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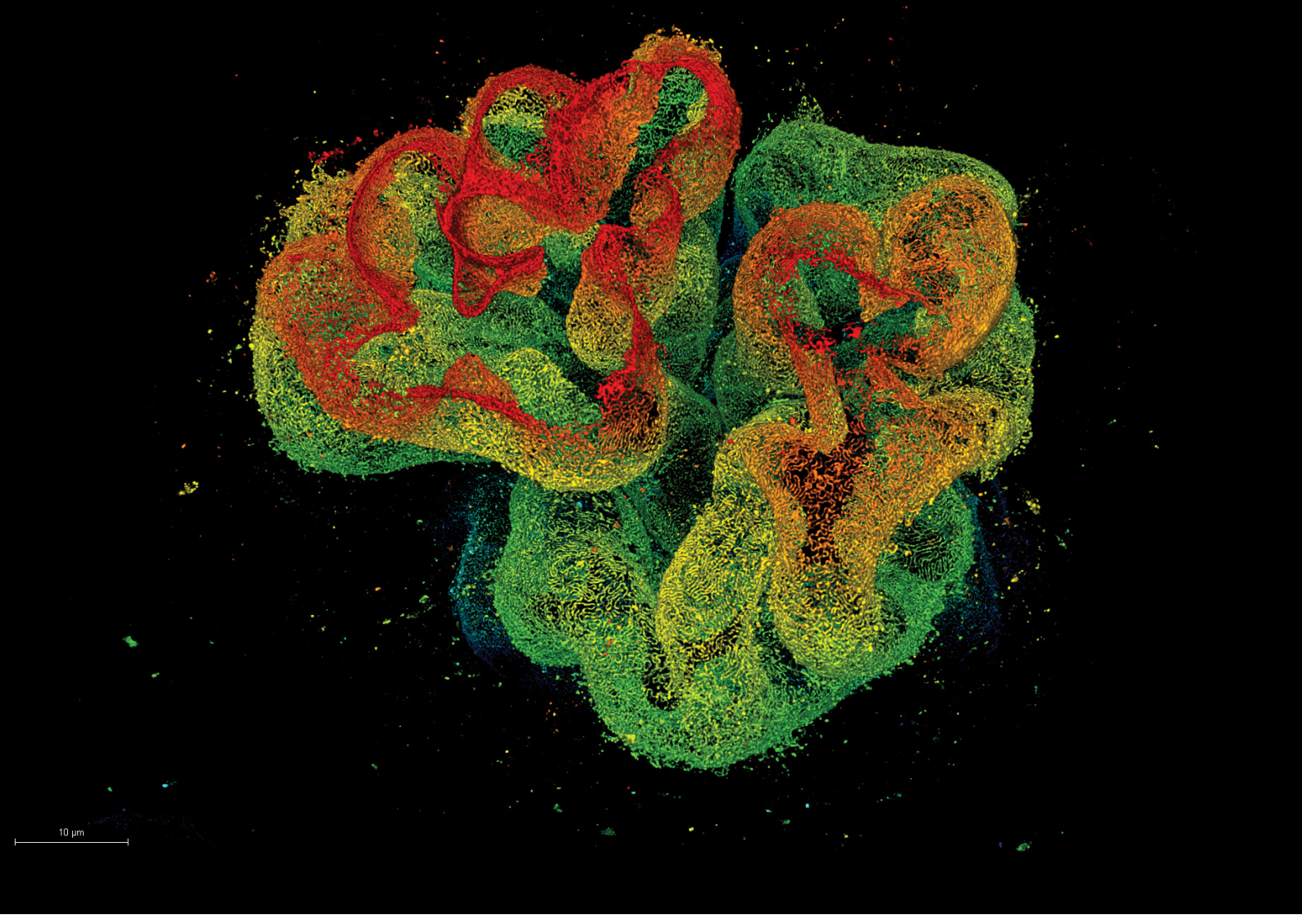
Application Report

THE GUIDE TO STED SAMPLE PREPARATION



The Guide to STED Sample Preparation

This Guide is designed for the TCS SP8 STED 3X nanoscope equipped with 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 SP8 STED 3X:

- The lateral resolution that can be achieved is below 50 nm (typically 30–40 nm, depending on the sample) 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 sample) 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 (592, 660, and 775 nm) and the spectral flexibility of the white light laser (WLL) for excitation.

