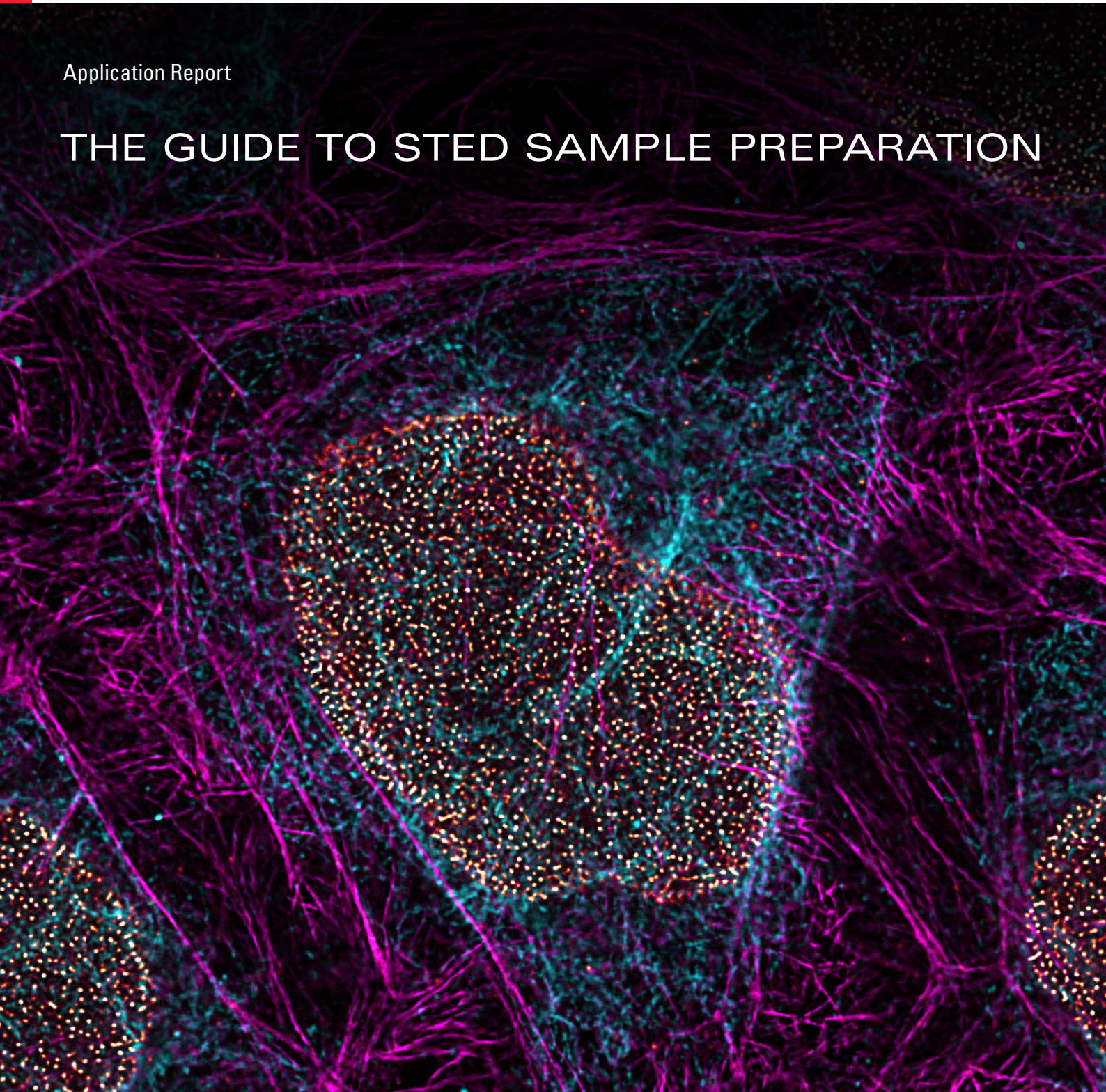


From Eye to Insight



Application Report

THE GUIDE TO STED SAMPLE PREPARATION



The Guide to STED Sample Preparation

This Guide is designed for the STELLARIS STED and STELLARIS 8 STED microscopes equipped with 589/592, 660 and 775 nm STED laser lines and STED WHITE objective lenses.

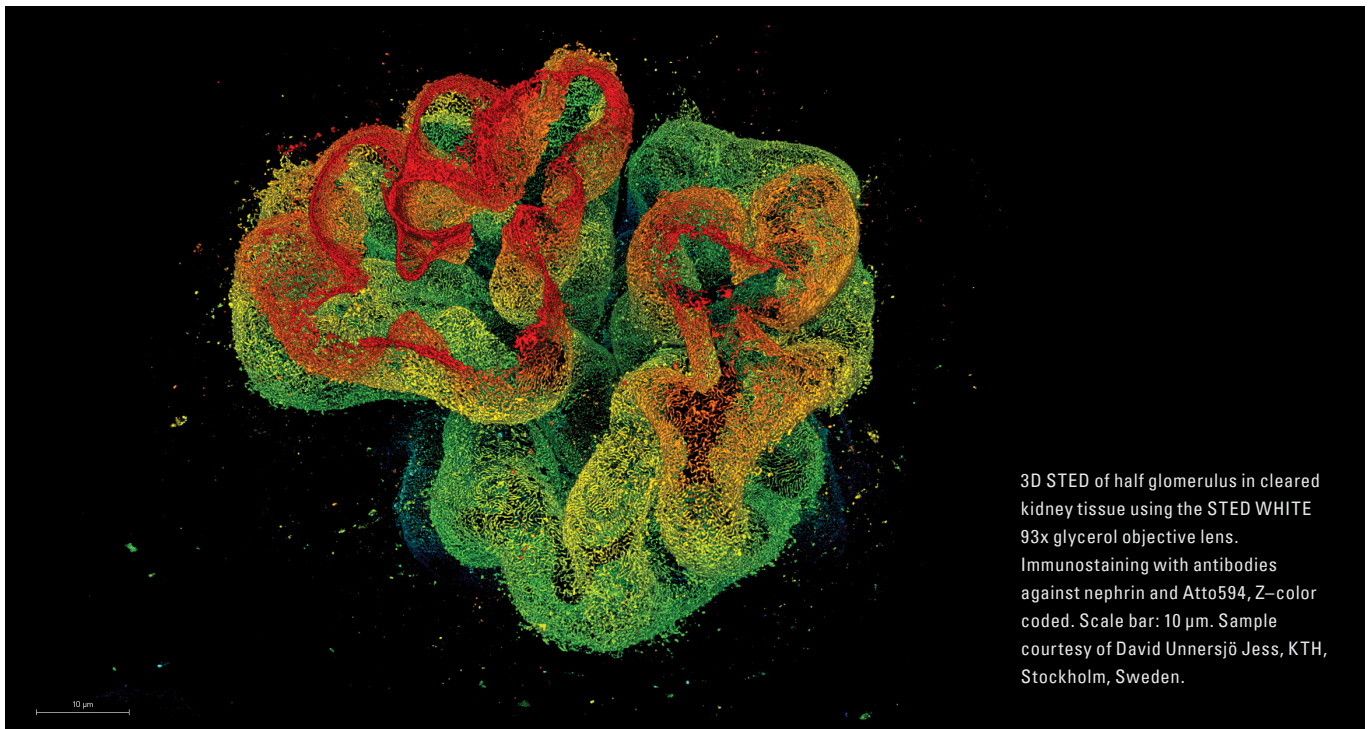
Stimulated emission depletion (STED) nanoscopy has revolutionized the life sciences bringing resolution well below the diffraction limit of confocal microscopy and with molecular specificity. STED is build on a confocal system, scanning the sample with an excitation beam together with a donut-shaped STED beam. In this way, fluorophores return to the ground state via stimulated emission and the effective observation volume is smaller than the diffraction-limited confocal volume.

