Feb.
2009
No. 32

CONFOCAL APPLICATION LETTER

reSOLUTION

Imaging with the Leica TCS STED – a Practical Guide

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

Leica
MICROSYSTEMS

Content

- Stimulated Emission Depletion – the basic principle

Practical aspects of STED imaging:

- Specimen
- Dyes and staining
- Sample preparation
- Image acquisition
- Tips & tricks

More than 130 years ago Ernst Abbe discovered that the investigation of specimens with a light microscope is limited by the nature of light itself. He found that objects that are closer than half the wavelength of visible light ($d=\lambda/(2NA)$) could not be optically separated. This established an apparently unbreakable resolution barrier of ~200 nm in far field light microscopy. However, Stefan Hell has recently broken this barrier. Using the concept of STimulated Emission Depletion (STED) in combination with confocal scanning microscopy, Hell has demonstrated that it is possible to image below the theoretical limits of resolution. This has allowed for the imaging of structures previously impossible to visualize using a far field microscope.

Cover:
Muscular protein titin - Isolated myofibrils from rats. Doublet (distance ~170 nm) resolved with STED. Both images (top: confocal/bottom: STED) are linearly deconvolved.

Courtesy Dr. Elisabeth Ehler, Kings College London, UK

Stimulated Emission Depletion – the basic principle

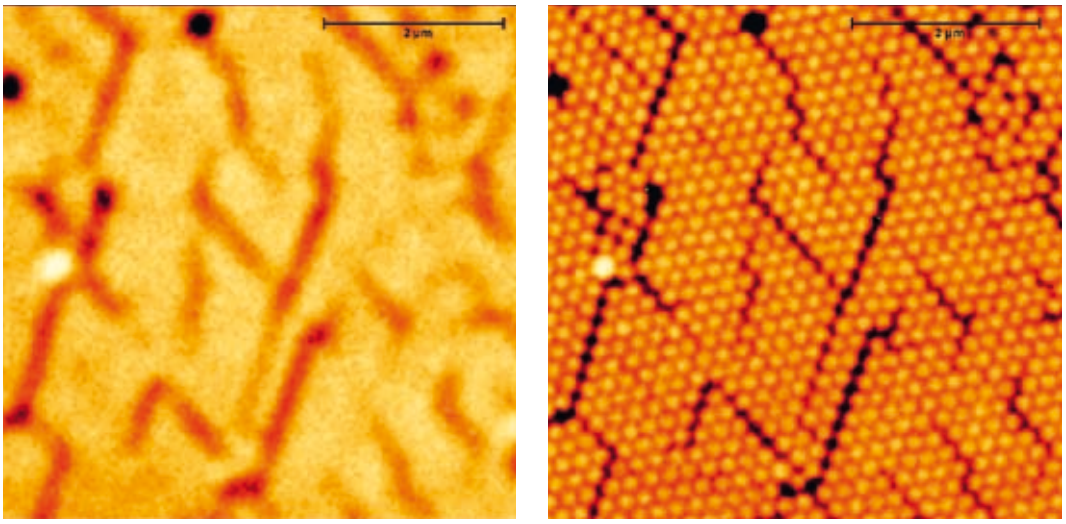


Fig. 1: Layer of fluorescent nano-beads imaged under optimal confocal conditions (left) and with the same settings with the STED-depletion laser turned on (right).

A conventional laser scanning microscope excites fluorophores in a sample with a diffraction limited spot resulting in ~200 – 250 nm lateral resolution under optimal conditions (High NA objectives, short wavelength of excitation light).

With a Leica TCS STED microscope the specimen is scanned by two pulsed laser beams that are tightly synchronized. The 640 nm wavelength laser excites the sample just as in a conventional confocal system. The excitation laser pulse is directly followed by a ring-shaped pulse from a Ti:Sapphire infrared laser. This ring is created from a pair of modulated beams (**Fig. 2a**). The wavelengths of these beams, 730 – 780 nm, do not excite the fluorophores in the sample (ATTO 647N or ATTO 655, **Fig. 6**, p. 7). Instead, the paired beams stimulate the excited molecules back to the ground state, **without emitting detectable fluorescence**. The initial process called "stimulated emission" was described by A. Einstein in the beginning of the 20th century and set the basis for the development of lasers.

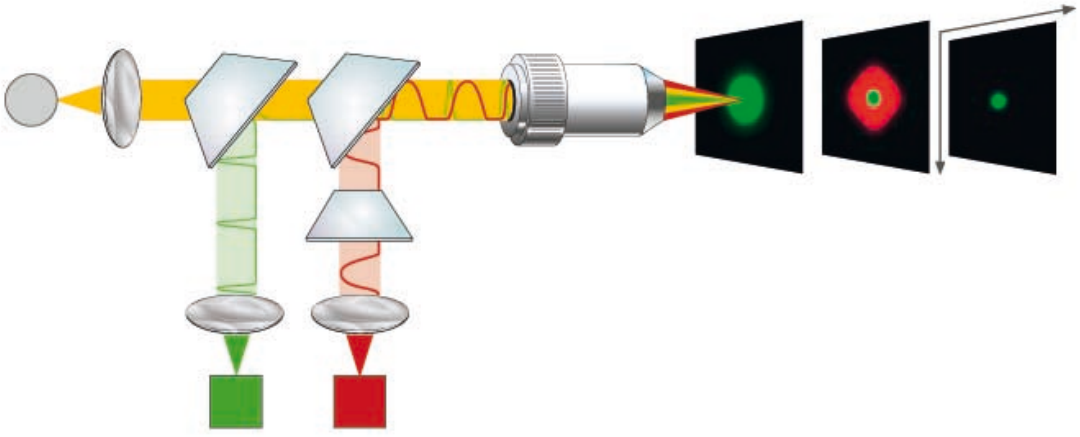


Fig. 2a: Coupling of STED excitation laser pulses (green) and STED depletion beam pulses (red) into the SP5 scanner to create the doughnut shaped fluorescence depletion for resolution enhancement.

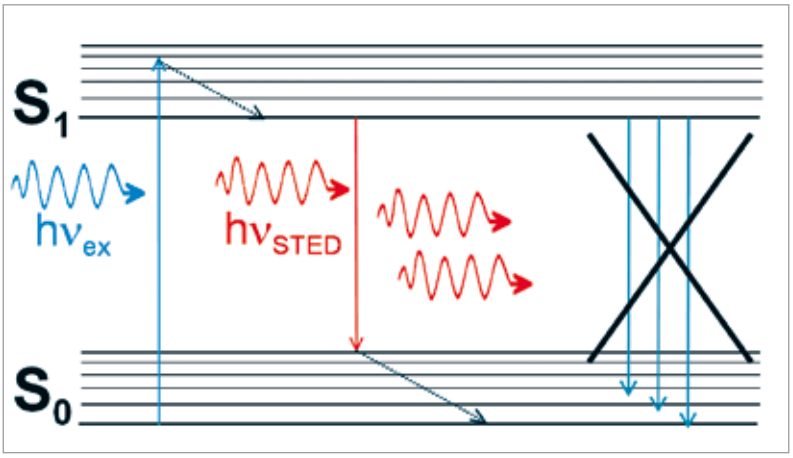


Fig. 2b: Process of stimulated emission: the fluorophore is excited to an upper electronic level by taking up energy (*hν*) from the excitation laser (blue) and is forced to return to the ground state by the depletion laser energy. This results in the generation of a second photon with the same spectral properties as the stimulating one so that its signal can be filtered out spectrally.

