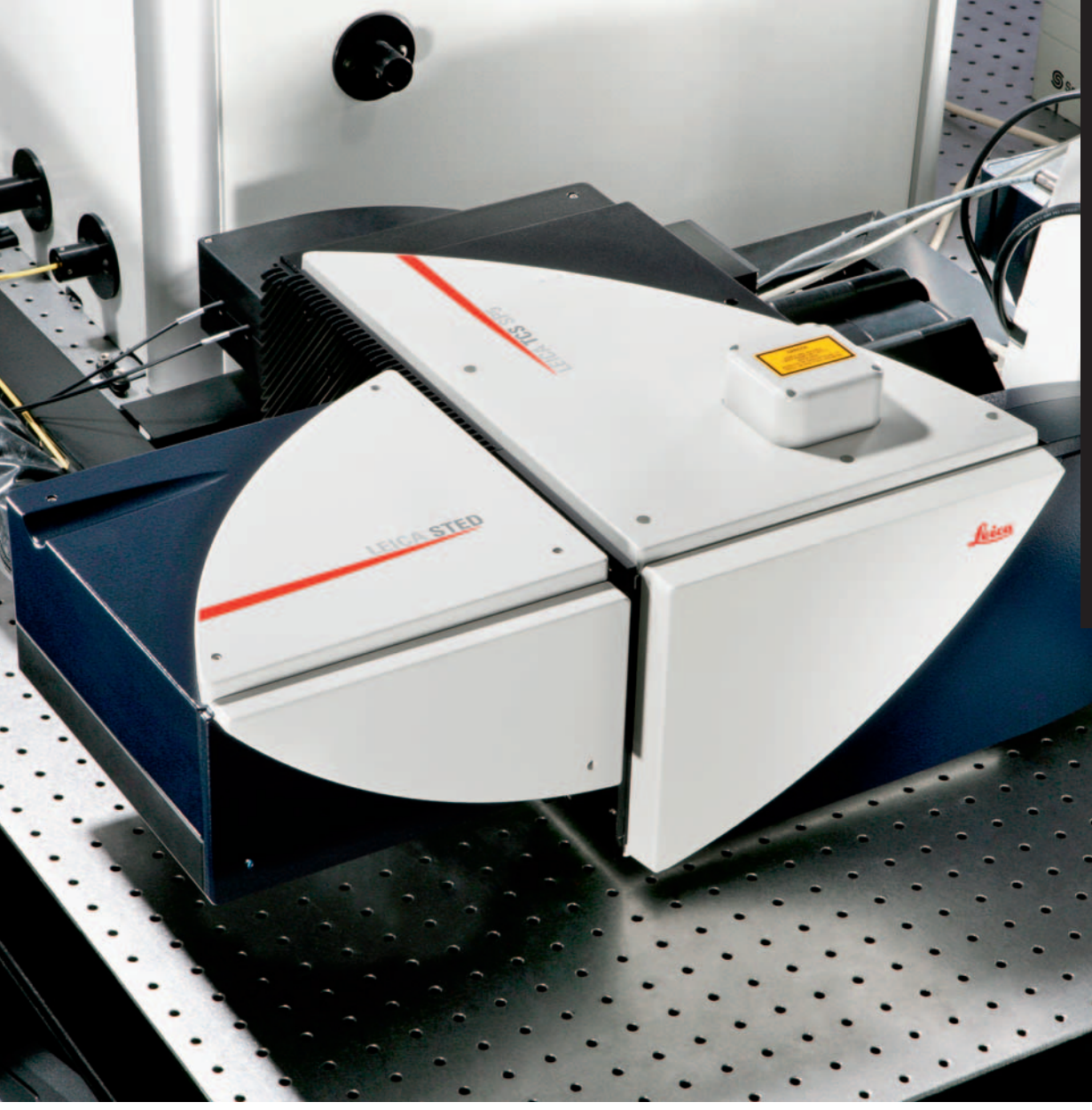
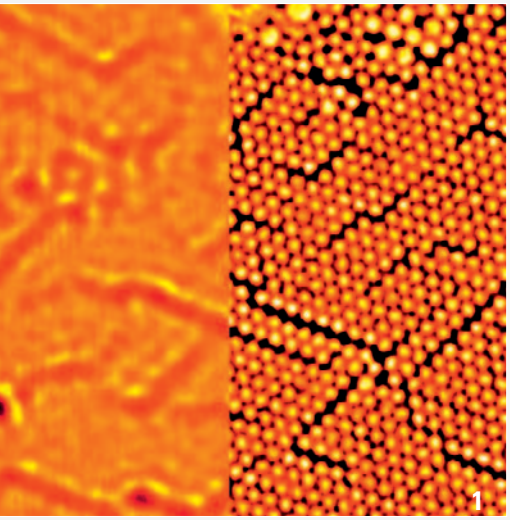


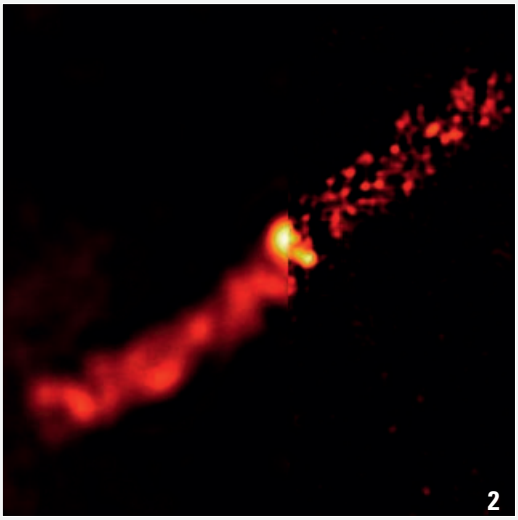
Leica TCS STED

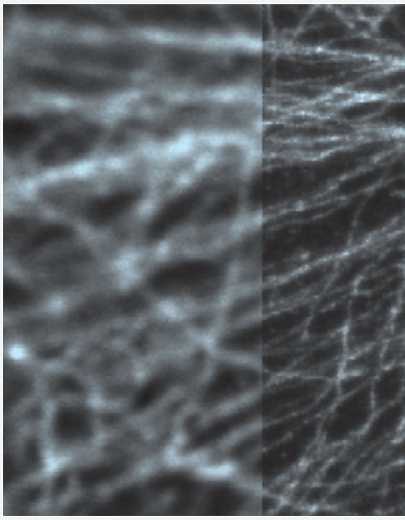
Beyond the Limits!