Title Page: 3D STED of half glomerulus in cleared kidney tissue using the STED WHITE 93x glycerol objective lens. Immunostaining with antibodies against nephrin and Atto594, Z–color coded. Scale bar: 10 µm. Sample courtesy of David Unnersjö Jess, KTH, Stockholm, Sweden.

Quick Start

General Recommendations
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 as mounting medium

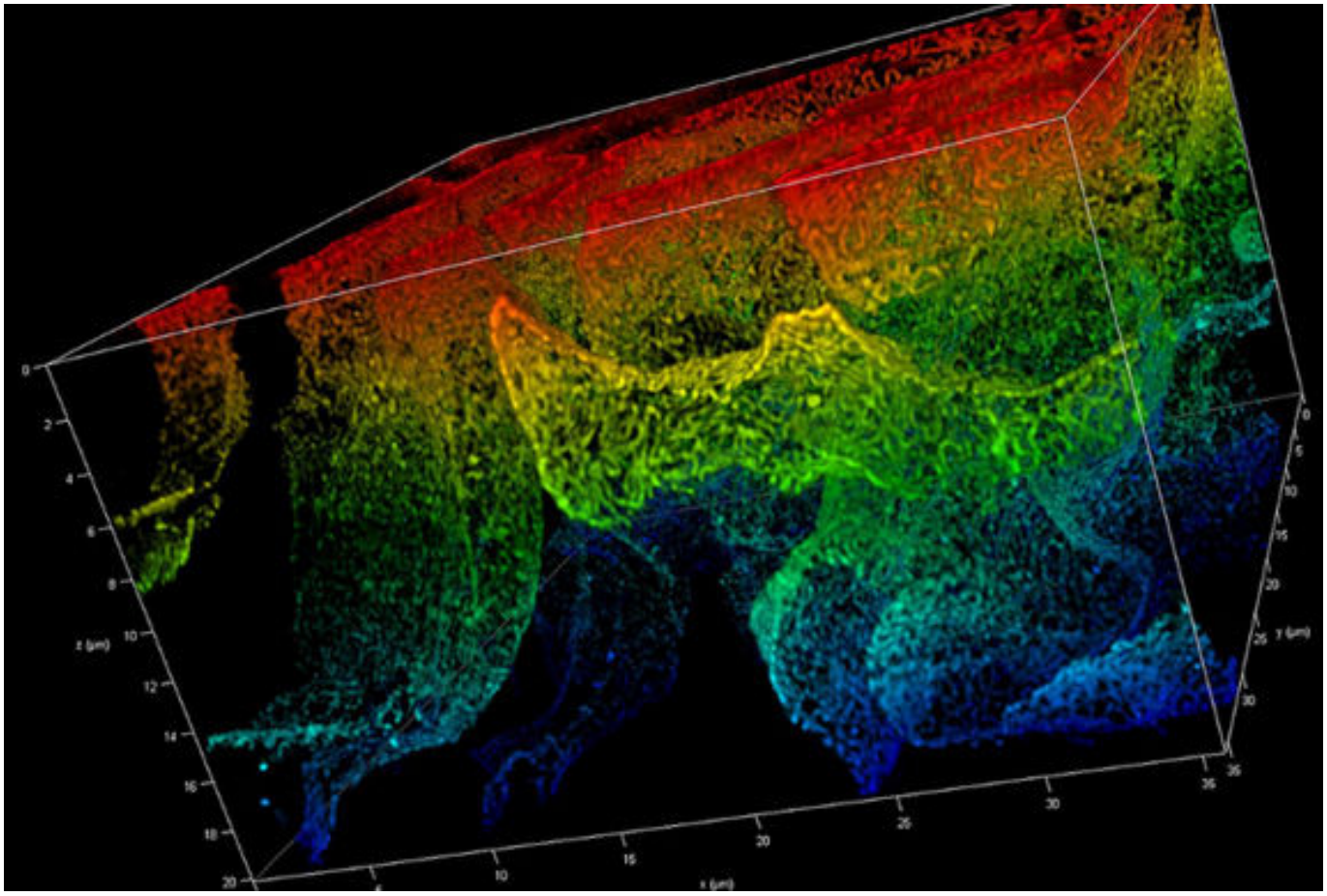
Top 3 fluorescent labels for single color STED imaging[1]