STED nanoscopy works in 2D (generating the donut-shaped beam with a vortex), and also in 3D (combining the vortex with a z-phase mask).

The following guide describes the main points for successful STED imaging.

Using the STELLARIS STED or STELLARIS 8 STED:

- > The lateral resolution that can be achieved is below 50 nm (typically 30-40 nm, depending on the sample and fluorophore) using the vortex donut;
- > Confocal optical sectioning is intrinsically present. Combined with the high-end STED WHITE objective lenses, it is possible to image 3D structures deep inside the sample (e.g. tissue);
- > The axial resolution can be pushed below 130 nm (typically 100 nm, depending on the sample and fluorophore) combining the vortex with the z-phase mask;
- > The resolution can be tuned in all three dimensions to match the needs of the application;
- > Fast image acquisition is possible (several images per second);
- > Live-cell nanoscopy is possible, using fluorescent proteins or other fluorescent tags, thanks to the highly sensitive spectral HyD detector;
- > A broad range of suitable fluorophores can be imaged, enabled by the option of three different STED lasers (589, 660, and 775 nm) and the spectral flexibility of the white light laser (WLL) for excitation.



Title page: STED for Cell Biology: Multicolor fixed-sample TauSTED Xtend 775; Vimentin AF 594 (cyan), Phalloidin ATTO 647N (magenta) and NUP107 CF680R (glow). Sample Courtesy of Brigitte Bergner, Mariano Gonzales Pisfil, Steffen Dietzel, Core Facility Bioimaging, Biomedical Center, Ludwig-Maximilians-University, Munich, Germany.

Quick Start

General Recommendations

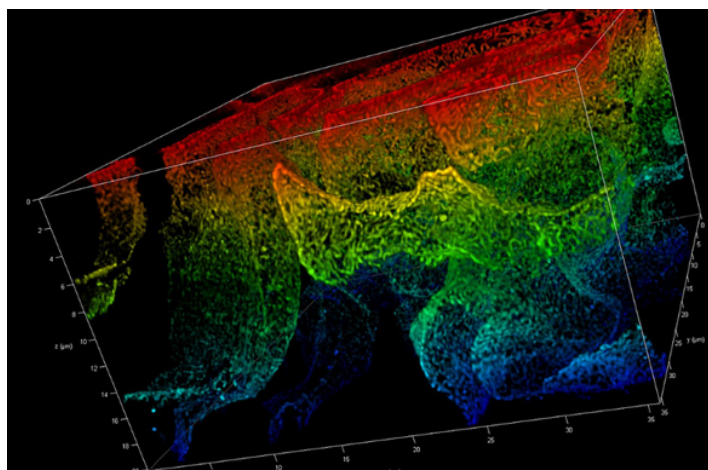
- > The dyes and procedures outlined in this guide apply to STED in general, and to TauSTED Xtend as well.
- > Samples should be bright and photostable in confocal mode before starting with STED
- > Prepare samples only on #1.5 or #1.5H glass coverslips
- > Avoid flaming coverslips to clean them, as carbon particles may deposit
- > Avoid DAPI for counterstaining and mounting media containing DAPI
- > Match the refractive index (RI) of mounting medium and objective lens immersion medium as closely as possible
- > Respect the curing time of hardening mounting media, e.g. at least 24h for Prolong Diamond
- > Avoid Vectashield and Abberior Mount as mounting medium

Top 3 fluorescent labels for single color STED imaging

	STED 589/592	STED 660	STED 775
Fixed samples	CF 488A	AF 555	STAR 635P
	AF 488	CF 555	ATTO 647N
Live cells	Citrine / mVenus / mNeonGreen	SPY555	SiR / SPY650

Top 3 fluorescent label combinations for dual color STED imaging

Fluorescent label #1			Fluorescent label #2			STED (nm)
Name	Exc. (nm)	Em. (nm)	Name	Exc. (nm)	Em. (nm)	
CF 440	440/515	475 – 510	StarGreen	493/519	523 – 580	589/592
AF 532	532	520 – 565	TMR	580	590 – 650	660
STAR 580	580	600 – 630	STAR 635P	635	655 – 750	775



Nephrin in cleared kidney section. 3D STED at a depth of 130 to 150 μm into the tissue, using the STED WHITE 93x glycerol objective lens. Immunostaining performed with ATTO 594. Sample courtesy of David Unnersjö-Jess, KTH, Stockholm, Sweden.

STED WHITE objective lenses

STED works for a large variety of samples: cells cultured in monolayer, cell spheroids, tissue sections, and even whole organisms, e.g. nematodes (*C. elegans*) and insects (e.g. larvae of *D. melanogaster*). The required depth of imaging is very dependent on the sample and the optical quality and chromatic correction of the objective lens can affect dramatically the STED performance.

The STELLARIS STED and the STELLARIS 8 STED systems are equipped with a dedicated family of high-end objective lenses: the STED WHITE class. The chromatic correction of the STED WHITE class delivers excellent performance all over the visible spectral range and at different depths (Figure 1).