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

Fig. 3: Extended form of Abbes law of diffraction. (**d** = distance; λ = wavelength, **NA** = numerical aperture, I_s = saturation intensity of depletion laser, i.e. the power that is necessary to halve the population of the excited state (this value depends on dye molecules and wavelength, I = maximum intensity of depletion laser)

The term implies a theoretical infinite resolution for $I \rightarrow \infty$.

Because of the doughnut-shaped beam profile, this depletion process occurs exclusively in the outer area of the excited spot, leaving the inner region unaffected. The remaining excited molecules emit their fluorescence and are detected. Because the depopulation of the excited state scales nearly exponentially with the intensity of the depletion laser, virtually all excited molecules in the ring-covered area are depleted from the excited state. The size of the inner area determines the resolution and depends on the depletion laser power. The higher the power, the smaller is the spot size, which results in higher resolution. Theoretically, this process could be continued down to molecular level, but there are practical limitations such as dye stability, vibrations, sufficient signal, and quality of the minimum. With the TCS STED it is possible to get resolution values of less than 90 nm (FWHM) routinely, assuming correct sample treatment and imaging parameters. This increase in resolution is purely optical, without employing image processing such as deconvolution or center of mass determination.

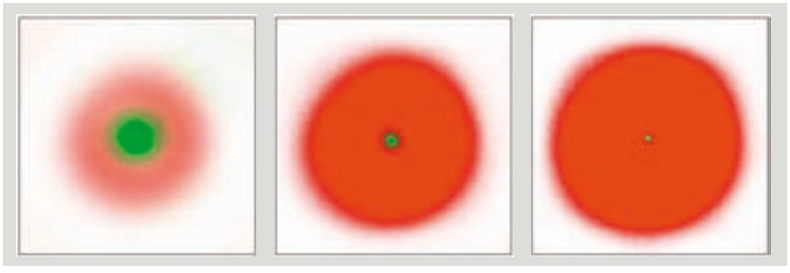


Fig. 4a: The effective fluorescent area (green) decreases with increasing depletion laser power (red).

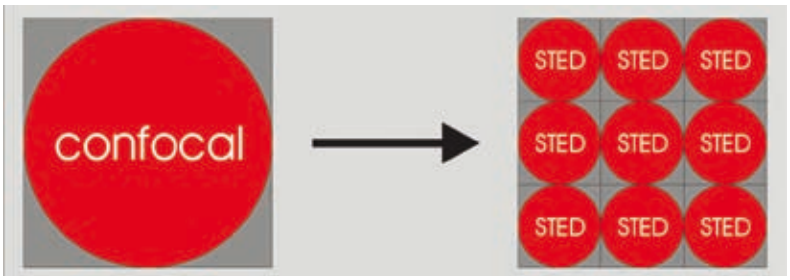


Fig. 4b: 3-fold reduction of the scanning spot size in x and y yields 9-fold more accurate sampling.

Practical Aspects of STED Imaging

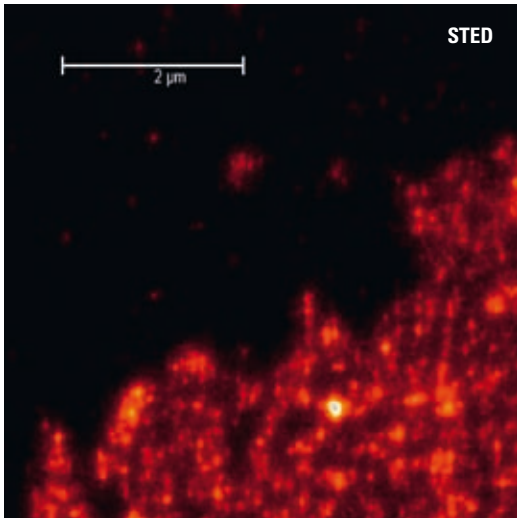
Specimen → Staining → Mounting → Image acquisition → Data analysis

The following sections describe the steps to acquire a high quality STED image. These steps are not significantly different from conventional confocal microscope practice. This starts with the appropriate labeling of the structure of interest by a STED-suitable fluorescent dye. Subsequently, the sample needs to be **embedded** correctly, before finally **images** can be **acquired** and **analyzed**. Each step influences the quality of the final result. The guide will start by discussing the selection of appropriate specimens to use in order to achieve good results with the Leica TCS STED. (Data analysis will not be discussed here.)

Specimen

STED microscopy has already been used successfully to answer questions from the fields of neuroscience, membrane biology and intracellular transport, where a variety of specimens have been investigated (see suggested readings, p. 19).

The criteria for a sample to result in a good STED image are not different from the ones in standard fluorescence microscopy: best results are obtained from **bright samples** with **high contrast**. The process of Stimulated Emission Depletion leads to a reduction of the area from which fluorescence is emitted, thus resulting in a signal intensity decrease. To obtain a good signal/noise ratio in the resulting STED image, one should start with a suitable confocal image. As an estimate for the TCS SP5, a good STED signal can be expected if one gets saturation of the internal photomultiplier (1250 V) with ~ 10% of the Helium-Neon 633 nm laser.



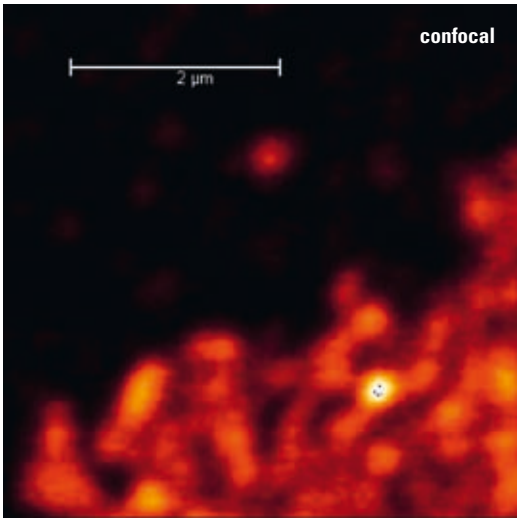


Fig. 5: Clustering of **Syntaxin 1A Nanodomains on the basolateral Membrane of PC12 Cells**: The nanoresolving power of STED microscopy allows for the determination of size and density of these biological entities.

Sample: courtesy of Prof. Dr. Thorsten Lang, University of Bonn, Germany

Dyes and Staining

STED microscopy is based on fluorescence. Consequently, the structure of interest has to be labeled specifically with an appropriate fluorophore. Careful control of the spectral conditions and dyes is necessary to get the best results with the STED microscope. The lasers used in the Leica STED, i.e. the pulsed 640 nm laser for excitation and the Ti:Sa-IR-Laser for depletion (STED-wavelengths ~730-780 nm) make the ATTO 647N and ATTO 655 the dyes of choice for STED experiments. These dyes are also suitable for standard fluorescence microscopy. In general, all fluorescent dyes are susceptible to stimulated emission but the vast majority cannot be used for sub-diffraction imaging due to their optical properties such as: Cy5, that can be depleted but is bleached after one scan.