Living up to Life

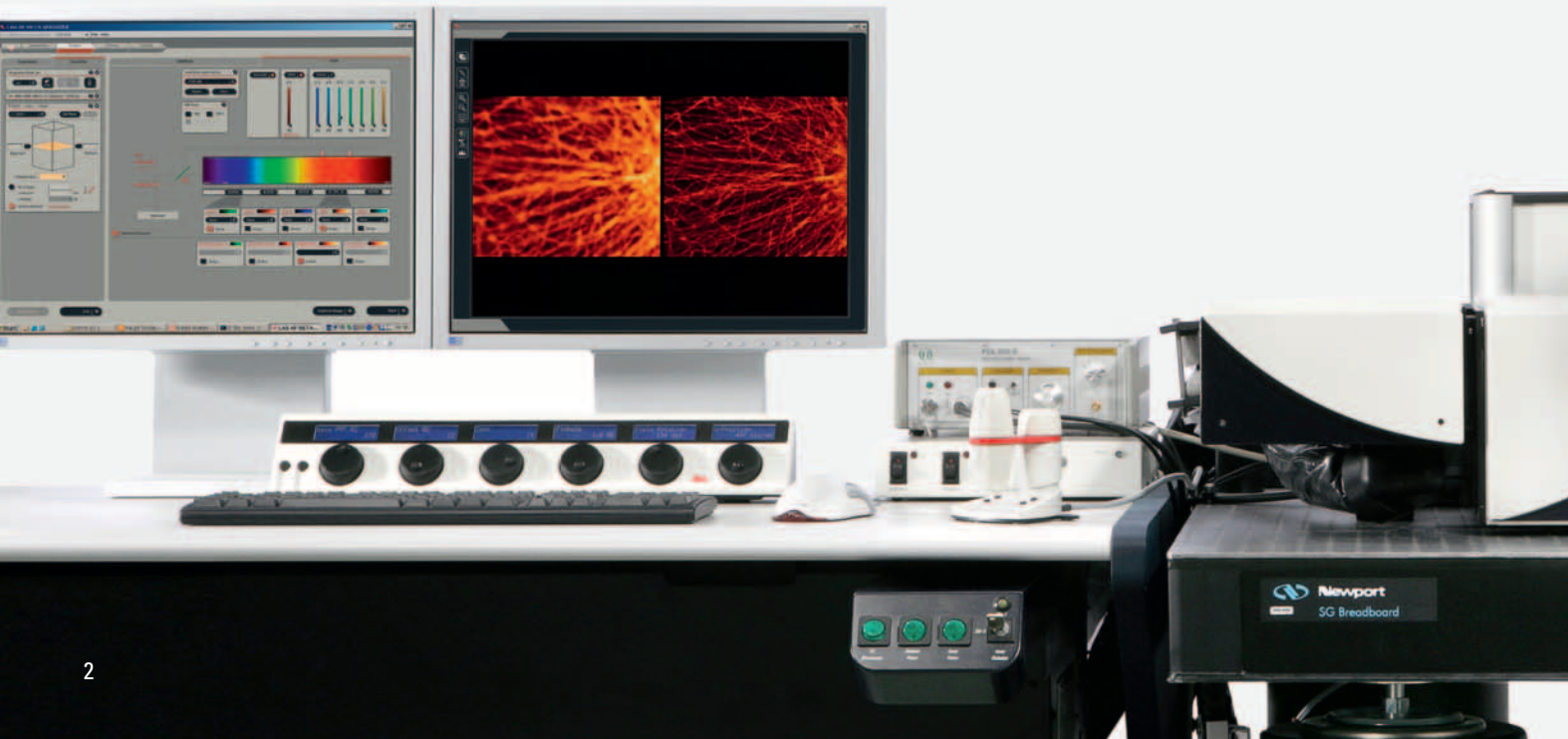
Leica
MICROSYSTEMS

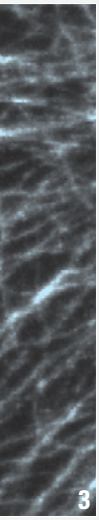






- Sharper Focus for New Insights
- Easy Access to Superresolution
- Combined Power of STED and Confocal!
- Upgrade to STED – Anytime!

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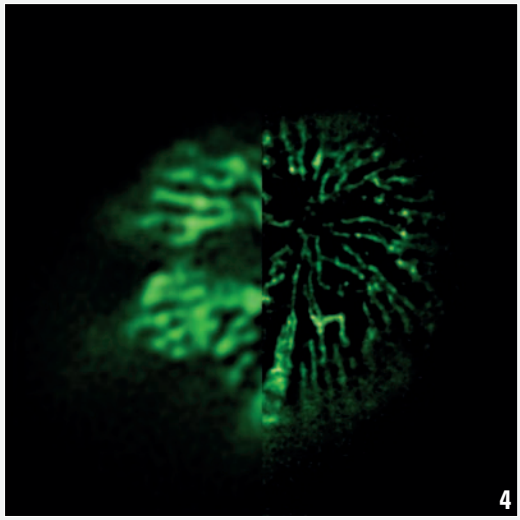
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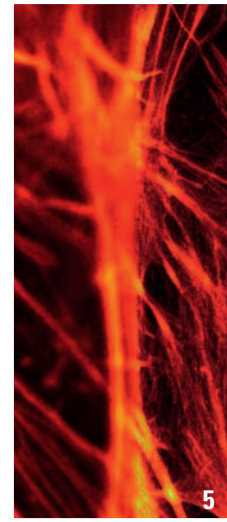

5

Substantial progress has been achieved in life sciences such as structural biology, basic medical research, cell biology and biochemistry over the last few years – linked with a strong increase in the application of fluorescence microscopy based methods. The precise characterization of cells and large cellular compartments has now become standard practice.

Leica TCS STED

Beyond the Limits!

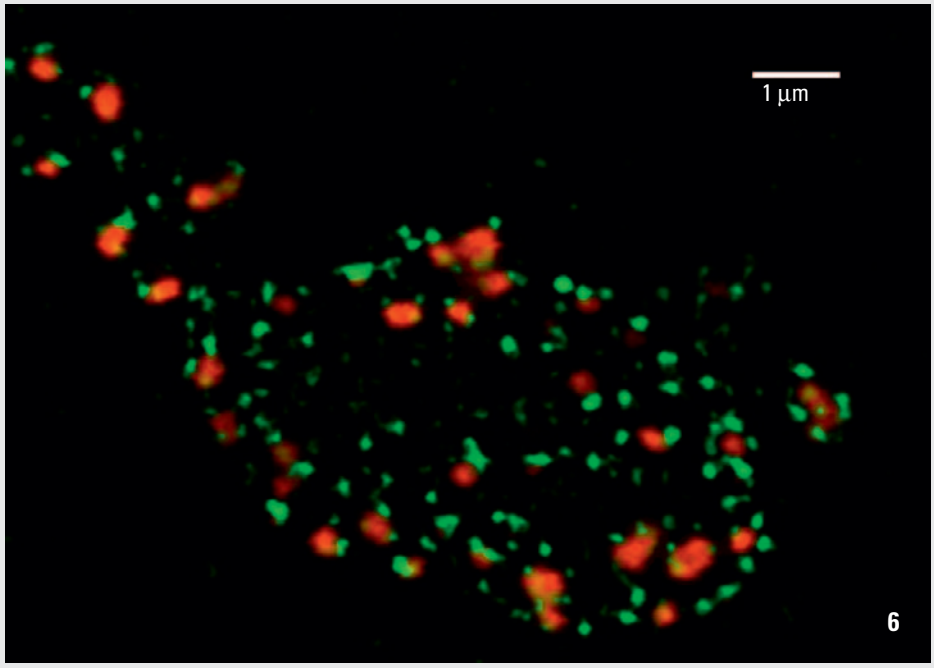
Nevertheless, as soon as it comes to the analysis of smaller structures, for instance viruses, membrane vesicles, etc., researchers are faced with the well known Abbe barrier of about 200 nm lateral resolution. The effort to bypass this limit ranges from laborious and expensive (e.g. electron microscopy) to indirect and sophisticated fluorescence measurement solutions.

With the integration of the groundbreaking STED concept into the approved broadband confocal platform Leica TCS SP5 we have created a new class of microscope, the Leica TCS STED. Its superresolution capacity allows confocal imaging with a resolution 2 to 3 times higher than could ever be achieved in a conventional scanning microscope – without compromising on usability. We call this: superresolution at a mouse click.