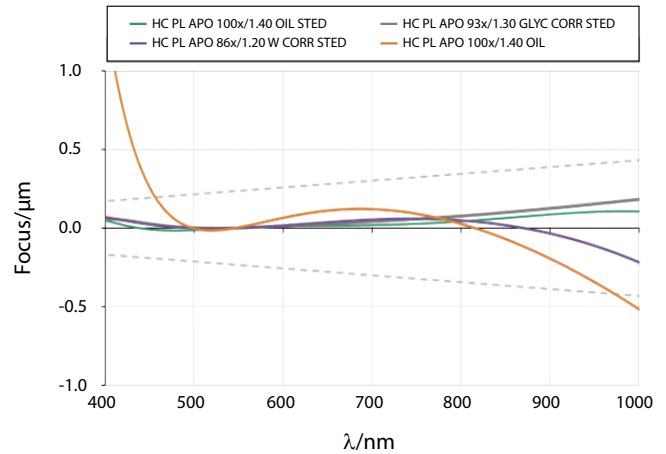


Figure 1. STED WHITE Class objective lenses: Chromatic correction over the 400 – 1000 nm wavelength range.

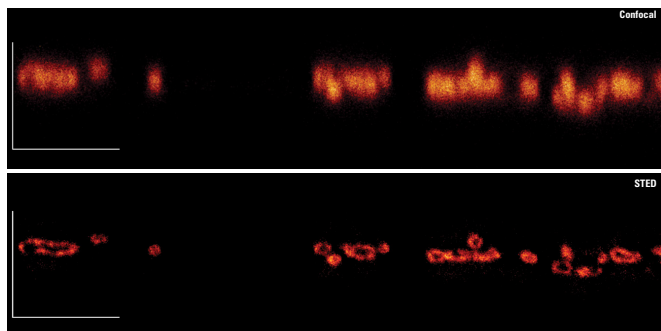
Depending on the application, three different objective lenses are available:

	HC PL APO 100x/1.4 OIL STEDWHITE	HC PL APO 93x/1.3 Glyc motCORR STEDWHITE	HC PL APO 86x/1.2 W motCORR STEDWHITE
Magnification	100x	93x	86x
Free working distance	130 μm	300 μm	300 μm
Immersion medium	Oil	Glycerol	Water
	Type F imm. $n_e^{23}=1.518$	Type G imm. $n_e^{23}=1.45$ Glycerine solution $n_e^{37}=1.46$	Water $n=1,33$
Application	Fixed cell samples	Deep tissue, fixed samples, live-cell	Live-cell, FCS

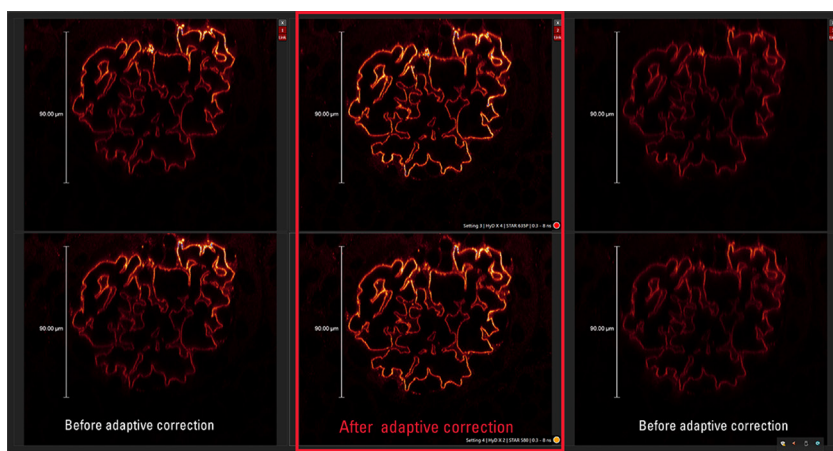
The 100x oil STED WHITE is the lens of choice for standard fixed samples and for structures close to the coverslip, and for imaging up to 30 μm deep in the sample. It gives the highest resolution based on its numerical aperture. For live-cell imaging and 2D/3D Deep STED Nanoscopy, the 93x Glyc STED WHITE is the lens of choice. The motorized correction collar allows adjustment to varying coverslip thickness, changes in temperature, and optimizing for signal from deep inside the specimen.

For applications in aqueous media and STED-FCS, the 86x W STED WHITE is the recommended lens.

It is important to match the refractive index of the sample and objective lens immersion medium as closely as possible. Please check the section “Sample mounting and substrate considerations” for additional information.



3D STED cross section of Cos 7 cells showing mitochondria labeled with antibodies against Tom20. Confocal (top) and STED (bottom) fluorescence corresponds to ATTO 549. Scale bar 10 μm . Sample courtesy of Urs Ziegler and Jana Doehner, ZMB, University of Zurich, Switzerland.



motCORR – correct adjustment of the motCORR minimizes spherical aberration and optimizes signal.

Choosing a fluorescent label for STED

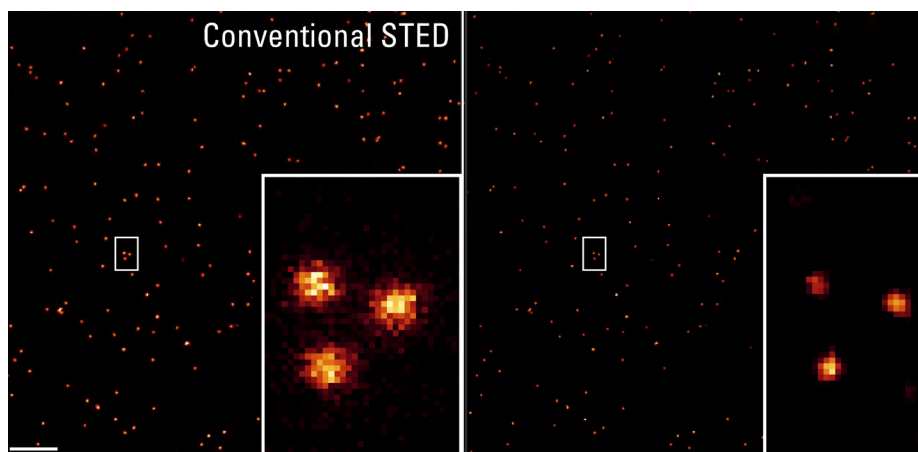
The palette of fluorescent labels suitable for STED imaging has grown over the years and it is possible to perform multicolor STED all over the entire visible spectrum. A selection of fluorescent labels and a rating of their STED performance can be found in Table 1. It is highly

recommended to choose a fluorescent label from this list and evaluate the STED performance *in situ*. Fluorescent labels with similar spectral properties as those listed in the table are probably also suitable for STED imaging. However, their performance may differ.