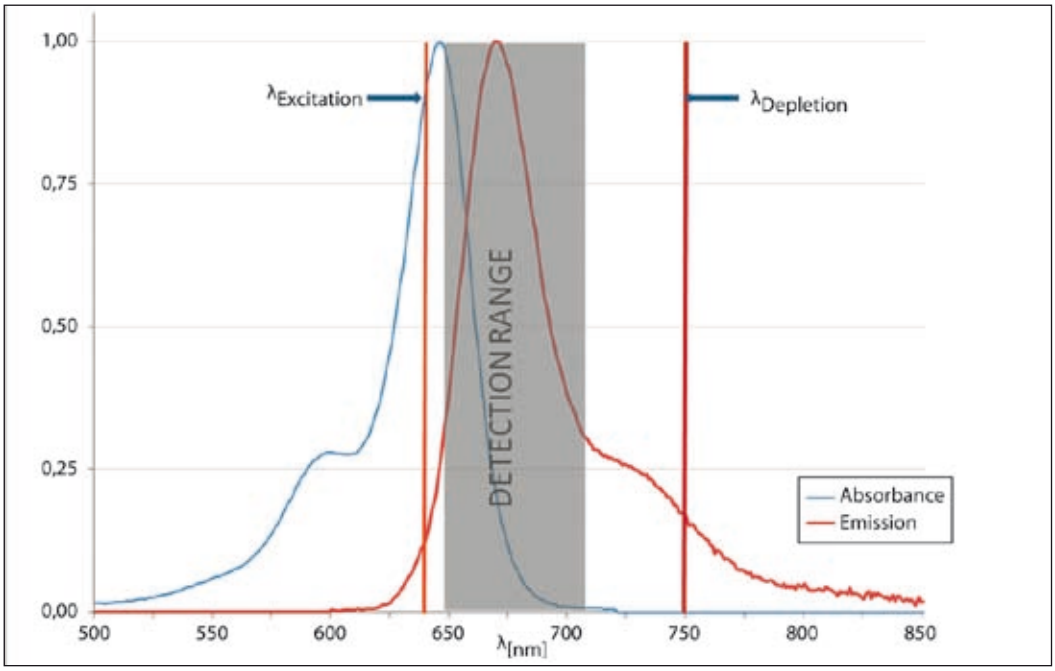


Fig. 6: Normalized excitation and emission spectrum of ATTO 647N. For STED excitation and depletion wavelength are at 640 and 750 nm respectively. The depletion wavelength can be tuned.

Criteria for a good STED dye:

- high cross section for stimulated emission
- no excitation at depletion wavelength
- photostability at depletion and excitation wavelength
- low Triplet-state population
- low non-linear photobleaching
- reactive group usable for coupling (e.g. to antibodies)

➡ **best choices for Leica TCS STED: ATTO 647N and ATTO 655**

Sample Preparation

The preparation of the sample is identical for STED and confocal microscopy. Well-known standard techniques such as direct and indirect immunofluorescence, FISH (Fluorescence In Situ Hybridization) and other labeling techniques (e.g. actin cytoskeleton labeling with phalloidin) can be used.

Imaging in the sub-100 nm range requires one to consider the size of the label itself. In immunofluorescence (IF) the specificity of antibodies (Ab) is utilized to fluorescently label biological structures of interest. In direct IF the dye is coupled directly to the primary antibody that specifically binds the structure. In indirect IF, secondary antibodies that recognize epitopes on the primary antibodies carry the dye. The size added to the structure of interest by the labeling procedure is usually larger in indirect IF, and here more preparation steps are necessary. However, the signal intensity is increased, due to the binding of several secondary antibodies, each with several fluorophores, to one primary antibody. Indirect immunofluorescence is normally preferred because one secondary antibody can be tagged to numerous primary antibodies, which greatly amplifies the signal.

Fig. 7: Direct and Indirect Immunofluorescence
While direct IF brings the fluorescent dye closer to the structure of interest, the indirect IF allows signal enhancement by the attachment of multiple secondary antibodies carrying fluorophores.

Mounting

Particularly when imaging with high NA objective lenses not only the microscope, but also the embedding medium, the cover slip and the immersion medium have an important impact on image quality and obtainable penetration depth. This is mainly due to the effect of refractive index (mis-) matching.

Changes in the refractive index along the optical path induce aberrations, leading to less resolution, and less signal, resulting in lower efficient depletion. This compromises the achievable resolution. The Leica TCS STED is equipped with a special 100x 1.4 NA oil objective optimized for cover slips of 170 µm thickness.

Oil (Leica Type F Immersion Fluid, n_e^{23} = 1,518) is used as the immersion medium. Therefore the refractive index of the embedding medium should be as close as possible to 1.518. Good STED results have been obtained with standard embedding media such as glycerol, mowiol and vectashield. Superresolution images could be obtained easily from planes 10 – 15 µm deep inside the sample, depending on its nature. A recent publication explores the possibilities of a new mounting medium, TDE, which provides perfect refractive index matching from water to oil. Initial results have shown that the STED dyes ATTO 647N and ATTO 655 perform nicely in TDE, paving the way for STED imaging far deeper inside the sample.

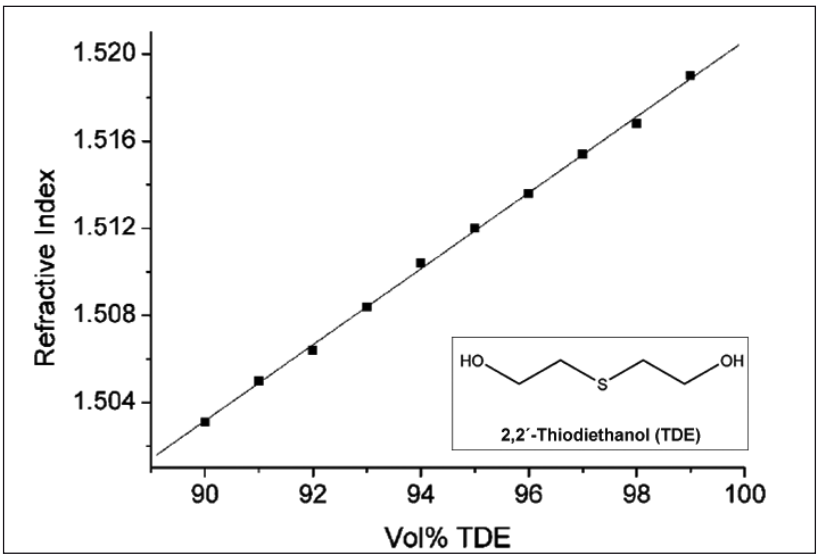


Fig. 8: 2,2' – Thiodiethanol. The refractive index of TDE/H_2O mixture can be freely (and linearly) adjusted from the refractive index of H_2O (1.332) to the refractive index of oil (1.518) by changing the ratio between the two constituents. Therefore the use of TDE/H_2O allows perfect matching of the refractive index of the embedding medium to the immersion medium, giving access to super resolution imaging deeper than 15 µm inside the sample. The characteristics of TDE/H_2O as embedding medium have been explored in detail in a recent publication (Staudt et al., Microsc Res Tech 2007).