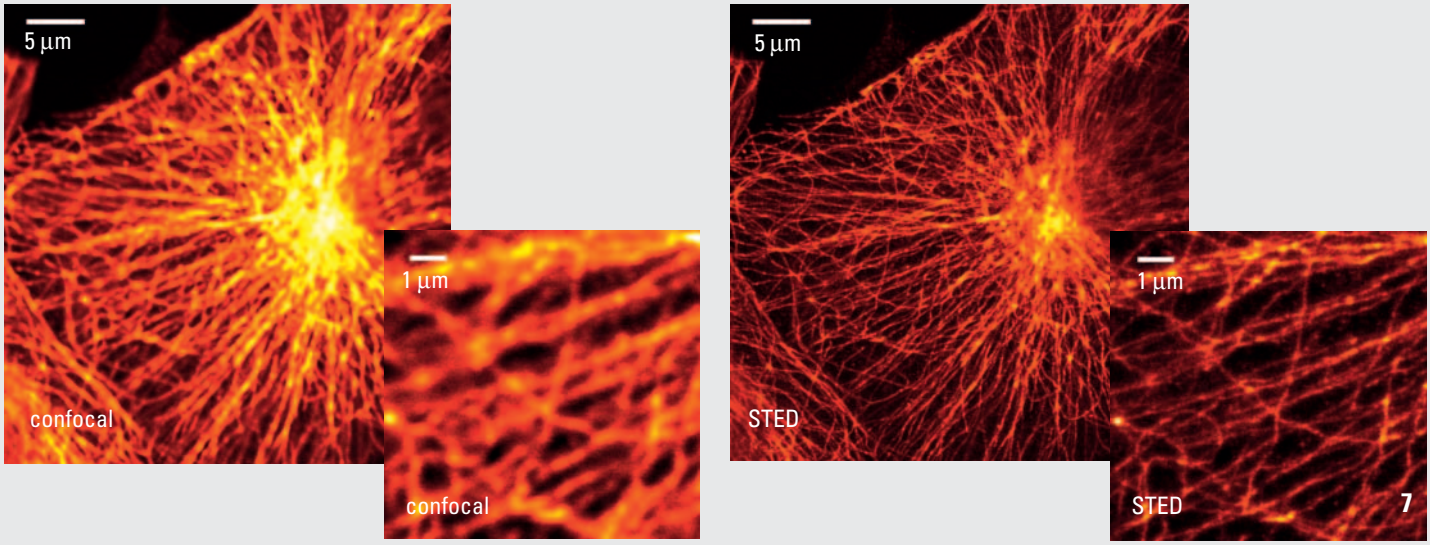
Some Applications of STED Microscopy

Neuroscience

Unveiling the molecular organization of presynaptic active zones is relevant for the understanding of the nervous system. STED microscopy was applied for distribution analysis of active zone proteins liprin (green, STED) and bruchpilot (red, confocal) which is conserved between flies and mammals (Wagh et al., Neuron, 2006; Kittel et al., Science, 2006).

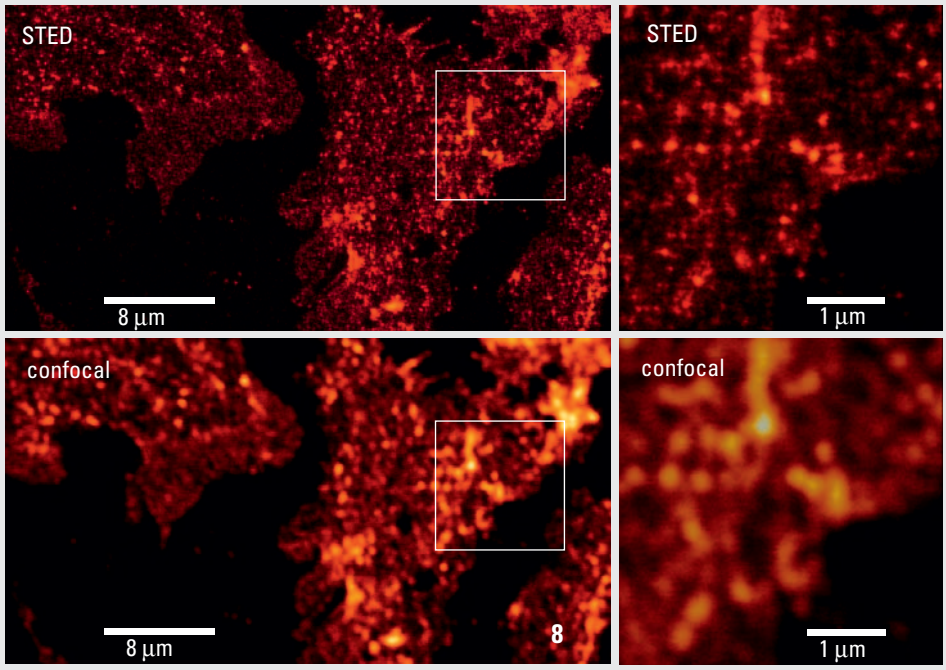


Cell Architecture



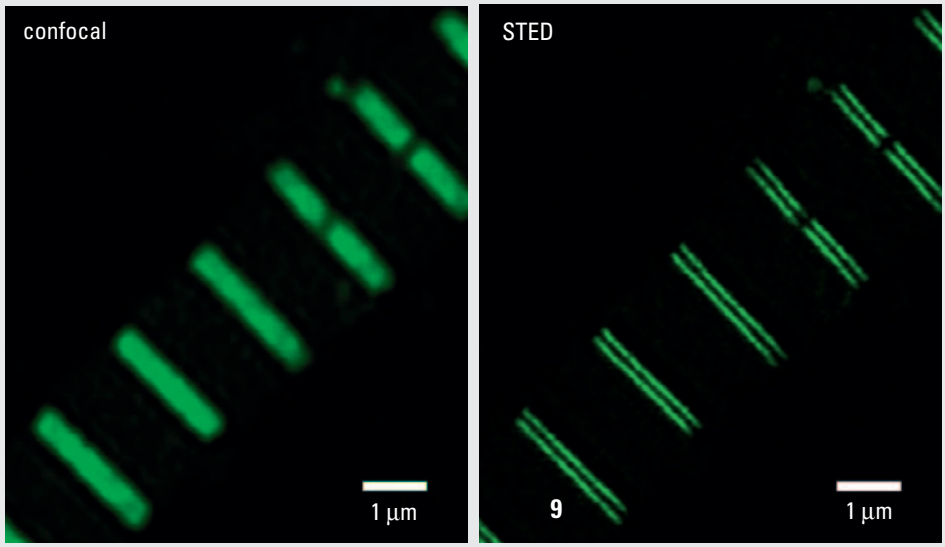
Cellular structures depend on the presence of cytoskeletal proteins, such as actin and tubulin. STED allows discriminating single fibers with significantly higher resolution compared to conventional confocal microscopy.

Membrane domains



Analyis of the spatial distribution of syntax-in STED STED within the basal plasma membrane of PC12 cells. STED microscopy allowed the py investigation of cluster density and the determination of average cluster sizes of 50 – 60 nm. [Science, Sieber JJ., 2007]

Physiology: Muscle Research



Rat myofibrils stained with antibodies against an N-terminal epitope in the titin molecule (clone T12), which lies at the edges of the Z- disc in the sarcomere.

And more:

- Synapse formation
- Neuron-Glia Interaction
- Active zones
- Membrane biology
- Micro-/Nanodomains
- Virology
- Vesicle transport
- Cell morphology
- Signal transduction
- Receptor studies
- Bacteriology

Sharper Focus for New Insights

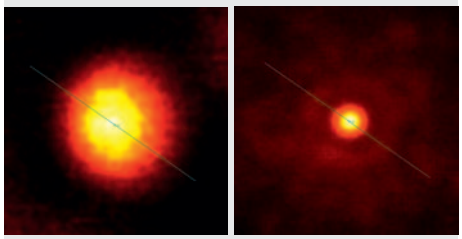
"To break a barrier it´s good to have a competent partner such as Leica."