Fluorophore	Exc. (nm)	STED (nm)	Performance
AF 488	496	589/592	very good
ATTO 488	496	589/592	excellent
CF 440	500	589/592	very good
CF 488A	440	589/592	excellent
DyLight 488	490	589/592	excellent
FITC	493	589/592	excellent
Star Green	488	589/592	very good
STAR 488	493	589/592	excellent
LBL-Dye A488	503	589/592	excellent

Fluorophore	Exc. (nm)	STED (nm)	Performance
OG 488	495	589/592	excellent
		660	very good
Chromo 488	488	589/592	excellent
		660	moderate
Chromo 505	488 / 514	589/592	excellent
		660	moderate
AF 532	532	660	excellent
AF 546	556	660	very good
AF 555	555	660	excellent
CF 555	555	660	excellent
Cy3	554	660	excellent
DyLight 550	562	660	very good
JF549	549	660	very good
ATTO 565	564	660	excellent
		775	very good
TMR / TRITC	554	660	excellent
		775	moderate
AF 568	578	775	very good
AF594	590	775	excellent
AF 633	632	775	very good
AF 647	650	775	moderate
ATTO 590	593	775	very good
ATTO 594	603	775	excellent*
ATTO 647N	646	775	excellent*
ATTO 633	630	775	very good
ATTO 655	663	775	very good
CF 640R	642	775	excellent
CF680R	680	775	excellent
JF(X) 646	646	775	excellent
SiR	652	775	excellent
STAR 580	587	775	very good
Star Orange	589	775	very good
STAR RED	638	775	excellent
STAR 635P	638	775	excellent

Table 1. Fluorescent labels for **single color STED**.



STED and DNA origami imaging: TauSTED 775 delivers resolutions <30nm on GATTA-Bead R whose nominal size is 23 nm. Scale bar: 1 μ m.

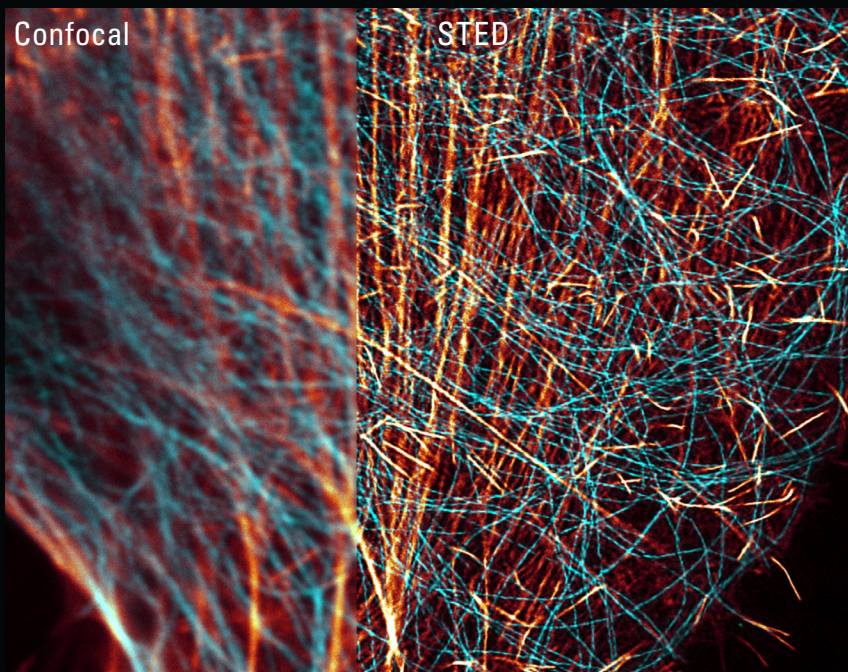
Multicolor STED is a very powerful tool to study molecular proximity with nanoscopy resolution. The STED donut determines where the fluorescence originates in space; therefore, imaging with a single STED line ensures that the different channels (colors) are intrinsically aligned. For this reason, applications requiring proximity/co-localization analysis are done using a single STED line and fluorescent labels with different emissions. It is also possible to implement a sequential workflow with

multiple STED lines, but this approach may require additional image analysis (i.e. post-processing image registration), as it is the case for channel overlay in classical co-localization experiments.

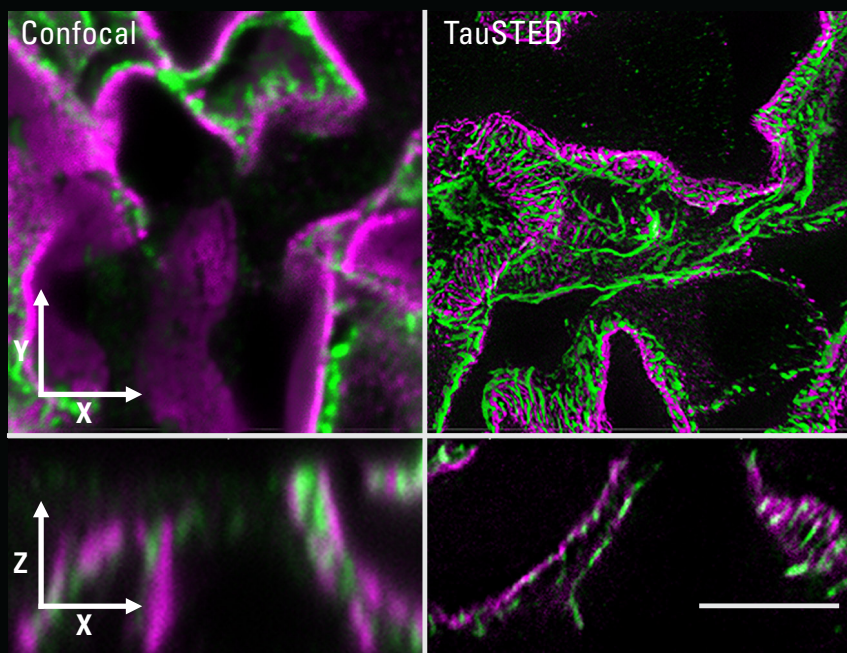
A selection of label combinations for dual and triple color STED are listed in Table 2 and Table 3. These combinations were tested for minimal cross-talk in the detection channels.

Name	Exc. (nm)	Em. (nm)	Name	Exc. (nm)	Em. (nm)	
BD Horizon V500, STAR 440SX	458/470	475 - 510	OG 488, Chromo 505	514/520	523 - 580	589/592
CF 440	440	460 - 500	Star Green	510	520 - 575	589/592
AF 532	514	520 - 565	TMR, TRITC, Cy3	580	590 - 650	660
AF 514, OG 488	505	515 - 565	TMR, TRITC, Cy3	580	590 - 650	660
AF 594, ATTO 594	590	600 - 630	STAR 635P, ATTO 647N	635/650	655 - 750	775
STAR 580	575	585 - 640	STAR 635P, ATTO 647N	635/650	655 - 750	775
STAR Orange	580	590 - 630	STAR 635P, ATTO 647N	640/650	655 - 750	775

Table 2. Fluorescent label combinations for **dual color STED** (single STED line). Slight adjustment of excitation lines and detection range may be required for optimal spectral separation.