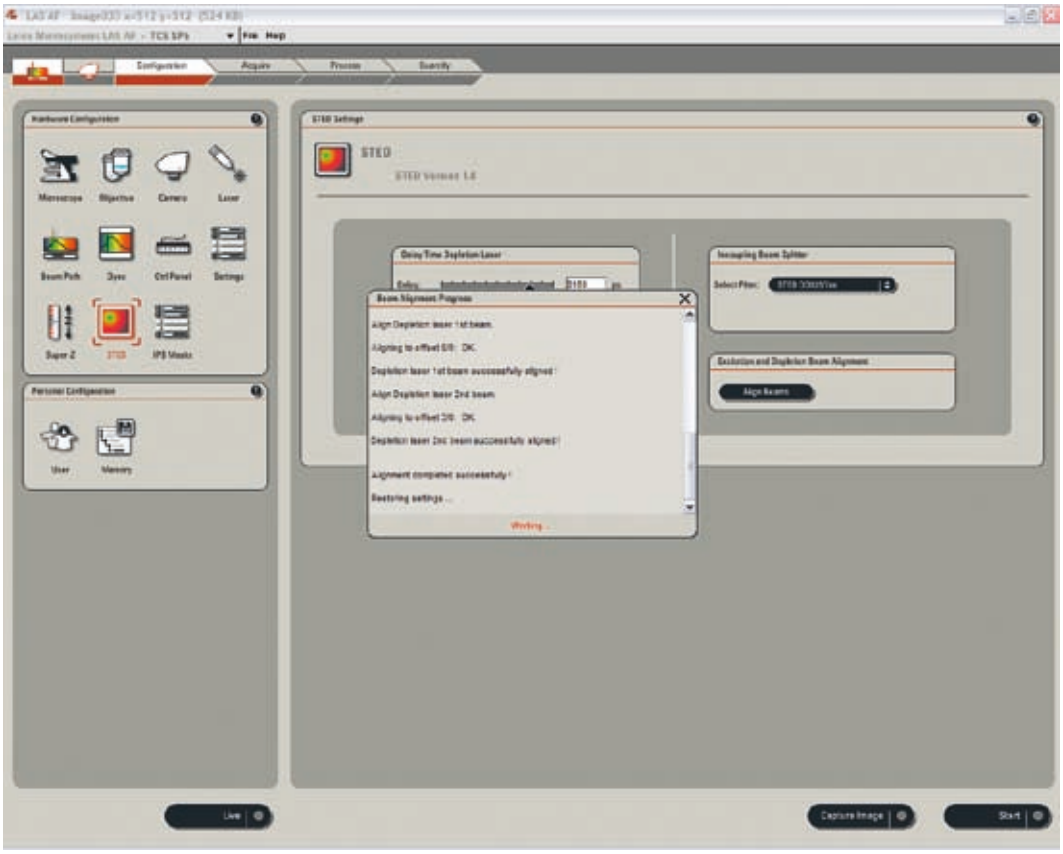
Image Acquisition

The operation of the Leica TCS STED is controlled completely by LAS AF software, just like the Leica TCS SP5. Because of the full integration of the STED-specific settings and operating steps, the access to super resolution STED images is as easy as taking conventional confocal images. The quick overview outlined below explains what to consider when acquiring data with the Leica TCS STED. Important points are listed in the box Tips and Tricks, p 13.

The auto alignment:
A perfect lateral overlay of the excitation spot and depletion laser is essential to perform STED microscopy successfully. As described before, the process of depletion must omit the inner region of the illuminated area to allow for fluorescence emission without compromising the resolution enhancement.

A fully automated alignment routine optimizes this by adjusting the involved laser beams with motorized optical elements. This routine should be carried out when starting up the system and then every hour thereafter to ensure optimal alignment. This is necessary because the effects of minor thermal drifts cannot be excluded completely in a complex system with many different sources of power, i.e. heat.

The alignment is started by one mouse click and takes ~ two minutes. Technically, the alignment is conducted on an internal target slide with a special reflective surface. This slide is inserted into the intermediate image plane. The sample is not illuminated during this process and does not have to be removed. Reflection images of the target slide surface are recorded with the individual lasers and compared (internal references: 594 nm, then 640 nm excitation laser, and last the 710 nm IR laser beams). Deviations are analyzed by a correlation algorithm and compensated for by moving the beam deflecting mirrors until a perfect overlay is achieved. No reference sample is needed. Experiments can be continued immediately - all settings are automatically restored.



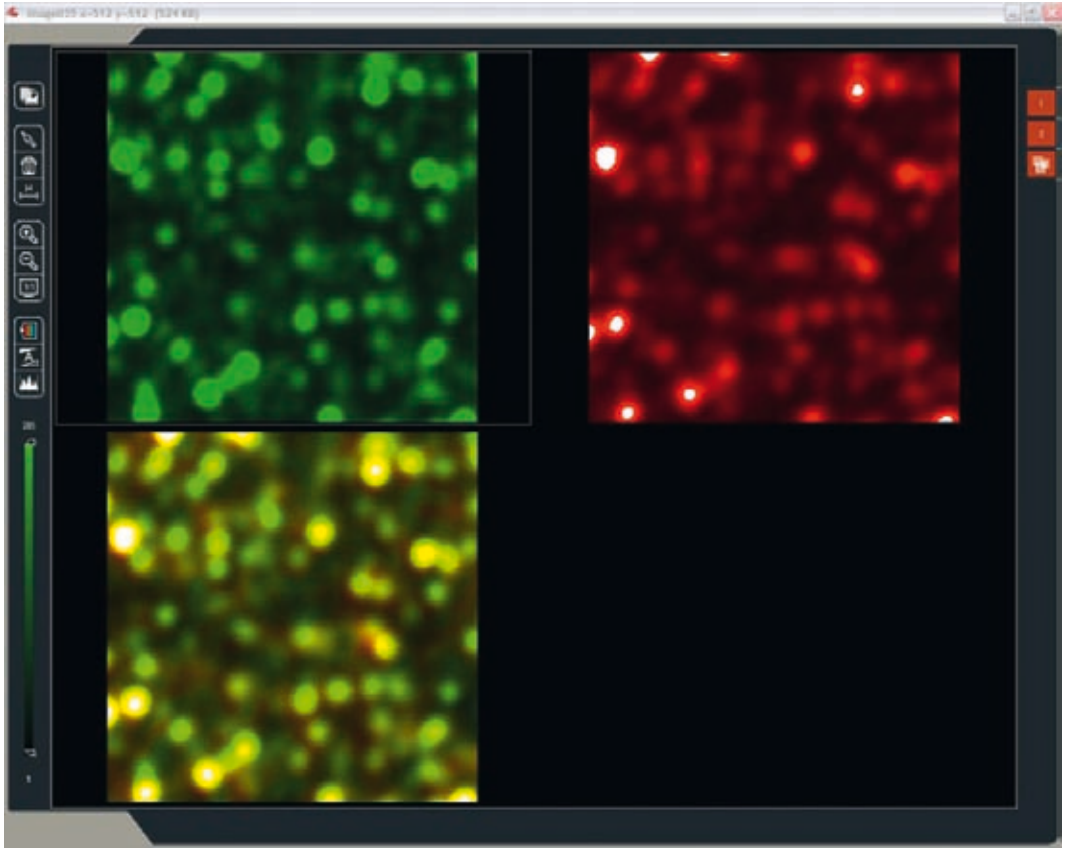


Fig. 9: Alignment procedure to ensure the perfect overlay of the excitation and depletion laser
Top: Parameters accessible under **Configuration**.
Bottom: Image of the target slide surface, consisting of a reflective sand sputtered surface (top left: 640 nm, top right: 710 nm, bottom left: overlaid image after auto alignment).

