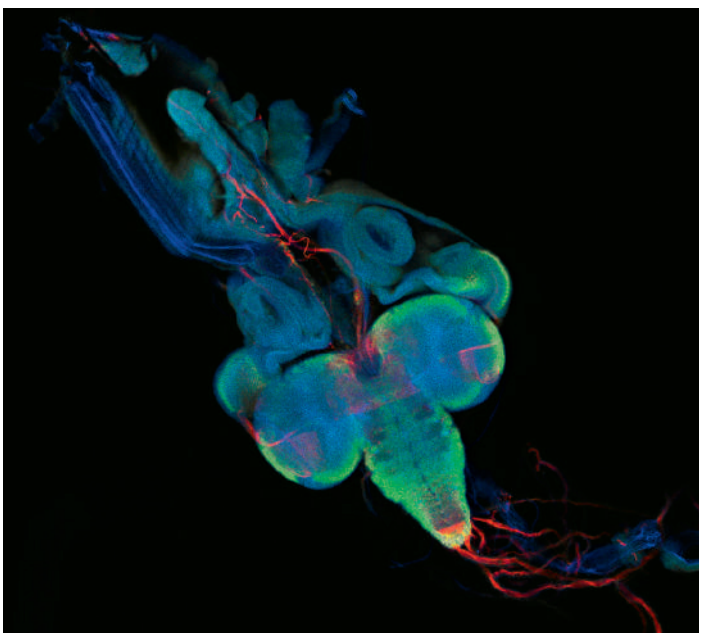
Leica TCS SP5 MP Features

Detection System
- Specific stands
- Specific objectives
- 20 x water immersion objective
- High efficiency photon collection
- Combination of RLD and TLD
- Up to 4 NDDs simultaneously

Scanning System, Laser Ports
- True single point confocal scanner
- Up to 8k x 8k per image
- Up to 250 images/sec
- UV, Vis and IR in one system
- IR specific port
- EOM attenuation

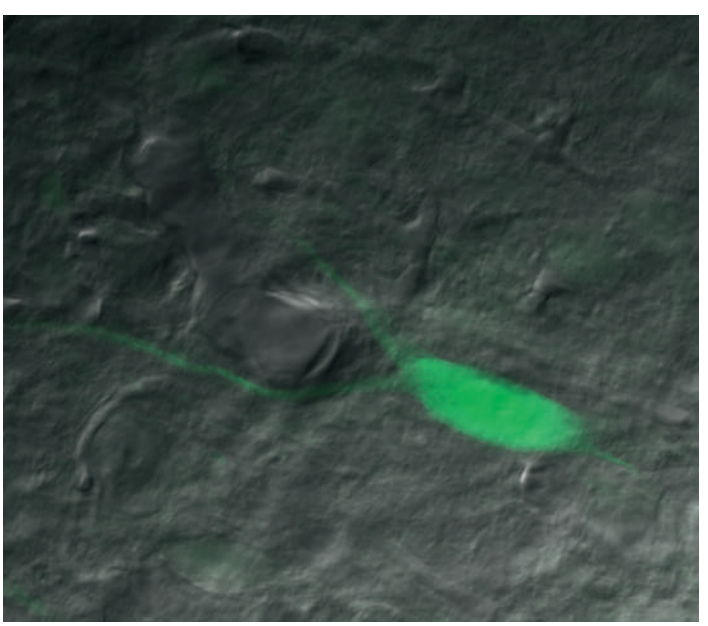
FLIM – FCS – FCCS
- APD (avalanche photo diode) for maximum sensitivity (quantum efficiency up to 80 %) for low light imaging and FCS
- Dual channel FCS and FCCS
- Both, spectral and external FLIM

Advantages of Leica TCS SP5 MP
- Deep penetration
- Lowest sample damage
- Real time imaging
- High resolution mapping
- Ready for integrated analytics



Drosophila larvae

Courtesy of Dr. Christoph Melcher, Forschungszentrum Karlsruhe, Germany



Mouse brain slice, Overlay of IR-SGC and fluorescence

Courtesy of Dr. Thomas Kuner, Institute of Anatomy and Cell Biology, Heidelberg, Germany