	STED 592	STED 660	STED 775
Fixed samples	OG 488	AF 555	STAR635P
	AF 488	ATTO 542	ATTO 647N
Live–cells	Citrine / mVenus	TMR	SiR

Top 3 fluorescent label combinations for dual color STED imaging[1]

Fluorescent label #1			Fluorescent label #2			STED (nm)
Name	Exc. (nm)	Em. (nm)	Name	Exc. (nm)	Em. (nm)	
STAR 440SX	458/470	475 – 510	OG 488	514/520	523 – 580	592
AF 532	532	520 – 565	TMR	580	590 – 650	660
STAR 580	580	600 – 630	STAR 635P	635	655 – 750	775

[1]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.



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 Atto594. 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 SP8 STED 3X is 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 penetration depths (Figure 1).

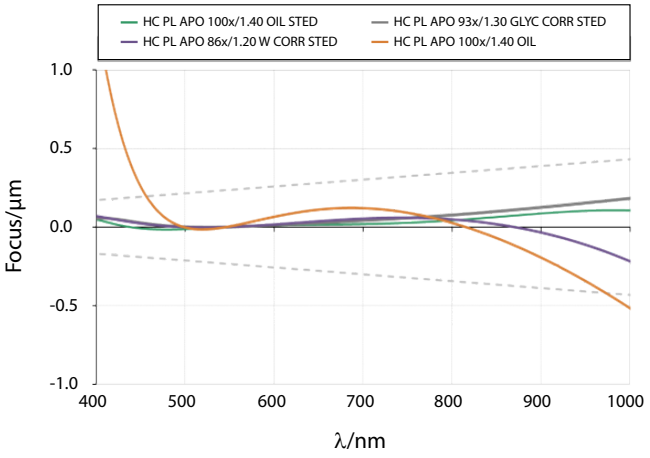


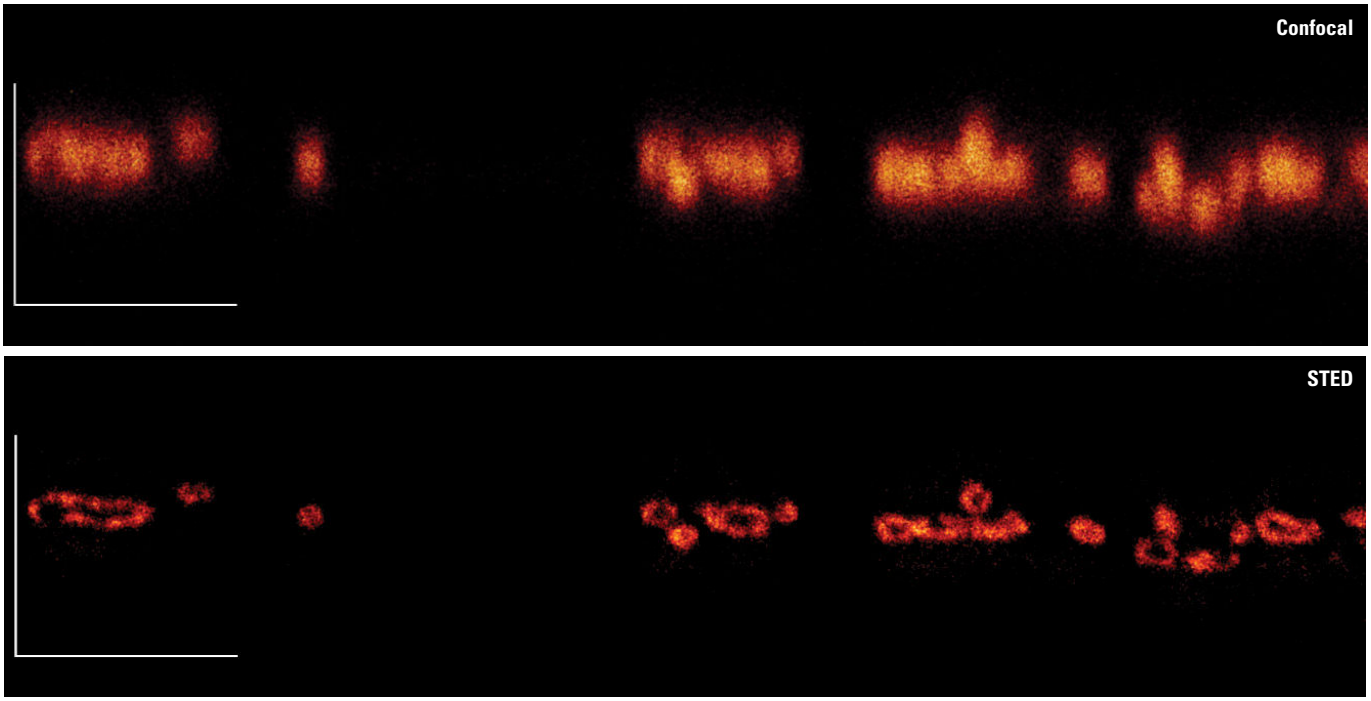
Figure 1. STED WHITE Class objective lenses: Chromatic correction over the 400 – 1000 nm wavelength range.