The workflow (Camera > Confocal tab > STED tab):
The STED dyes ATTO 647N and ATTO 655 fluoresce in the far red spectral range, hardly visible for the human eye (sensitivity at 680 nm <5% compared to 550 nm). Thus it is difficult to select an appropriate area of the fluorescent sample through the eyepieces without a counter stain such as Alexa 488. For convenience, the system is equipped with a fully software integrated CCD-camera, the DFC 360FX, so that one can find the focal plane and navigate on the sample. Having found a suitable area of the sample it is then possible to toggle over from **Camera** to **Confocal** mode.

The higher resolution and the reduced signal intensity of STED images require scanning with more pixels (i.e. larger format) at a slower scan speed, and/or with higher averaging compared to their confocal counterparts. To account for this and to deliver an optimized workflow, two independent acquisition settings can be accessed under **Acquire**: the **Confocal** and the **STED** tab, between which one can toggle easily.

To avoid undersampling, the format is adjusted automatically when the user switches from the **Confocal** to the **STED** tab. The **Confocal** tab is used to select the optical plane, the field of view etc. in a fast live scan and to obtain confocal images. The **STED** tab provides control of the STED laser and access to a second set of imaging parameters that can be fine-tuned for STED data acquisition (e.g. scan speed, averaging). When changing to the **STED** tab, the format is adjusted automatically to avoid undersampling due to pixels that are too large.

The most convenient workflow is to start with fast imaging using the CCD-camera (filter for ATTO dyes: Y5) to find the focal plane and to select the area of interest by moving the xy-stage. After that, the user switches to Confocal mode and chooses the precise field of view and optical plane that should be imaged. To take a STED image, the user toggles over to the **STED** tab, and then, after making fine adjustments as needed, captures the STED image. Subsequent imaging can be performed very fast, due to the separate storage of the now-defined confocal and STED settings.

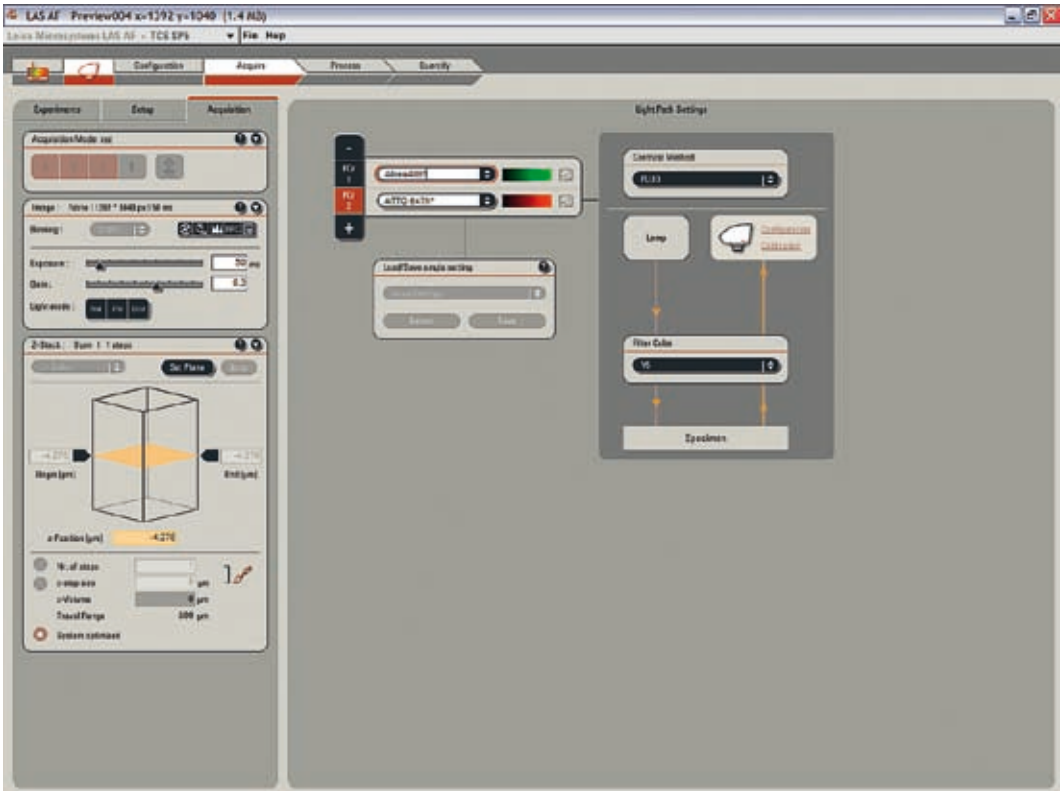


Fig. 10: User interface of the DFC 360FX. Different filters are available, binning, illumination time and gain can be adjusted.

Things to consider acquiring STED images

Improved resolution requires the use of smaller pixel sizes to avoid undersampling. Shrinking the area where the fluorescence signal is emitted from, as is done in STED microscopy, further results in less signal per pixel compared to standard confocal microscopy. The Leica TCS STED is equipped with two different types of detectors – Photomultiplier Tubes (PMTs) and Avalanche Photo Detectors (APDs) – to allow imaging of the full range from very bright to rather dim samples.

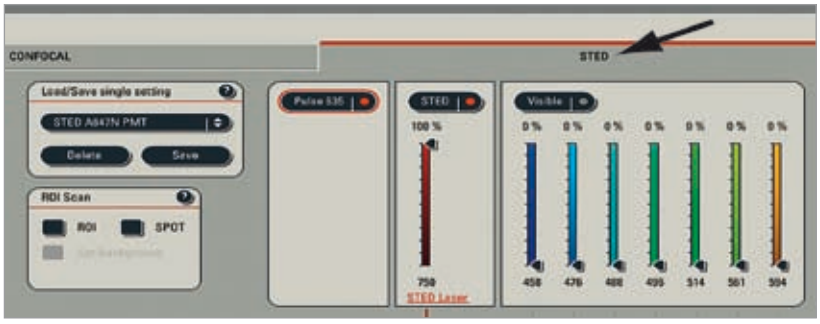


Fig. 11: The Confocal and the STED tab: In scanning mode two different settings of acquisition parameters can be easily accessed by toggling between the STED and the Confocal tab.