Two-color TauSTED Xtend imaging of mammalian cells labeled with CF 440 - Phalloidin (glow) and immunostained against tubulin, StarGreen (cyan).



STED for kidney disease research: Multicolor 2D and 3D TauSTED 775 of non-cleared mouse kidney immunostained for synaptopodin (green) and nephrin (magenta). The STED WHITE glycerol lens provides optimal adaptive correction to resolve the proteins within the highly compact tissue architecture. Scale bar: 10 μm . Sample courtesy of Victor Puelles, Milagros Wong, and Jan Czogalla, Universitätsklinikum Hamburg-Eppendorf, Germany.

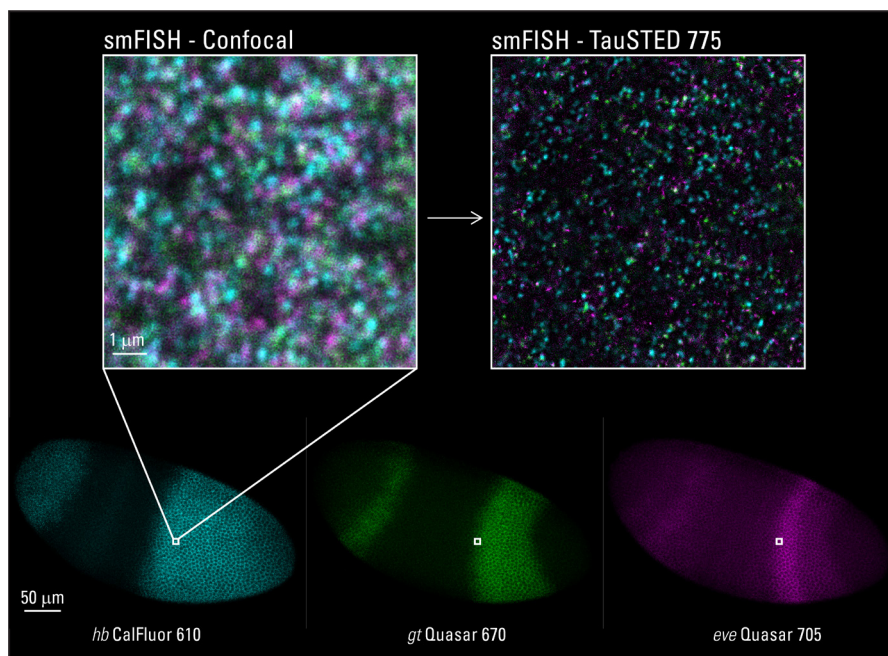
STED: 589/592 nm			660 nm			775 nm		
Fluorescent label #1			Fluorescent label #2			Fluorescent label #3		
Name	Exc. (nm)	Em. (nm)	Name	Exc. (nm)	Em. (nm)	Name	Exc. (nm)	Em. (nm)
OG 488	488	500 - 545	TMR, TRITC	550	560 - 635	ATTO 647N, STAR 635P	640	650 - 750
OG 488*	470	475 - 525	AF 532	532	538 - 550	TMR, TRITC	580	590 - 650
AF 514*	480	490 - 535	AF 546	540	545 - 580	AF 594	590	600 - 650
TRITC	550	560 - 590	ATTO 594	600	610 - 640	ATTO 647N, STAR 635P	660	665 - 750
AF 568	550	560 - 570	AF 594, ATTO 594	580	590 - 625	SiR, STAR 635P	640	650 - 750
AF 594	585	595 - 630	ATTO 647N	640	650 - 690	CF 680R**	690	700 - 750

Table 3. Fluorescent label combinations for **triple color STED** (single and multiple STED lines).

Slight adjustment of excitation lines and detection range may be required for optimal spectral separation.

* Multi-color images can be acquired by using either the fitting STED laser only or both STED lasers frame/stack sequentially.

** Very low STED power needed.



STED for Developmental Biology: smFISH* of RNA in a *Drosophila* embryo whole-mount preparation. Probes are directly labeled and there is no signal amplification. Top: Three color TauSTED 775 captures the signal from *hb* CalFluor 610 (cyan), *gt* Quasar 670 (green), and *eve* Quasar 705 (magenta). Bottom: confocal imaging of the whole *Drosophila* embryo. Sample courtesy of Tom Pettini, University of Manchester, UK.*Single molecule in-situ hybridization.

The sample of interest may contain additional fluorescent markers, for example a counterstain for confocal use only. In that case, the emission of the counterstain should fall outside the detection range of the fluorophores used for STED to avoid interference. Furthermore, the STED line may excite the markers and cause photobleaching; it is recommended to image those markers before STED imaging.

Please note: DAPI and Hoechst (typical DNA counterstains) may have a negative influence on image quality in terms of background, especially when using the 589/592 nm STED line. Avoid their use whenever possible. If nanoscopy images of DNA/nuclear staining are required, we recommend Picogreen (ThermoFisher) for the STED lines 589/592 nm and 660 nm, and SiR / SPY650-DNA (Spirochrome) for STED applications with the 775 nm laser.

The next section is about immunofluorescence labeling, but the fluorophore selection discussed here is valid for other labeling strategies (e.g. polypeptide tags such as SNAP and Halo, click chemistry reactions, organelle markers).

Immunofluorescence labeling considerations

After selecting the fluorescent label(s) for STED, the next step is to perform the labeling and evaluate the quality *in situ*. As a rule of thumb, one needs to have an excellent confocal image quality – in terms of high signal/noise ratio – before switching to STED mode.

The recommended approach for immunofluorescence labeling is to perform two experiments, A and B, sequentially or in parallel:

A) Evaluation of STED for the selected label(s) in the cellular / tissue environment

The labeling properties of fluorophores and antibodies can change with the microenvironment (pH, ionic strength, redox state). Therefore, it is recommendable to perform a control labeling in the sample of interest with a well-established primary antibody (e.g. an antibody against alpha-tubulin for cell samples). This step helps to check that the fluorescent label behaves as expected *in situ*.

B) Immunofluorescence labeling on the structure of interest

Using a standard protocol as starting point (see below), optimize the labeling for the structure(s) of interest. It is useful to perform a series of experiments with increasing concentrations of primary and secondary antibodies.