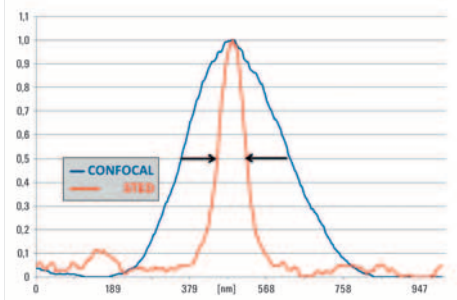
Prof. Stefan W. Hell
Max Planck Institute for Biophysical Chemistry, Göttingen, Germany

$$\Delta x \approx \frac{\lambda}{2n\sin\alpha\sqrt{1+\frac{I}{I_s}}}$$

Lateral resolution of a STED microscope can be approximated by the upper equation

confocal STED





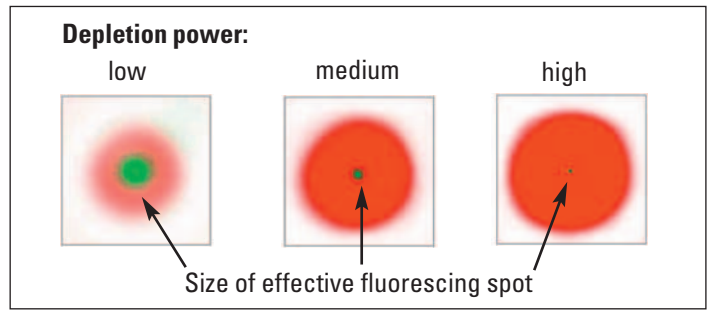
Averaged images of fluorescent nanobeads (35 nm) to determine the Full Width at Half Maximum (FWHM)

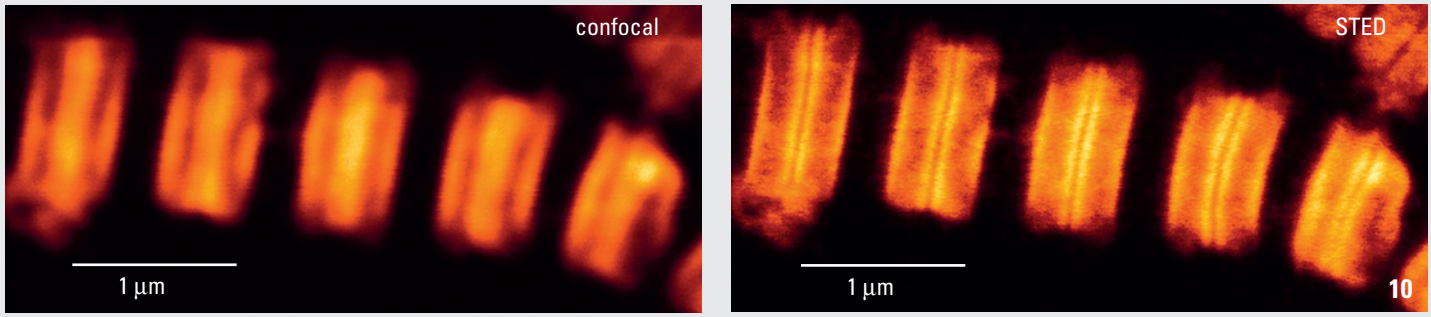
Think of a light microscopist as a painter – the tinier the details he is interested in, the finer the brush he uses. Leica TCS STED technology delivers the "finest brush" ever in far field microscopy. It enables you to acquire images richer in detail than you ever thought possible.

With Stefan Hell's award-winning invention of Stimulated Emission and Depletion (STED) technology, a new chapter in fluorescence microscopy has begun. In a Leica TCS STED microscope the sample is illuminated by two pulsed laser beams, tightly synchronized. The 635 nm wavelength excites the fluorophores of the sample the same way a conventional confocal system does. The excitation laser pulses are directly followed by a ring shaped illumination of a Ti:Sapphire Infrared laser. This pulse inhibits the fluorescence in the outer regions of the illuminated spot.

The result: A smaller fluorescence spot that allows much more accurate scanning than with other methods using focused light. The fluorescing area is practically decreased at least 5 fold-equivalent to increased resolution. STED microscopy is in principle not limited by diffraction any more. A wealth of unknown details is revealed, setting the stage for new insights. Synapse formation, vesicle transport, receptor – ligand interactions – just some examples of applications that can be observed directly in the intact specimen with this new superresolution microscope. STED technology opens completely new horizons for your research – this is real "Visual Biochemistry".

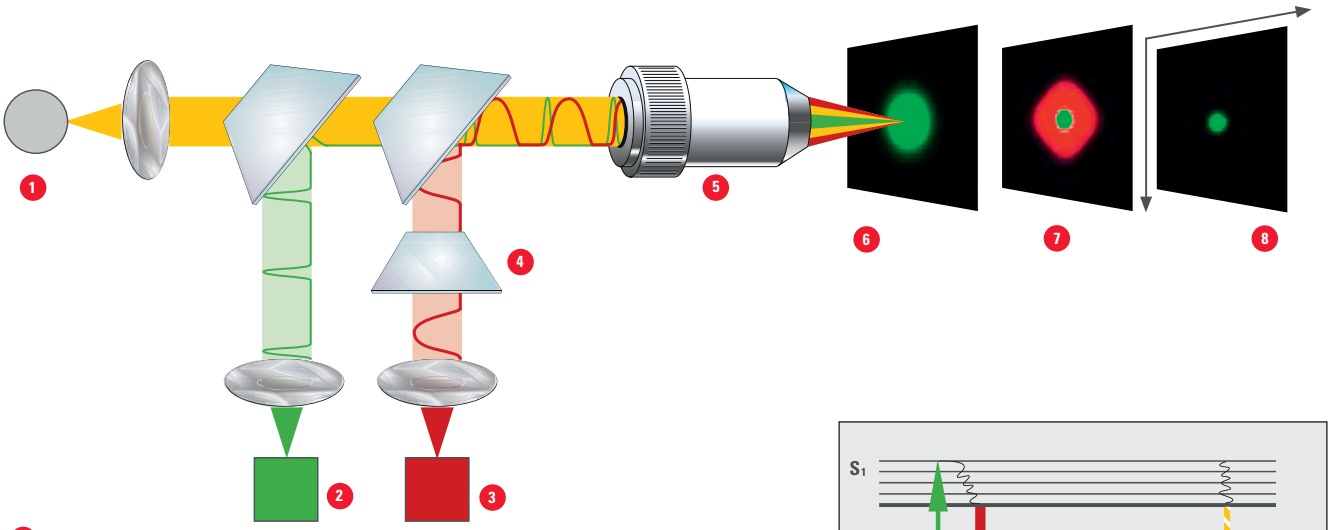
The future oriented and promising technology has been realized in an ultra precise and Leica patented device in combination with our state-of-the-art broadband confocal, the Leica TCS SP5. The result is a highly stable and easy to use system, ready for the challenges of tomorrow: The Leica TCS STED.

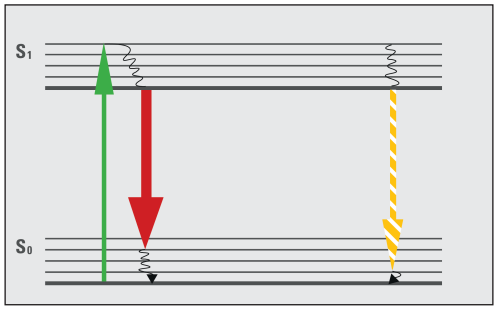




Myosin heads in slow skeletal muscles. Characteristic A-Band clearly visible.