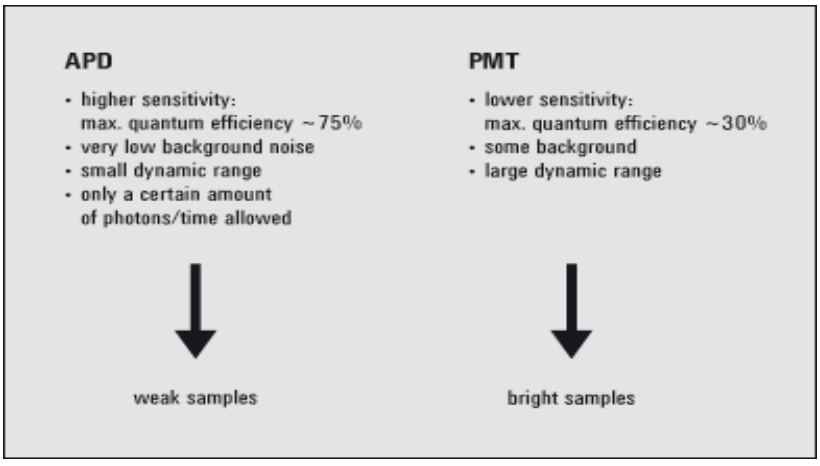


Fig. 12a: Pros and Cons of APDs and PMTs.

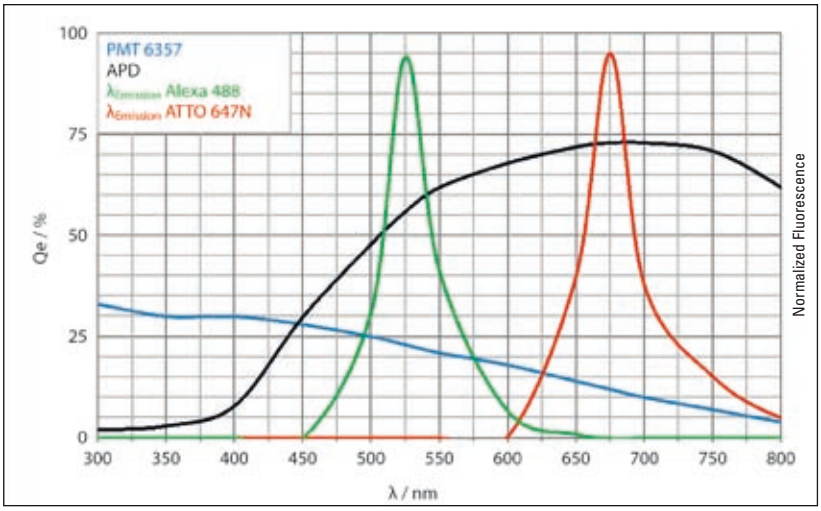


Fig. 12b: Comparison of detector sensitivity.

The scan speed and therefore image acquisition time depends on the signal/noise ratio. 100 Hz and 4 times line averaging is an appropriate setting to image a biological sample of average intensity, resulting in an overall scan speed of ~20 seconds/frame at 512 x 512 pixels. Images have also been acquired at a much faster rate up of to 19 frames/s, depending on the sample (achievable S/N ratio) under investigation.

It is recommended to use a higher rather than a lower excitation intensity to obtain the optimal signal/noise ratio and to rule out potential excitation by the Ti:Sa IR laser. The pulsed 640 nm laser diode is controlled manually. To ensure optimal pulse timing a certain intensity value (usually 5.6 – 5.7) should not be exceeded. Furthermore, the excitation should not be saturated, meaning that a moderate increase in excitation should still be reflected by an intensity increase in the image.

Images with STED resolution are recorded with the active STED laser (shutter open, red light at the slider). As soon as the STED laser is inactive, the images exhibit confocal resolution. The slider allows the user to freely adjust the intensity of the depletion laser. As derived from the modified Abbe equation, higher resolution is obtained with higher depletion power, but other effects such as reduction of signal (eventually lead to worse S/N ratio) and re-excitation have to be considered as well. Therefore the optimal depletion and excitation power should be determined individually for each sample.

The Leica TCS STED grants access to resolution beyond the diffraction barrier in xy while still providing confocal resolution in z. Therefore it is easy to image 3D stacks with increased spatial information in each optical section.

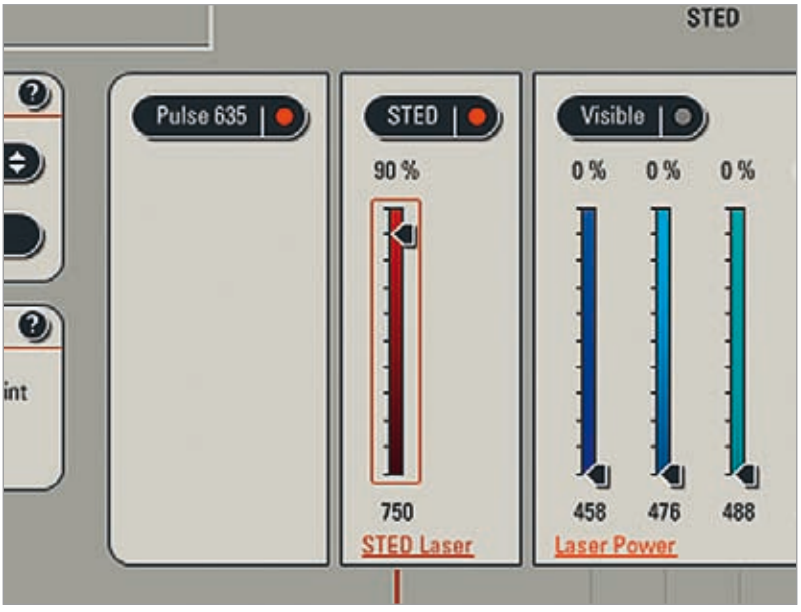


Fig. 13: Intensity adjustment for depletion laser. The slider allows adjusting the resolution of the recorded image by changing the depletion effect (proportional to laser power).

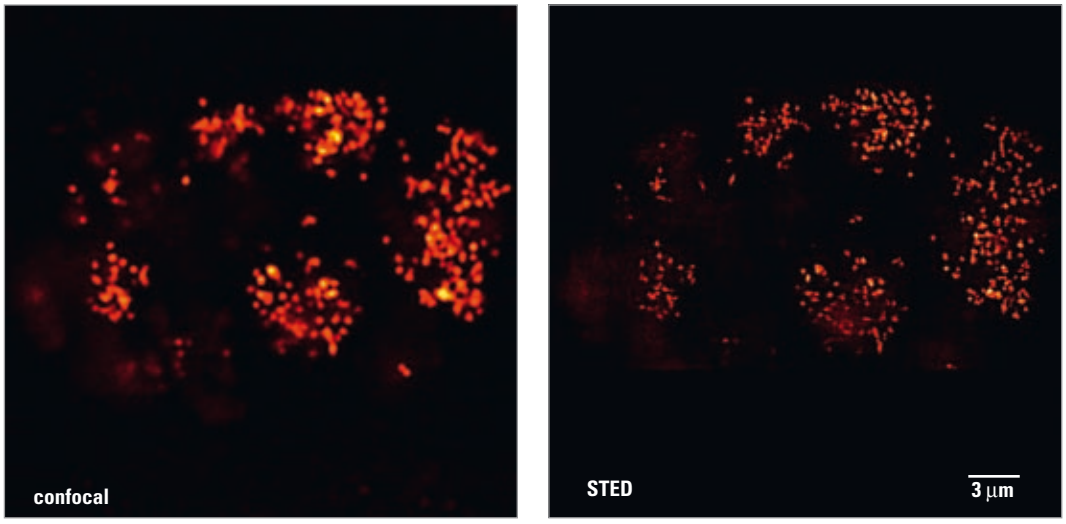


Fig. 14: Replication foci (= chromosome territories) in a cell nucleus visualized with Fluoresence In Situ Hybridization (FISH) revealing amount and size of clusters. Confocal (left) and STED image (right) were processed by the same linear deconvolution algorithms.