As mentioned above, the S/N ratio provides a good estimate for the quality of the staining. The labeling density plays an important role in signal intensity and increasing the antibody concentration can enhance sample quality. Therefore, it is advisable to test the staining with **2 to 5-fold higher secondary antibody concentrations than the typical working (confocal) concentrations** to ensure optimal labeling density.

Standard immunofluorescence labeling protocol



WARNING – Hazardous substances

The substances listed below are toxic and harmful to the environment and human health. Observe the safety data sheets of the mentioned substances and take necessary safety precautions to protect you, other persons and the environment.

Each action performed in immunofluorescence labeling has a distinct influence on sample quality. The following protocol serves as a starting point for mammalian cells. If the sample of interest has already a well-established staining protocol, that can be tested directly and maybe even optimized with the following recommendations.

Reagents:

- > **Phosphate buffer saline (PBS), pH 7.4**
- > **2% Paraformaldehyde (PFA) in PBS**
- > **0.1% Triton in PBS**
- > **Bovine Serum Albumin (BSA)**

Procedure:

All steps are performed at room temperature.

1. Rinse 3x with PBS.

Cells should be washed, culture medium removed by rinsing several times. Tissues should be dissected and cleaned from parts that could hinder image acquisition. Use established lab protocols, if they are known to work. Samples must be treated gently and quickly.

2. Fix with 2% PFA in PBS for 15 min.

Fixation is a critical step, as it defines how well the structure(s) will be preserved. This step becomes even more critical at the resolution STED provides, and thus should be addressed with care. PFA is a common fixative, but it is not always the best performing one. Literature search and optimization may be required, along with the recommended guidelines from the primary antibody manufacturer. Alternatively, a 5 min incubation with ice-cold (-20°C) 100% methanol can be used. Methanol fixation does not require subsequent permeabilization steps (steps 5 and 6 can be ignored). Recently, a new fixation method based on glyoxal was reported and may be an alternative. For details, refer to Richter et al., *EMBO J.* 2018 Jan 4; 37(1): 139 – 159.)

3. Rinse 3x with PBS.

Remove most of the fixative quickly for the following steps.

4. Wash 3x with PBS (5 min each).

Remove the rest of the fixative for the following steps.

5. Permeabilize with 0.1% Triton in PBS for 10 min.

Crucial step to reveal epitopes to primary antibodies. Lower concentrations / shorter incubation times may preserve the structure better, but compromise labeling density. Higher concentrations / longer incubation times may make the epitope more accessible to antibodies but also deteriorate the structure. Some fixatives – e.g. methanol – do not need extra permeabilizing steps.

6. Rinse 3x with PBS.

Remove permeabilizing agents.

7. Block with 2% BSA in PBS for 1h.

Blocking can be performed with different agents, normally consisting of inert proteins that bind to non-specific binding partners which would otherwise bind to antibodies and increase the unspecific labeling of fluorescent dyes. It is also advisable to use blocking agents while incubating with antibodies, as e.g. the serum helps in preserving the cellular structure. Thicker tissues might require longer incubation times.

8. Incubate with primary antibody for 1h.

Higher antibody concentrations may be helpful for increasing labelling density for better STED imaging. Longer incubation times may give better results, but also may increase background, so it needs to be checked. In thicker samples (e.g. whole mounts), incubation may take up to days. Alternatively, the incubation can be done at 4°C overnight.

9. Wash 3x with PBS (5 min each).

Washing steps are important, especially when using high concentration of antibodies. 5 minutes is the absolute minimum for washing steps here. Longer washing times (10 – 20 min) can deliver better results. Previous rinsing steps may speed up the process.

10. Incubate with secondary antibody for 1h.

Antibody concentration may need optimization in terms of S/N ratio. A good starting point is a 1:100 dilution for commercially available fluorescently labeled antibodies; for other antibody sources, a good starting point is 5x higher concentration than the recommended one. Thicker tissues need longer incubation times.

11. Wash 3x with PBS (5 min each).

Remove unbound antibodies from sample. Longer and more washing steps will increase the quality of the labeling. Previous rinsing steps may speed up the process.

12. Mount.

See “Sample mounting and sample substrate considerations” section.

13. Store at 4°C until imaging.

As mentioned above, the staining should look crisp and bright when observed through the ocular. The staining should yield good S/N in confocal images.

An important point to consider for STED imaging in combination with immunofluorescence is the size of the tag. The size of an antibody molecule (IgG approximately 10 nm) may become the limiting factor for labeling density and the size of the structure measured with STED. If size becomes an issue, a different approach using smaller tags, such as F(ab)-fragments or fluorescently labeled nanobodies, may be more adequate. Alternatively, polypeptide tags expressed with the target protein through transfection or genome-editing techniques, or a fluorescent tag directed to the structure of interest (e.g. phalloidin fluorescent conjugates for actin) can deliver excellent STED image quality.

Sample mounting and substrate considerations

Optimal optical performance requires an environment with a homogeneous index of refraction to enable a high penetration depth and to minimize aberrations. Hence, the mounting medium should have a refractive index that matches the immersion medium of the objective lens. Furthermore, autofluorescence derived from the mounting medium should be negligible upon application of the STED laser (589 / 592, 660 or 775 nm).

Changes in the refractive index due to sample composition –for example, myelin and fat in tissue samples– may distort the shape of the focal spot and affect the STED performance. In such cases, optical clearing can help. Details of STED combined with **optical clearing** are described in Unnersjö-Jess et al., *Kidney International*, 2015. Also, clearing with the SeeDB2 protocol has been used to map the neuronal circuitry (Meng–Tsen Ke et al., *Cell Reports*, 2016).

Prolong Gold and Prolong Diamond anti-fade reagents (Thermo Fisher Scientific) are good starting options for mounting samples for STED and can be used for cells in culture unless a different mounting medium is required. It is very important to follow the indications about the curing times from the manufacturer (for example, we recommend to wait at least 24 h before imaging samples mounted in Prolong Gold / Diamond).

In some cases, the mounting medium can alter the fluorescent properties of the labels. For example, VectaShield (Vectorlabs) affects the fluorescence quantum yield of large Stoke's shift dyes and should be avoided in those cases; mounting media containing DAPI should be avoided in general. The 2,2-Thiodiethanol (TDE) can affect the behavior of fluorescent proteins and some green dyes. For a list of dyes working in TDE, please refer to Staudt et al., *Microscopy Research and Technique*, 2007.

For home-made mounting medium options, very good results were obtained with glycerol-based recipes as described below:

A Mixtures of glycerol / water-based solutions

Mixtures of PBS and glycerol produce mounting media with refractive indices that can be adjusted between 1.33 and 1.47. They are easy to prepare and suitable for longer sample storage at -20°C . The media are particularly useful in combination with the 93x STED WHITE glycerol lens. Adding antifading reagents, e.g. DABCO (2.5 %) or NPG (4 %), can help when higher photostability is needed. Antifading reagents containing p-phenylenediamine decrease STED performance and should be avoided.