The STED process



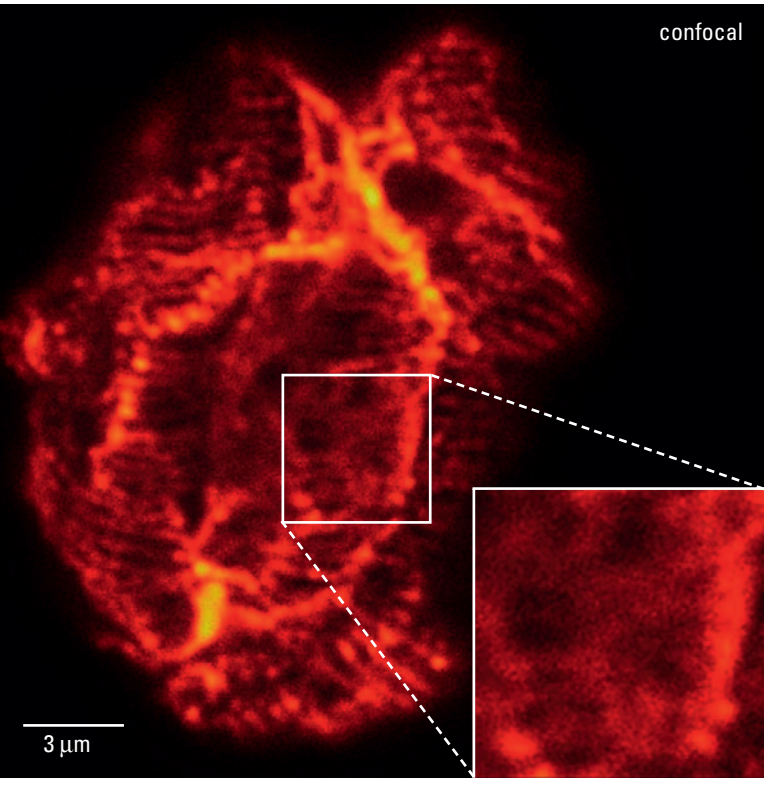


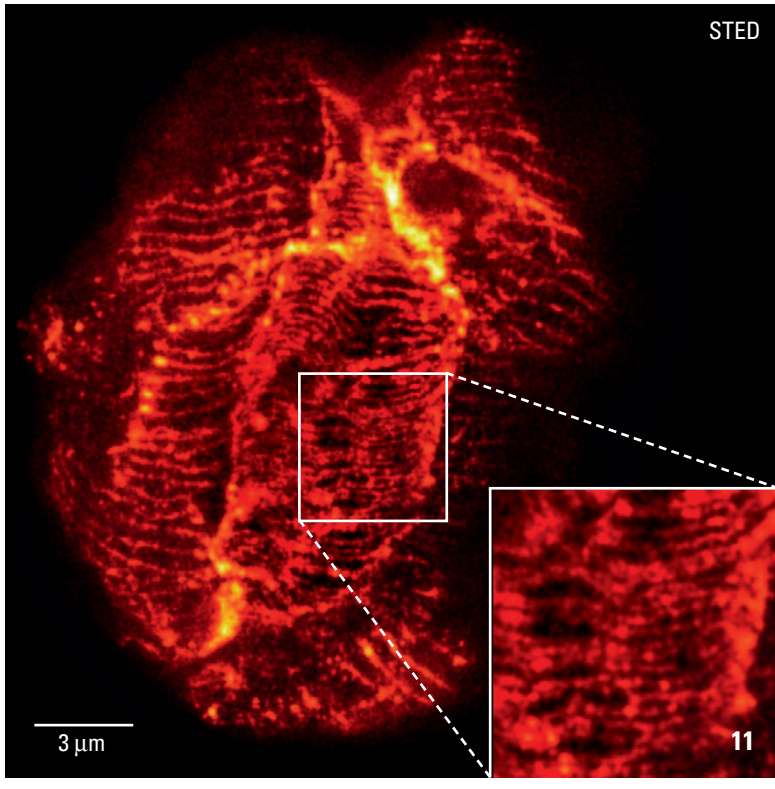
1. Detector
2. Excitation laser (635 nm)
3. Depletion laser (725 – 850 nm)
4. Phase filter
5. STED objective
6. Focused excitation spot
7. Overlay
8. Resulting fluorescence spot

Diffraction unlimited resolution – functioning principle of the STED Microscope

The key of resolution enhancement is the downsizing of the fluorescent spot. This is achieved by **ST**imulated **E**mission **D**epletion (**STED**): The fluorophores in the sample are excited by the pulses of the 635 nm laser (green). These pulses are directly followed by a pair of perpendicularly polarized beams from a red shifted stimulating light pulse (725 – 850 nm) – the STED pulse (red). It induces a depletion of the excited dye molecules before they can leave the excited state by emitting detectable fluorescence photons. Due to the doughnut shaped point spread function (7) of the depletion light the fluorescence inhibition by the STED process applies only to the outer regions of the spot. The inner part of the doughnut remains unaffected from this depletion process.

Page 6, bottom right: The totality (i.e. saturation) of fluorescence reduction in the outer regions is important for resolution improvement. The size of the remaining fluorescent area depends on the depletion efficiency, that is controlled by laser power, sample, staining etc.





Microtubular organization in toxic dinoflagellate *Karenia brevis*

Easy Access to Superresolution

The STED functionality is fully integrated into the Leica Application Suite (LAS AF). This user friendly and ergonomic platform has become a highly approved tool for microscopists. As soon as you are familiar with Leica LAS AF you are able to operate the STED system without needing to study new workflows. Your benefit? Time for your important research.

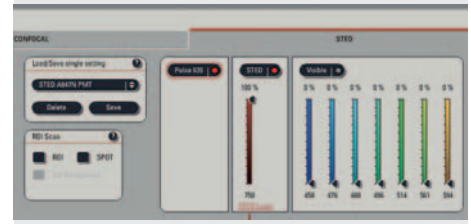
Click on a tab and work in confocal or STED mode. Depletion laser settings, scan speed, scan format and more can be saved in Leica's standardized Instrument Parameter Settings (IPS). Storage and recall of numerous dye and sample specific settings is achieved a click of the mouse.

Start in the confocal mode, adjusting the necessary parameters such as zoom, scan field, detectors and their sensitivity. According to your specific sample and needs, use the internal photomultipliers or switch to avalanche photodiodes. Move on to the STED tab, where the specific parameters are already preadjusted. Fine tune the settings like excitation laser power and detector gain if desired. Then capture the STED image with just one button.

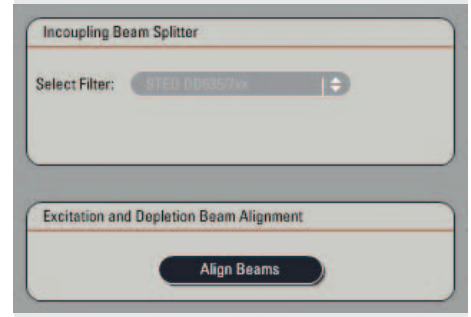
This concept minimizes undesired bleaching during the preadjustment process. The user deals with the relevant parameters only. It simplifies image acquisition and ensures maximal working efficiency. It's as simple as that!

Auto-Aligning for Reproducible Results and Ease of Use

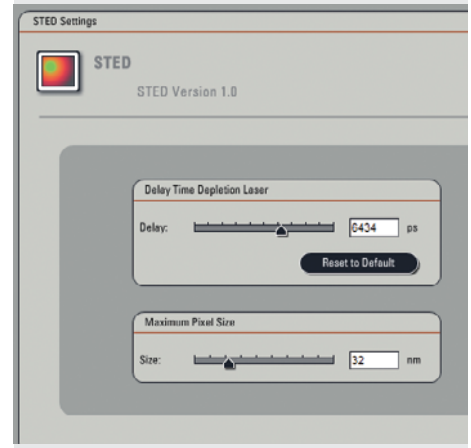
Time and space are important: Perfect synchronization of the laser pulses and nanometer accuracy of the beam alignment are a must for maximum depletion efficiency, equivalent to best resolution. In the Leica TCS STED this is realized by a patented and software controlled auto-alignment routine. Complex adjustments are history – calibration at a mouse click.