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

	HC PL APO 100x/1.4 oil STED WHITE	HC PL APO 93x/1.3 Glyc motCORR STED WHITE	HC PL APO 86x/1.2 W motCORR STED WHITE
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
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, with excellent performance up to 30 µm deep into 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 Atto594. Scale bar 10 µm. Sample courtesy of Urs Ziegler and Jana Doehner, ZMB, University of Zurich, Switzerland.

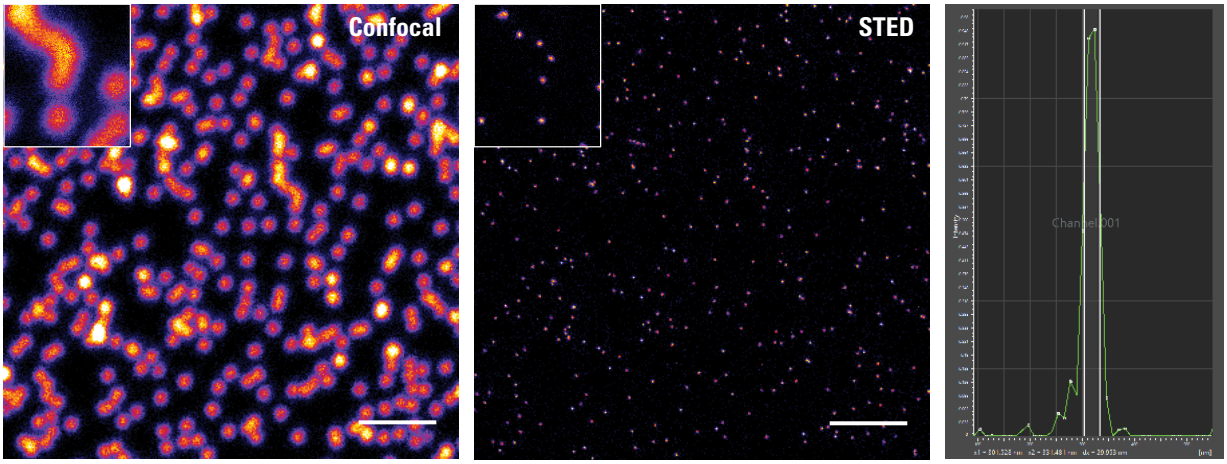
Choosing a fluorescent label for STED

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*.

Fluorophore	Exc. (nm)	STED (nm)	Performance[1]
AF 488	488	592	excellent
DyLight 488	488	592	excellent
STAR 488	488	592	excellent
ATTO 488	488	592	very good
FITC	488	592	very good
STAR 440SXP	458 / 470	592	very good
OG 488	488 / 514	592	excellent
		660	very good
Chromeo 488	488	592	excellent
		660	moderate
Chromeo 505	488 / 514	592	excellent
		660	moderate
AF 532	532	660	excellent
AF 555	555	660	excellent
Cy3	550	660	excellent
AF 546	546	660	very good
DyLight 550	550	660	very good
ATTO 565	565	660	excellent
		775	very good
TMR / TRITC	554	660	excellent
		775	moderate
AF 594	594	775	excellent
ATTO 647N	647	775	excellent*
ATTO 594	594	775	excellent*
SiR	635	775	excellent
STAR RED	635	775	excellent
STAR 580	580	775	excellent
STAR 635P	635	775	excellent
AF 568	568	775	very good
AF 633	633	775	very good
ATTO 633	633	775	very good
ATTO 655	655	775	very good
ATTO 590	590	775	very good

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

[1]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. *Hydrophobic, may require labeling optimization to suppress background.