B Mowiol mounting medium

Take 6 g of glycerol (analytical grade) and add 2,4 g of Mowiol powder, 6 ml of H_2O , 12 ml of 0.2 M TRIS buffer pH 8, and stir the solution ca. 4 h. After that, let the solution rest for additional 2h. Heat the mixture in a water bath for 10 min at 50°C and then centrifuge at 5000 g for 15 min. Finally, take the supernatant and store aliquots at -20°C . Mowiol can be used for storing samples at -20°C . DABCO (2.5 %) can be added for higher photostability.

The material and thickness of the substrate supporting the sample constitutes a critical point in terms of the optical STED performance. All Leica objective lenses with coverglass correction are corrected for #1.5 coverslips, thickness: 0.170 ± 0.01 mm (e.g. Hecht-Assistent, cat. number 1014/2424 or Menzel). To ensure optimal STED performance, it is recommended to go even further with quality and only use high precision glass coverslips / bottom cell culture chambers, #1.5H (thickness 0.170 ± 0.005 mm). For glass coverslips, this option is available from, e.g., Marienfeld-Superior. For glass bottom cell culture chambers, the following types have shown good results: Lab-Tek Chamber Slide System (Thermo Fisher Scientific), Ibidi μ -slides / μ -dishes (Ibidi GmbH), and Mattek glass bottom dishes (MatTek Corporation).

The most common mounting media for STED applications are summarized as follows (Table 4):

Mounting Medium	Provider	Comment
Glycerol	e.g. Sigma–Aldrich	RI adjustable in combination with water-based solutions (~1,43 – 1,47).
Mowiol ¹	e.g. Calbiochem	Excellent for long–term sample storage at –20 C.
Prolong Gold ¹ Prolong Diamond ¹	Thermo Fisher Scientific	Excellent for long-term sample storage at -20 C. Follow instructions for adequate curing time before imaging samples RI 1,47 after curing
EverBrite Mounting Medium EverBrite Hardset Mounting Medium	Biotium	RI 1,46 RI 1,46 after curing
TDE	e.g. Sigma–Aldrich	RI adjustable (1,33 – 1,52). Affects fluorescence yield of fluorescent proteins and green dyes ²

Commonly used Mounting Media for STED Microscopy.

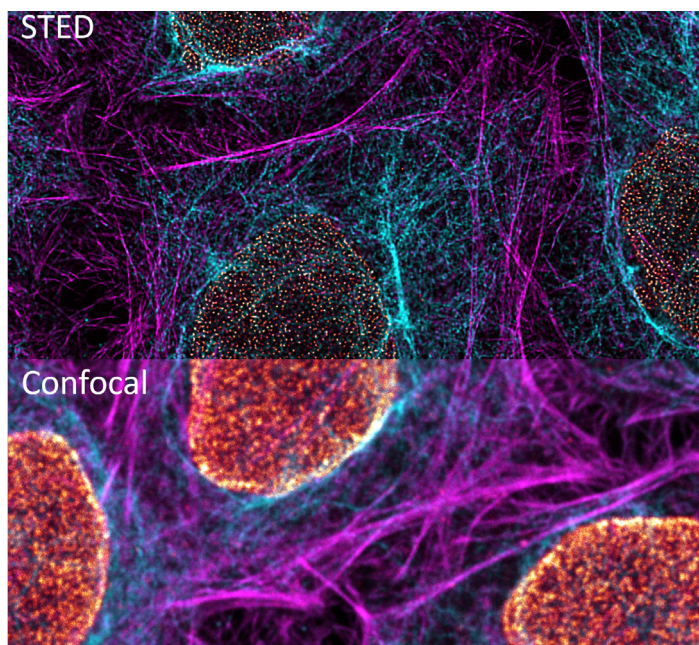
¹Recommended mounting media for cellular applications.

²Staudt et al., 2,2-Thiodiethanol: A New Water Soluble Mounting Medium for High Resolution Optical Microscopy, Microscopy Research and Technique, 2007.

Please note: The use of #1 coverslips significantly decreases image quality, both in terms of loss of resolution and signal intensity, and, therefore, should be avoided.

If the sample substrate or the sample itself absorbs in the STED beam wavelength (589 / 592, 660, or 775 nm), it may be destroyed during imaging.

This same problem is common for substrates, such as polymer coverslips and resins used for embedding, and samples containing natural pigments. Therefore, it is crucial to avoid these substrates, if possible, and areas of the sample with strong STED absorption as much as possible.



Triple color, 2D STED imaging TauSTED Xtend 775; Vimentin AF 594 (cyan), Phalloidin ATTO 647N (magenta) and NUP107 CF 680R (glow). STED (top) and confocal (bottom) comparison.

Live-cell STED imaging

STED has extended its range of applications to imaging dynamic processes in live-cell and organisms with nanoscopy resolution. The live cell capability is significantly improved by TauSTED, which utilizes reduced light dose and delivers high image quality and resolution. TauSTED Xtend enables even more gentle live-cell imaging as it allows to further reduce the light intensities, maintaining resolution and image quality. To get the best STED performance, a fundamental step is, again, selecting the most appropriate fluorescent label. For live-cell imaging, there are two main strategies to choose from, namely using fluorescent proteins (FPs) or using organic fluorophores. In the case of FPs, a straightforward approach is the expression of the protein of interest fused to a fluorescent protein (FP). The options for FPs suitable for STED are summarized below (Table 5)

Name	Excitation (nm)	STED (nm)	Performance*
mTFP ¹	462	589/592	excellent
mTurquoise ²	434	589/592	very good
eGFP	488	589/592	very good
EmGFP	487	589/592	very good
mNeonGreen	506	589/592	excellent
LanYFP	513	589/592	excellent
eYFP	513	589/592	excellent
		660	moderate
Venus	515	589/592	excellent
		660	moderate
mCitrine	516	589/592	excellent
		660	moderate
DsRed	558	660	very good
mRFP ¹	584	660	very good
mStrawberry	574	660	very good
mCherry	587	775	moderate
iRFP713	690	775	moderate

Table 5. Fluorescent proteins suitable for STED imaging

*Rating based on internal and external feedback. It may not apply to all experimental situations, due to changes in signal intensity / stability (microenvironment, degree of labeling, mounting medium) and STED efficiency.