Courtesy of Dr. Marion Cremer, LMU Munich, Germany

Tips & Tricks

- Use cover slips of 170 µm thickness and Leica Type F Immersion Fluid (Cat. Nr. 11513 859).
- Match the refractive index of the embedding medium to the one of oil (n_e^{23} = 1,518) to gain maximal penetration depth.
- Perform auto alignment at start up of system and every hour thereafter.
- Choose the appropriate detector for your sample (bright sample: internal PMT No. 4; weaker sample: external APD).
- Stay close to optical axis (apply panning only within zoom 3 region).
- Do not saturate excitation / do not use excitation above a certain value (5.7).
- In general full depletion power yields maximal resolution increase. But the optimal depletion power to use with a certain sample should be determined individually.
- If you want to scan faster, select a smaller region with a higher zoom value, resulting in a smaller scan field (e.g. selection of zoom 18 (scan field ~8 x 8 µm) that can be recorded with 256 x 256 format.
- Further increase in speed can be achieved by scanning rectangles rather than squares without compromising needed pixel size (e.g. 512 x 256 scan field).
- Correct data sampling according to the Nyquist theorem (~2,5-fold oversampling, STED pixel size 20 – 32 nm).

Correct data sampling*

Data acquisition with a scanning microscope implies the digitization of the fluorescence signal emitted from the sample. This means that a quasi-continuous signal is converted into a subdivided one, with the pixels as the associated entities. According to the Nyquist theorem, a reliable digitized representation of the data is only assured if the sampling frequency is at least two times higher than the highest spatial frequencies in the sample, which in imaging translates to the highest possible resolution. In practical terms of confocal microscopy, correct sampling is granted by pixel sizes at least 2.3 times smaller than the theoretical resolution.

*This section does not only apply for STED but for confocal imaging, too.

Example:

- Objective: HCX PL APO 100x/1.4 Oil
- Excitation wavelength: 488 nm
- According to Kino: $FWHM_{xy}$ = 0.4 * exc / NA, multiplied by 1,5 – 1,7 (depending on Stokes shift of the dye)
- FWHMxy ≈ 210 nm Required pixel size for accurate sampling: 210 nm/2.3 = 91 nm

A pixel size of maximally 90 nm is sufficient for correct representation of data recorded under these settings. If this value is surpassed, no

Pixel size: 34 nm

Pixel size: 136 nm

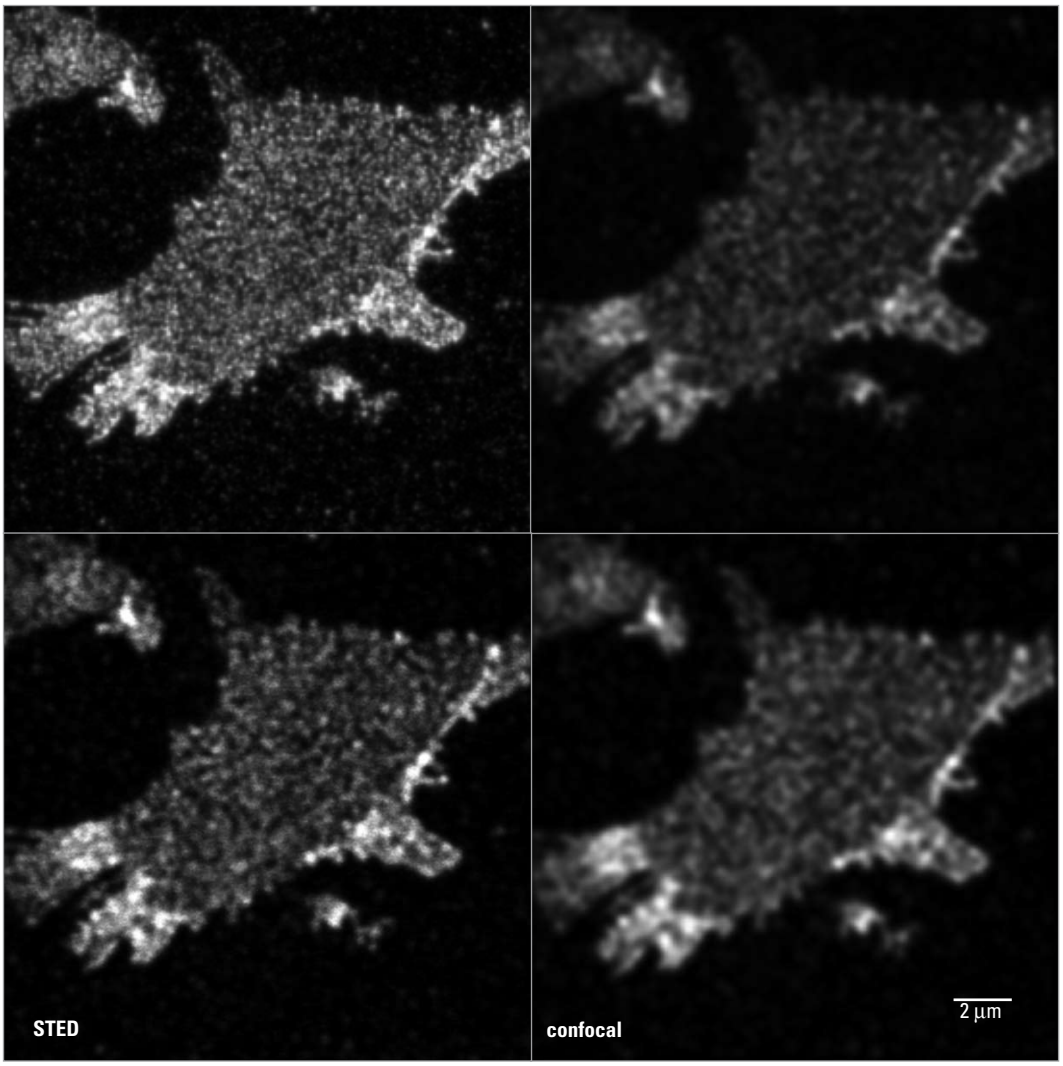


Fig. 15: The effect of undersampling. Syntaxin sheets: top: pixel size 136 nm bottom: 34 nm. The difference between confocal (right) and STED image (left) diminishes due to undersampling.

further information is gained (known as empty magnification), but the time needed for scanning is increased and photobleaching becomes a more severe problem, too. If the sample is scanned with pixels that are too large, i.e. undersampling, it is not represented correctly and information is lost. This can cause not only disappearance of details, but can also introduce artifacts. The latter is known as aliasing, where shapes in the specimen are completely changed.