Intuitive user interface with easy toggle between STED & confocal mode



STED beam alignment automatically done (duration ~ two minutes)



STED settings can be fine tuned manually if desired

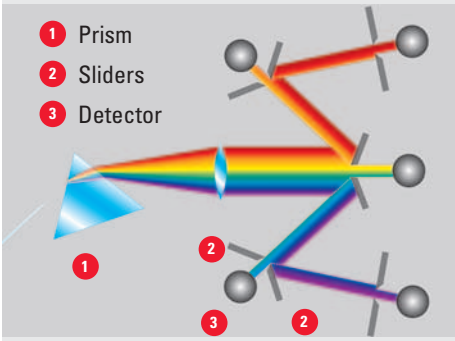
Leica TCS SP5

High Speed Live Cell Imaging and High Resolution Morphology – All in One:

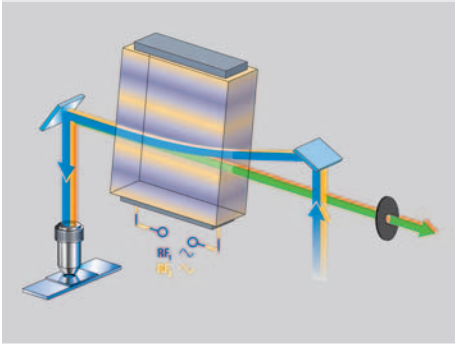
- 250 frames/sec - 64 mpx/image
- Fastest true confocal system

Leading in Multispectral Imaging:

- Acousto-Optical Beam Splitter, AOBS
- up to 5 spectral detectors
- ROI-spectrometer
- Intelligent and intuitive user interface: Leica Application Suite Advanced Fluorescence (LAS AF)
- Easy access, one interface for all
- Experiment wizards
- Fully integrated IR-laser with EOM



Spectral Detector, SP



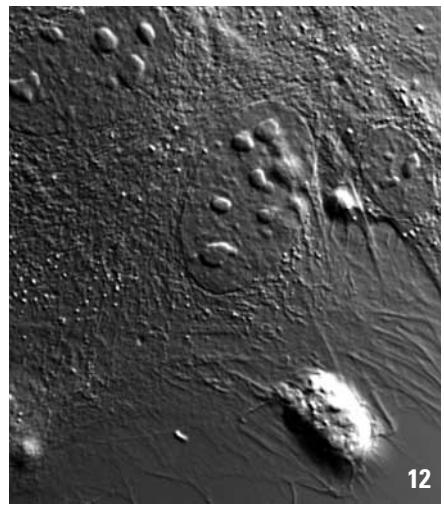
Programmable Acousto-Optical Beam Splitter (AOBS®)

Combined Power of STED and Confocal

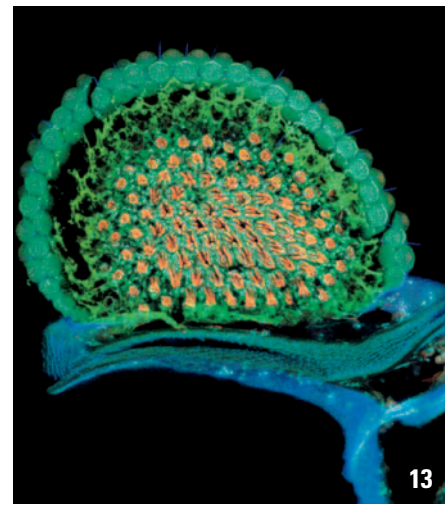
Find STED technology and the full versatility of the TCS SP5 combined in one system. The Leica TCS STED is not only a superresolution microscope but also a fully equipped multiphoton confocal system with up to five internal spectral detectors. Profit from the patented innovations of our broadband confocal, such as spectral detectors, the AOBS or the Tandem Scanner. With the fully integrated pulsed IR-Laser controlled by an electro-optical modulator you can do every kind of multiphoton experiment such as deep tissue penetration, ROI-scanning, etc.

With this combination of advanced technologies you are prepared for all future challenges – and you have two leading microscopy systems in one: The Leica TCS SP5 broadband confocal for high resolution and high speed imaging and the Leica TCS STED system for superresolution imaging. Toggle between the two worlds of resolution at your fingertips!

The powerful and highly versatile Leica TCS STED is ideal for imaging core facilities, even as a standalone confocal microscope.



Mouse fibroplasts
All contrast techniques (DIC, ICT) are available in the Leica TCS STED



Drosophila eye
Full range of multicolor experiments can be performed

Upgrade to STED – Anytime!

(Not yet) ready for STED? – Don't worry! STED is available as an upgrade for TCS SP5.

You might not need STED-resolution today but you think about using it tomorrow? We have the solution: You can upgrade your Leica TCS SP5 to STED – anytime. The necessary adaptations will be tailor-made for your present SP5 confocal microscope. The system grows with your demands. With a TCS SP5 you are ready for STED and prepared for the future.

The STED Workflow

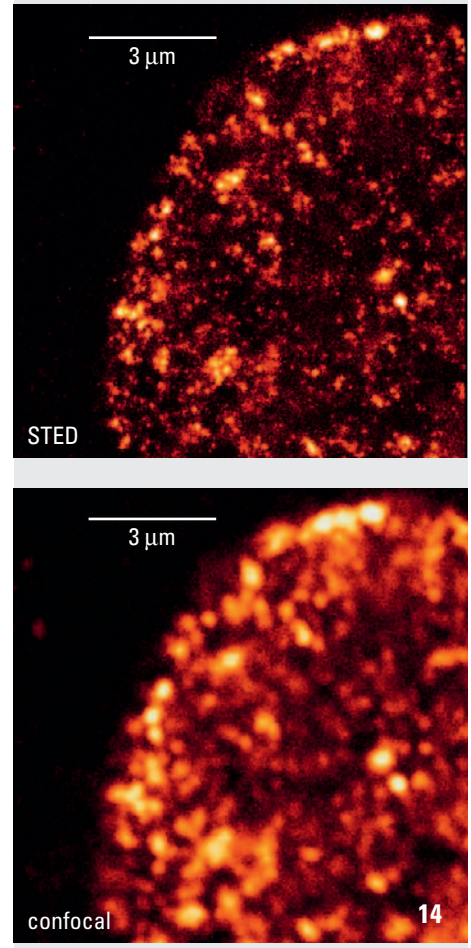
1. Place your sample on the microscope stage
2. Activate the CCD-camera
3. Select the area of interest in your sample
4. Go to confocal mode
5. Adjust your settings
6. Go to STED mode
7. Capture your image
8. That´s it!

Direct Optical Imaging – no Computational Artifacts

Until now, resolution in light microscopy was limited by diffraction as described by Ernst Abbe. Images of higher resolution have been obtainable only with computational methods such as deconvolution algorithms.

Today, these limitations are history. The groundbreaking improvement of a STED-microscope is superresolution achieved by an interplay of optics and photophysics. The image quality is independent of algorithm accuracy. It is purely optical. Excitation, depletion using the doughnut-shaped laser profile and emission are well understood and seamlessly integrated processes, This makes the system so easy to operate and its results scientifically reliable. Depending on the sample, particle distances of less than 70 nm have been clearly resolved.

Resolution improvement based on mathematical data processing can be applied additionally to STED images. Conventional deconvolution algorithms can be used, considering the STED-specific shape of the point spread function (PSF).