Organic fluorophores outperform FPs in STED imaging, especially in the red emission range, but have the drawback of being more difficult to target to the structure of interest, plus being non cell-permeable. However, developments in recent years have shown a growing interest and success in labeling approaches based on tags that can be combined with cell-permeable fluorophores for STED nanoscopy (Table 6). A smart approach is to clone the protein of interest with a small polypeptide (e.g. SNAP-tag, Halo-tag, Fluorogen-activating proteins) that reacts *in situ* with cell-permeable fluorescent tags. An example of this strategy that produces excellent STED results is the combination of Halo-tag and SiR-CA for single color STED applications. Dual color, live-cell STED imaging using SNAP-tag / ATTO 590BG

and Halo-tag / SiR-CA has been reported by Bottanelli et al., Nature Communications, 2016. Another interesting concept for live-cell STED with exchangeable fluorogenic probes was demonstrated by Spahn et al., Nano Letters, 2019. This approach enables whole cell, 3D, multi-color STED for extended periods of time and with excellent signal to noise ratio. Recently, Johnsson group developed a set of cell permeable, fluorogenic, STED compatible fluorophores, which allow wash-free, multicolor, live-cell nanoscopy. Wang. et. al. Nature Chemistry, 2020. The variants of SNAP-tag and Halo-tag in addition broaden enormously the range of possible cellular targets.

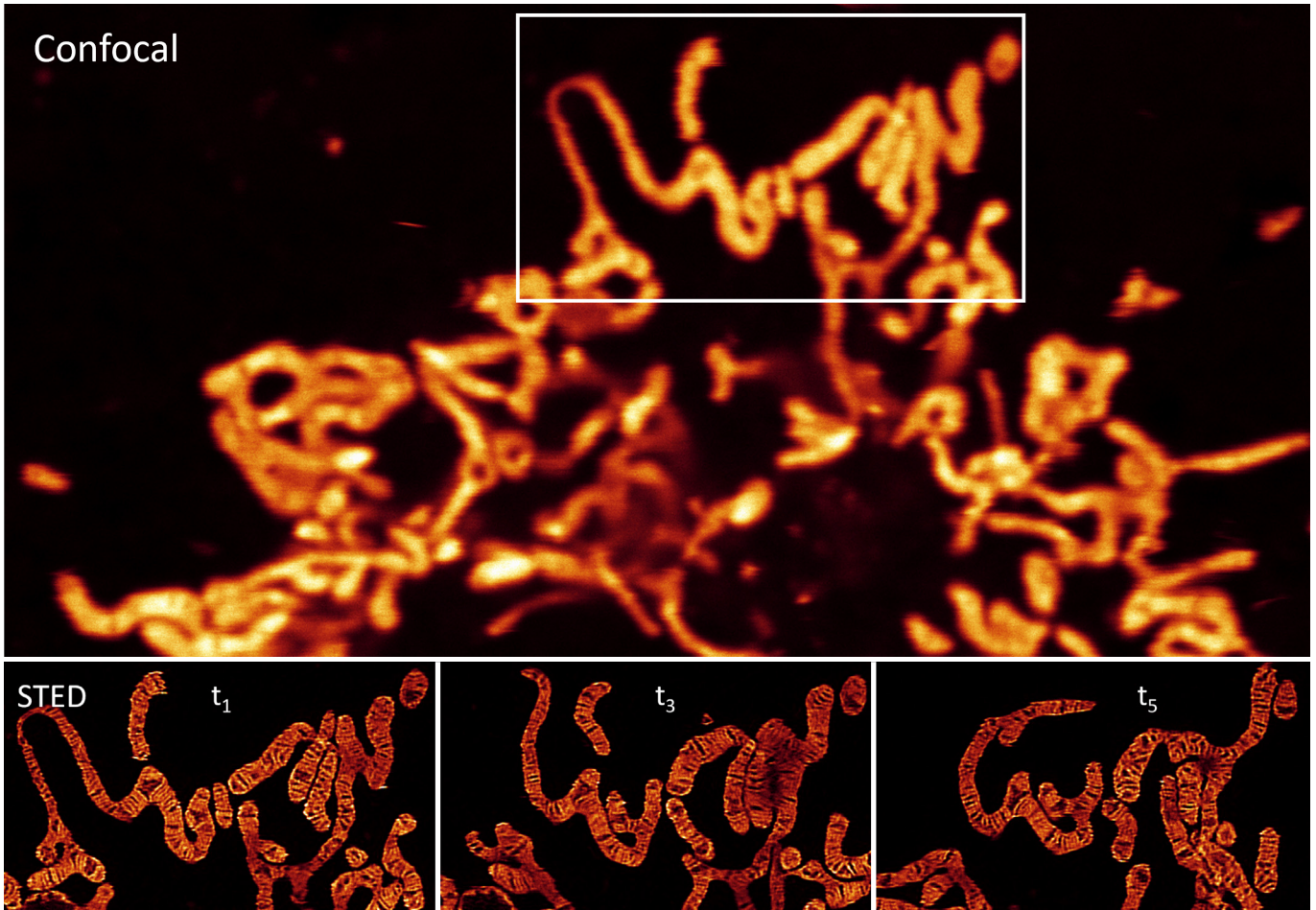
Name	Excitation (nm)	STED (nm)	Performance*
Tubulin Tracker Green	488	589/592	excellent
OG 488 BAPTA	494	589/592	excellent
SPY505-DNA ¹	512	589/592	excellent
TMR, Methyl Ester, Perchlorate (TMRM)	540	660	excellent
SPY555-DNA / -actin / -FastAct / -tubulin, SPY555-BG (for SNAP-tag) ¹	555	660	excellent
ATTO 590BG (for SNAP-tag) ²	590	775	excellent
Fluorogen Activated Proteins / Malachite Green	640	775	excellent
Nile Red / Nile blue	561	775	excellent
PK mitoOrange ¹	591	775	excellent
SiR-DNA / -tubulin / -actin, SiR-BG (for SNAP-tag) ¹	652	775	excellent
SPY595-DNA ¹	599	775	excellent
SPY620-DNA /-actin, SPY620-BG (for SNAP-tag) ¹	619	775	excellent
SPY650-DNA / -tubulin / -FastAct, SPY650-BG (for SNAP-tag) ¹	652	775	excellent

Table 6. Organic fluorescent labels for live-cell STED.

*Rating based on internal and external feedback. It may not apply to all experimental situations, due to changes in signal intensity/stability (microenvironment, degree of labeling, mounting medium) and STED efficiency.

¹commercially available from Spirochrome.

²commercially available from New England Biolabs Inc.



Live-cell TauSTED Xtend imaging with STED at 775 nm: mitochondria stained with PKmitoOrange in COS7 cells.