STED imaging on 23 nm Gattabeads labeled with Atto647N. Scale bar 2 µm. Images correspond to raw data. The profile on the right shows FWHM < 30 nm for a single bead. Sample available from Gattaquant GmbH, Germany.

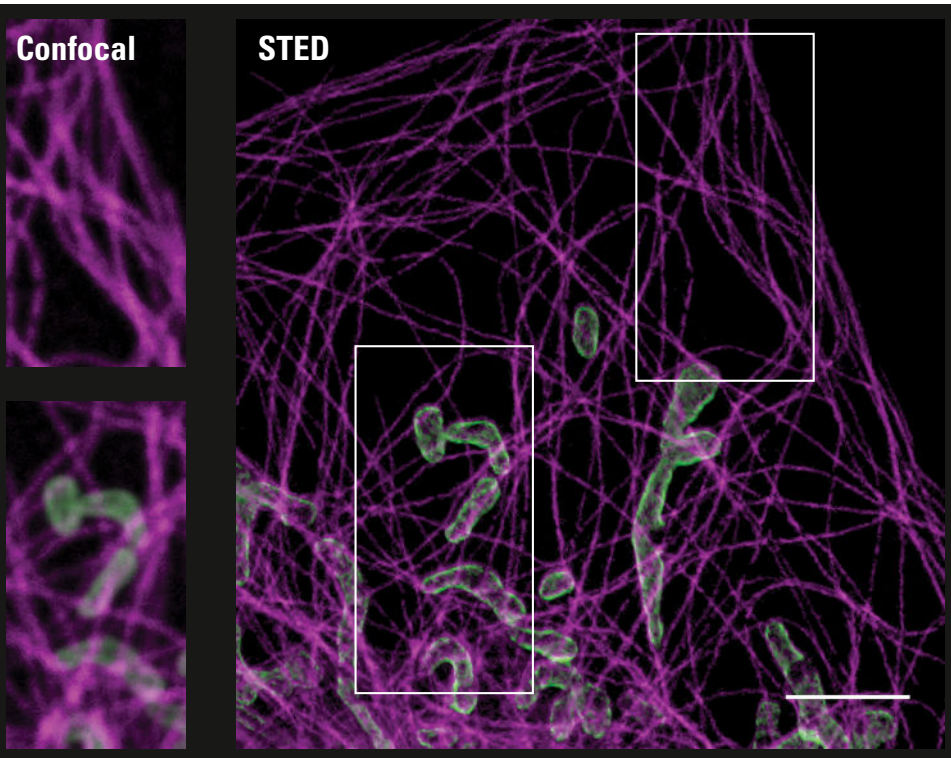
Multicolor STED is a very powerful tool to study molecular proximity with nanoscopy resolution. The STED donut determines where the fluorescence originates in space; therefore, imaging with a single STED line ensures that the different channels (colors) are intrinsically aligned. For this reason, applications requiring proximity/co–localization analysis are best 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.