For a given excitation wavelength and a certain numerical aperture the appropriate pixel size is a fixed value. The zoom option allows adjusting the pixel size to the specific imaging settings. By decreasing the zoom the pixel size increases, which can result in undersampling. STED exhibits a strong improvement in lateral resolution, thus requiring smaller pixels to avoid undersampling when working with the same zoom settings as in the confocal mode.

For convenience, this is automatically considered in the software when switching from the confocal to the STED tab by adjusting the format accordingly. The pixel value considered for this adjustment is found under **Configuration** and can be changed, if desired. The default setting of 32 nm is calculated by dividing 80 nm (achievable resolution in a Leica TCS STED system under very good conditions) by 2.5.

Suggested Readings

1. Abbe E. (1873) Beitraege zur Theorie des Mikroskops und der mikroskopischen Wahrnehmung. Arch. f. Mikr. Anat., 9:413–420.
2. Hell S.W. and J. Wichmann. (1994) Breaking the diffraction resolution limit by stimulated emission depletion microscopy. Opt. Lett., 19(11):780–782.
3. Hell S.W. (2003) Toward fluorescence nanoscopy. Nature Biotechnol., 21(11):1347–1355.
4. Westphal V. and S.W. Hell.(2005) Nanoscale resolution in the focal plane of an optical microscope. Phys. Rev. Lett., 94:143903.
5. Sieber JJ et al. (2006) The snare-motif is essential for syntaxin-clustering in the plasma membrane. Biophys J. Apr 15;90(8):2843-51.
6. Klar T.A. and S.W. Hell. (1999) Subdiffraction resolution in far-field fluorescence microscopy. Opt. Lett., 24(14):954–956.
7. Dyba M, S. Jakobs, and S.W. Hell. (2003) Immunofluorescence stimulated emission depletion microscopy. Nature Biotechnol., 21(11):1303 – 1304.
8. Westphal V et al. (2008) Video-rate far-field optical nanoscopy dissects synaptic vesicle movement. Science. Apr 11;320(5873):246-9.
9. Kittel T. et al. (2006) Bruchpilot promotes active zone assembly, Ca2+ channel clustering, and vesicle release. Science. May 19;312(5776):1051-4.
10. Willig KI et al. (2006) STED microscopy reveals that synaptotagmin remains clustered after synaptic vesicle exocytosis. Nature. Apr 13;440(7086):935-9.
11. Geumann U. et. al. (2008) SNARE Function Is Not Involved in Early Endosome Docking. Mol Biol Cell. Oct 8.[Epub ahead of printing]
12. Sieber JJ Science 2007 Anatomy and dynamics of a supramolecular membrane protein cluster. Science. 2007 Aug 24;317(5841):1072-6

Acknowledgements

1. Dr. Elisabeth Ehler, Kings College London, England
   (Titin T12(Rat myofibrils) ATTO 647N)
2. Dr. Thorsten Lang, Max Planck Institute forBiophysical Chemistry, Göttingen, Germany
   (Syntaxin-1 Membrane domains on plasma membrane sheets from PC12 cells, ATTO 647N)
3. Dr. M. Cremer, LMU Munich, Germany
   (Chromosomal substructures (replication foci), ATTO 647N)

“With the user, for the user”
Leica Microsystems

Leica Microsystems operates globally in four divisions, where we rank with the market leaders.

• Life Science Division

The Leica Microsystems Life Science Division supports the imaging needs of the scientific community with advanced innovation and technical expertise for the visualization, measurement, and analysis of microstructures. Our strong focus on understanding scientific applications puts Leica Microsystems’ customers at the leading edge of science.

• Industry Division

The Leica Microsystems Industry Division’s focus is to support customers’ pursuit of the highest quality end result. Leica Microsystems provide the best and most innovative imaging systems to see, measure, and analyze the microstructures in routine and research industrial applications, materials science, quality control, forensic science investigation, and educational applications.

• Biosystems Division

The Leica Microsystems Biosystems Division brings histopathology labs and researchers the highest-quality, most comprehensive product range. From patient to pathologist, the range includes the ideal product for each histology step and high-productivity workflow solutions for the entire lab. With complete histology systems featuring innovative automation and Novocastra™ reagents, Leica Microsystems creates better patient care through rapid turnaround, diagnostic confidence, and close customer collaboration.

• Surgical Division

The Leica Microsystems Surgical Division’s focus is to partner with and support surgeons and their care of patients with the highest-quality, most innovative surgical microscope technology today and into the future.

The statement by Ernst Leitz in 1907, “with the user, for the user,” describes the fruitful collaboration with end users and driving force of innovation at Leica Microsystems. We have developed five brand values to live up to this tradition: Pioneering, High-end Quality, Team Spirit, Dedication to Science, and Continuous Improvement. For us, living up to these values means: **Living up to Life**.

Active worldwide

Australia:	North Ryde	Tel. +61 2 8870 3500	Fax +61 2 9878 1055
Austria:	Vienna	Tel. +43 1 486 80 50 0	Fax +43 1 486 80 50 30
Belgium:	Groot Bijgaarden	Tel. +32 2 790 98 50	Fax +32 2 790 98 68
Canada:	Richmond Hill/Ontario	Tel. +1 905 762 2000	Fax +1 905 762 8937
Denmark:	Herlev	Tel. +45 4454 0101	Fax +45 4454 0111
France:	Rueil-Malmaison	Tel. +33 1 47 32 85 85	Fax +33 1 47 32 85 86
Germany:	Wetzlar	Tel. +49 64 41 29 40 00	Fax +49 64 41 29 41 55
Italy:	Milan	Tel. +39 02 574 861	Fax +39 02 574 03392
Japan:	Tokyo	Tel. +81 3 5421 2800	Fax +81 3 5421 2896
Korea:	Seoul	Tel. +82 2 514 65 43	Fax +82 2 514 65 48
Netherlands:	Rijswijk	Tel. +31 70 4132 100	Fax +31 70 4132 109
People’s Rep. of China:	Hong Kong	Tel. +852 2564 6699	Fax +852 2564 4163
Portugal:	Lisbon	Tel. +351 21 388 9112	Fax +351 21 385 4668
Singapore		Tel. +65 6779 7823	Fax +65 6773 0628
Spain:	Barcelona	Tel. +34 93 494 95 30	Fax +34 93 494 95 32
Sweden:	Kista	Tel. +46 8 625 45 45	Fax +46 8 625 45 10
Switzerland:	Heerbrugg	Tel. +41 71 726 34 34	Fax +41 71 726 34 44
United Kingdom:	Milton Keynes	Tel. +44 1908 246 246	Fax +44 1908 609 992
USA:	Bannockburn/Illinois	Tel. +1 847 405 0123	Fax +1 847 405 0164

and representatives in more than 100 countries

Order no.: **1593104019**

www.leica-microsystems.com

Leica
MICROSYSTEMS