Histone H3 in Hela Cell nuclei ATTO 655-conjugated antibodies from Active Motif

STED Features

- STED-Excitation: 635 nm diode laser
- Depletion: Infrared Laser Spectra-Physics MaiTai Broadband
  – 725 – 850 nm usable for STED or
  – full spectral range (710 – 990 nm) for conventional two photon microscopy
- XY-resolution (FWHM) 90 nm, depending on sample, embedding and staining
- Typical point object separation ~70 nm
- STED dyes:
  – ATTO 647N (750 nm depletion)
  – ATTO 655 (780 nm depletion)
- Z-resolution: confocal
- Auto beam alignment of excitation and depletion beam for long term stability
- STED coupling is occupying UV port of the Leica TCS SP5, UV stainings can still be excited using the two-photon laser in non-STED operation

Objective HCX PL APO 100x/1.4 Oil STED

Teaming Up for Best Results

Improved colocalization analysis

Separating neighbouring organelles, vesicles or protein clusters by using different fluorescent tags and application of colocalization analysis is an important approach in biomedical research.

The resolution enhancement achieved by Stimulated Emission Depletion brings a completely new level of accuracy to colocalization studies. The STED detector channel (PMT 4) can be easily combined with up to four spectral confocal channels of the Leica TCS SP5. This allows you to use all common dyes for multicolor imaging parallel to your diffraction unlimited imaging in the STED channel based on ATTO 647N or ATTO 655 dyes.

Maximum sample flexibility

Each sample is different, last but not least in brightness. Users examining a broader range of samples with different intensities require a system with maximum flexibility in sensitivity and dynamics.

The Leica TCS STED fully adapts to your needs with its perfectly harmonized highly dynamic spectral photodetectors and extremely sensitive avalanche photo diodes. Use the spectral internal detectors for bright signals to get the maximum dynamic range. When cells are less bright, just switch on the APDs – sensitivity at a mouse click.

This flexibility to work with different kinds of samples and staining intensities leads to maximal imaging freedom on the nanometer scale.

Dedicated optics enable highest resolution

For obtaining optimal STED efficiency, exact overlap of the focal plane from excitation and depletion laser is essential. The large spectral shift between excitation and depletion wavelength of up to 150 nm requires a dedicated objective. Our STED objective features perfect chromatic correction to get the highest resolution possible. Moreover, it works perfectly for standard confocal imaging.

Fast visualization for instant results

The Leica TCS STED is equipped with a fully integrated DFC 360FX CCD camera. This enables fast visualization of the STED-dye labeled samples – which emit fluorescence in the far red spectral range and are therefore invisible to the human eye. The integrated camera makes it easy to identify appropriate cells or cellular regions. As the air-cooled CCD camera is fully controlled by LAS AF, there is no need to employ any additional software for camera imaging.

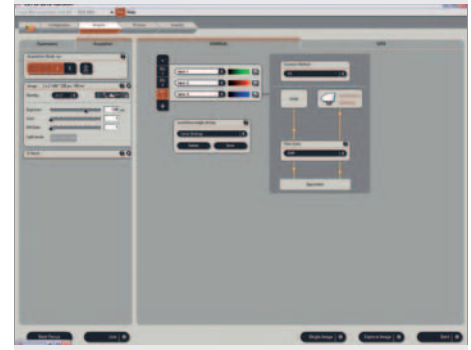
We talk science

Leica Microsystems assists you in driving your research by providing outstanding application support and consultancy. Our skilled bio-medical application specialists understand your experiments from sample preparation and basic imaging to advanced analytical protocols. They are available to support you before and after the installation of the system, ensuring efficient generation of top quality results.

With the new Leica TCS STED you conquer uncharted territories; the fundamentally improved resolution allows you to gather more information from your intact specimen than ever before. A very simple workflow, the full automation and perfect integration into the Leica TCS SP5 platform make STED technology a tool for everyday use. Enjoy the versatility of Leica confocal systems. Optimally adjusted components, such as the CCD camera, the STED objective or the avalanche photo diodes, provide you with a multitude of options – for full flexibility and maximum efficiency every day.

Confocal and Multiphoton Base System

- Inverted microscope Leica DMI6000 CS with fluorescence optical outfit Leica EL6000
- Spectral confocal laser scanning system Leica TCS SP5 (Tandem Scanner optional)
- Visible lasers with AOTF control
- AOBS® (Acousto-Optical Beam Splitter)
- Up to 5 spectral detector channels:
  - 4 confocal/two photon & 1 STED channel
  - 2 external APD (Avalanche Photo Detector) channels for highest sensitivity (1 usable for STED)



Leica DFC 360FX CCD camera
Full integration into LAS AF for accessibility with one click

Acknowledgements:

We gratefully acknowledge the following scientists for providing images and samples, respectively:

1 Colloidal crystal structure of fluorescent nanospheres ATTO 647N
Courtesy of Max-Planck Institute for Biophysical Chemistry, Goettingen, Germany

2 FtsZ-distribution in **bacillus subtilis** ATTO 647N
Courtesy of Phoebe Peters and Liz Harry, University of Technology, Sydney, Australia; Prof. Guy Cox, University of Sydney, Australia

3 Microtubular Network in Vero Cells β-Tubulin, ATTO 647N
Leica Microsystems CMS, Mannheim, Germany

4 Microtubular backbone of toxic dinoflagellate **Karenia brevis**.
Tubulin-ATTO 647N
Courtesy of Dr. Elisa Berdalet, Gisela Llaveria, Institut de Ciències del Mar (CMIMA-CSIC), Barcelona, Spain; Dr. Timo Zimmermann, Centro de Regulació Genòmica, Barcelona, Spain

5 Actin Fibers from Ptk cells F-Actin ATTO 647N
Leica Microsystems CMS, Mannheim, Germany

6 Neuromuscular junctions of *drosophila* larvae
Green: Liprin, ATTO 647N, red: Bruchpilot, Cy3
Courtesy of Prof. Dr. Stephan Sigrist and Dr. Werner Fouquet, University of Wuerzburg, Germany

7 Microtubular Network in Vero Cells β-Tubulin ATTO 647N
Leica Microsystems CMS, Mannheim, Germany

8 Membrane domains on plasma membrane sheets from PC12 cells
Syntaxin-1 ATTO 647N
Sample: courtesy of Dr. Thorsten Lang, Max Planck Institute for Biophysical Chemistry, Goettingen, Germany

9 Titin T12 in rat myofibrils. ATTO 647N
Courtesy of Dr. Elisabeth Ehler, Kings College London, England

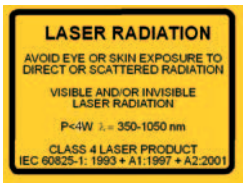
10 Rat myofibrils Myosin heads ATTO 647N
Courtesy of Dr. Elisabeth Ehler, Kings College London, England