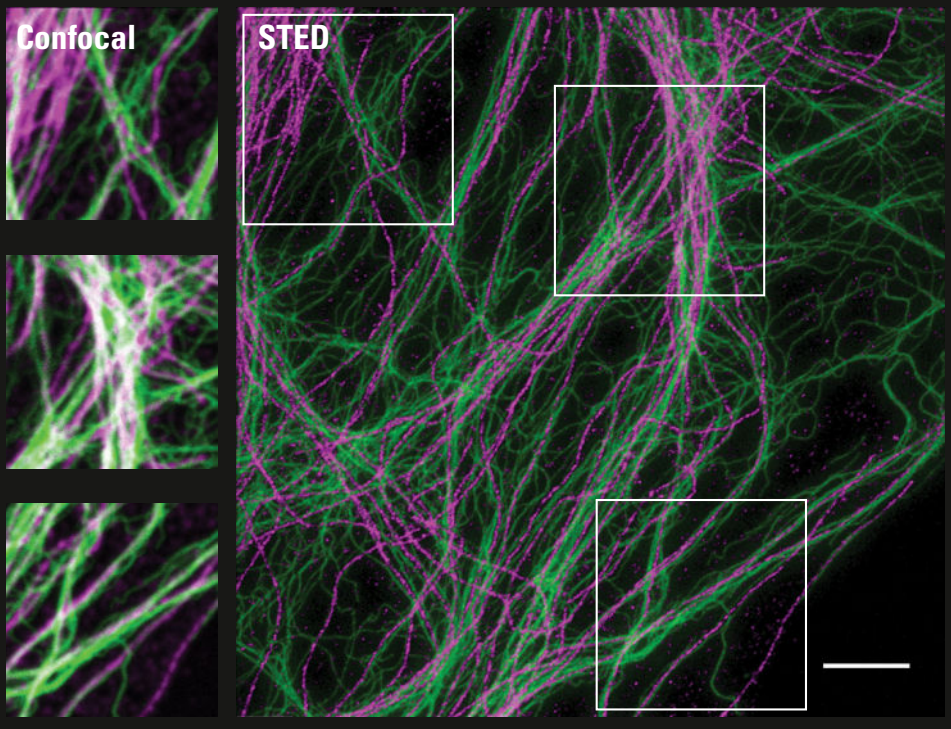
Fluorescent label #1			Fluorescent label #2			STED (nm)
Name	Exc. (nm)	Em. (nm)	Name	Exc. (nm)	Em. (nm)	
BD Horizon V500, STAR 440SX	458/470	475 – 510	OG 488, Chromeo 505	514/520	523 – 580	592
AF 532	514	520 – 565	TMR, TRITC, Cy3	580	590 – 650	660
AF 514, OG 488	505	515 – 565	TMR, TRITC, Cy3	580	590 – 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 600	590	600 – 640	STAR 635P, ATTO 647N	635/650	655 – 750	775

Table 2. Fluorescent label combinations for **dual color STED** (single STED line)[1]

1 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, sample aging) and STED efficiency. Slight adjustment of excitation lines and detection range may be required for optimal spectral separation.



2D STED imaging of Cos 7 cells showing mitochondria labeled with antibodies against Tom20 (Atto 594, green) and tubulin (STAR 635P, magenta. Scale bar 2 µm. Sample courtesy of Urs Ziegler and Jana Doehner, ZMB, University of Zurich, Switzerland.



2D STED imaging of mammallian cells labeled with antibodies against vimentin (Alexa Fluor 488, green) and tubulin (Alexa Fluor 647, magenta). Scale bar 2 µm. Sample courtesy of Leila Nahidiazar, University of Amsterdam, Netherlands.