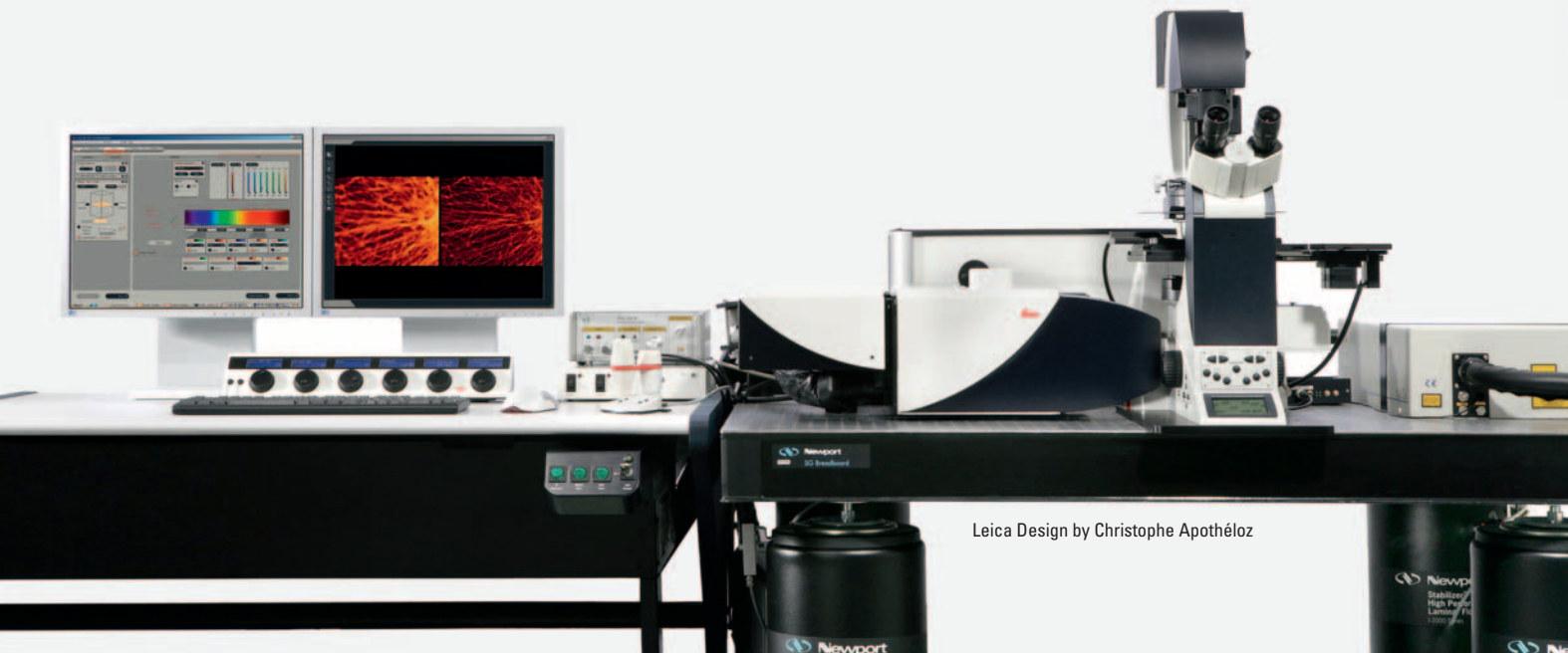
11 Microtubular backbone of toxic dinoflagellate **Karenia brevis**
Tubulin-ATTO 647N
Courtesy of Dr. Elisa Berdalet, Gisela Llaveria, Institut de Ciències del Mar (CMIMA-CSIC), Barcelona, Spain; Dr. Timo Zimmermann, Centro de Regulació Genòmica, Barcelona, Spain

12 Mouse fibroblasts transmitted light (DIC)
Courtesy of Dr. Günter Giese, Max Planck Institute for Medical Research, Heidelberg, Germany

13 **Drosophila melanogaster** (eye section)
Red: F-Actin, Cy3, blue: nuclei, DAPI; Green: pigmented cells, GFP
Courtesy of Anne Galy, IGBMC, Strasbourg-Illkirch, France

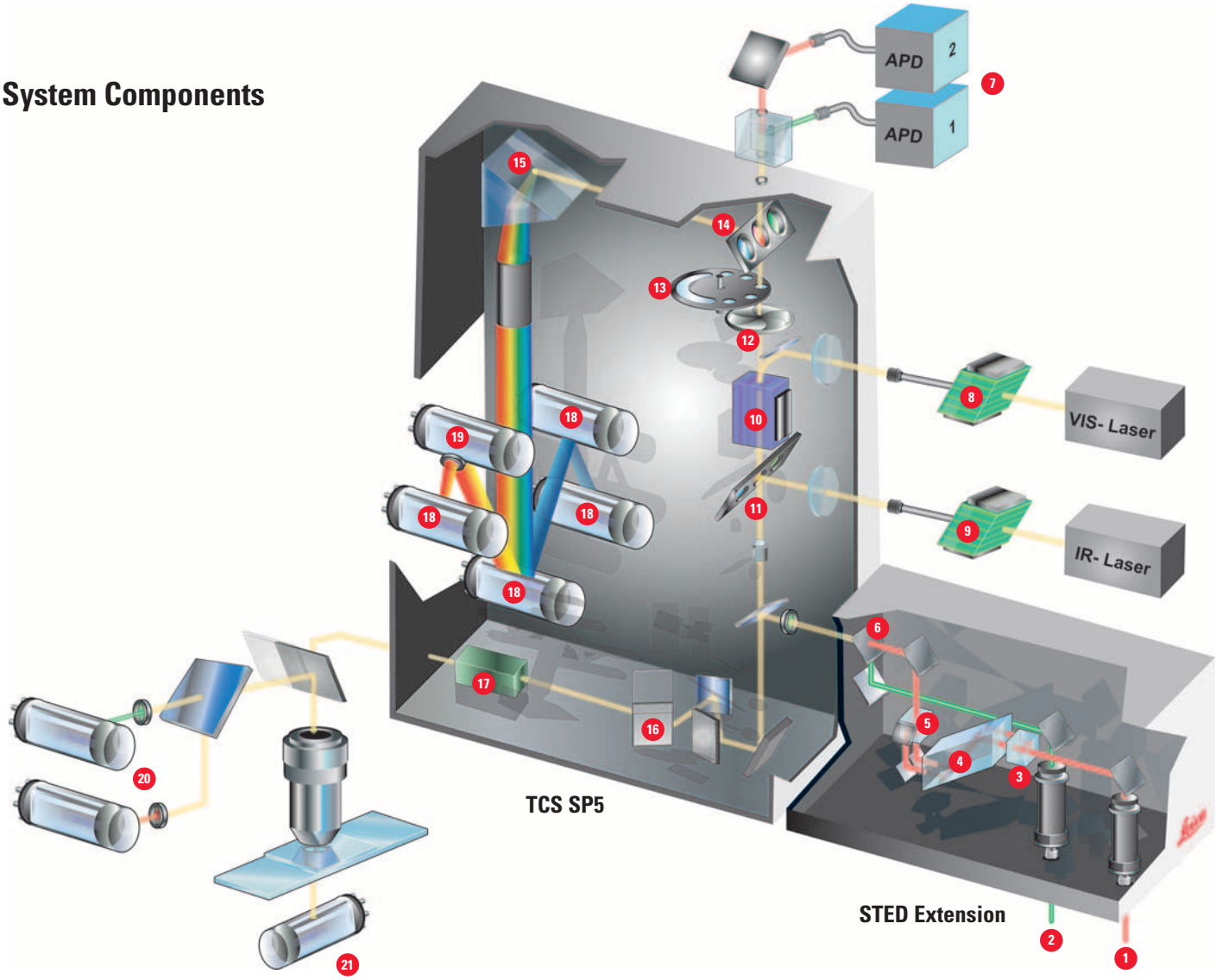
14 Histone H3 in Hela Cell nuclei
Courtesy of Dr. Brian Bennett
ATTO 655-conjugated antibodies from Active Motif





Leica Design by Christophe Apothéloz

System Components



1. Incoupling IR laser for STED
2. Incoupling 635 nm laser
3. Beam splitting prism
4. Phase filter
5. Beam combining prism
6. Dichroic LP650
7. Avalanche Photo Detectors (APD)
8. Visible range AOTF
9. IR EOM
10. AOBS
11. Multi function port
12. Confocal detection pinhole
13. Filter- and polarizer wheel
14. X1 – emission port
15. Spectrophotometer prism
16. Tandem Scanner
17. Field rotation optics
18. Photomultiplier 1,2,3 & 5
19. Photomultiplier 4 (STED)
20. Reflected light nondescanned detectors
21. Transmitted light detector

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