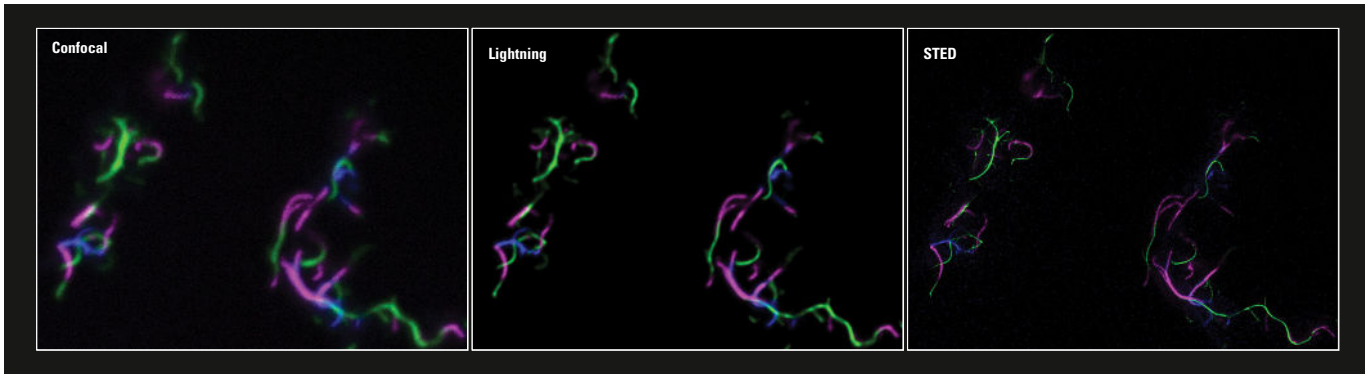
STED: 592 nm, 660 nm, 775 nm

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
AF568, AF555	550	560 – 570	AF 594, ATTO594	580	590– 625	SiR, STAR 635P	640	650 – 750

Table 3. Fluorescent label combinations for **triple color STED** (single and multiple STED lines)[1]

[1]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, sample aging) and STED efficiency. 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.



Bacteria flagella in three colors, imaged at confocal, super–resolution, and nanoscopy level. Sample courtesy of Dr. Erhard, Humboldt–University Berlin, Germany.

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 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 592 nm and 660 nm, and SiR–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 (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 the "3D Deep STED Nanoscopy" workflow: https://www.leica–microsystems.com/applications/life–science/3d–sted–nanoscopy/ and 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 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
TDE	e.g. Sigma–Aldrich	RI adjustable (1,33 – 1,52). Affects fluorescence yield of fluorescent proteins and green dyes[2]

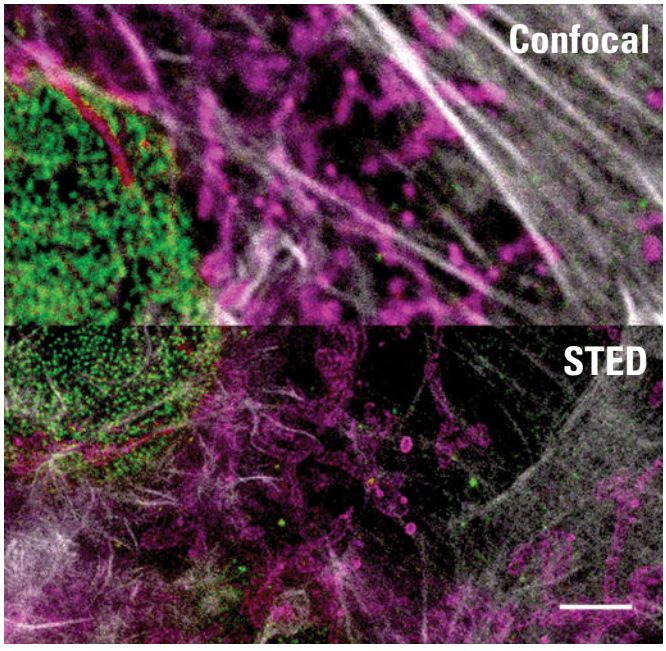
[1]Recommended mounting media for cellular applications.

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

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).

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 (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 of Cos 7 cells stained with antibodies against NUP153 (AF568, green), TOM20 (Atto 594, magenta), and with SiR–actin (SiR, gray). Confocal (top) and STED (bottom) comparison of a sample mounted in Prolong Diamond. Scale bar 2 µm. Sample courtesy of Urs Ziegler and Jana Doehner, ZMB, University of Zurich, Switzerland.

Live–cell STED imaging

STED has extended its range of applications to imaging dynamic processes in live cells and organisms with nanoscopy resolution. 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]	462/470	592	excellent
mTurquoise[2]	434/470	592	very good
eGFP	484	592	very good
EmGFP	487	592	very good
mNeonGreen	506	592	excellent
		660	moderate
eYFP	514	592	excellent
		660	moderate
Venus	515	592	excellent
		660	moderate
mCitrine	516	592	excellent
		660	moderate
DsRed/mRFP	558	660	very good
mStrawberry	574	660	very good
mCherry	587	775	moderate
iRFP	690	775	moderate

Table 5. Fluorescent proteins suitable for STED imaging

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 / ATTO590–BG and Halo–tag / SiR–CA has been reported by Bottanelli et al., Nature Communications, 2016. Recently, an 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.

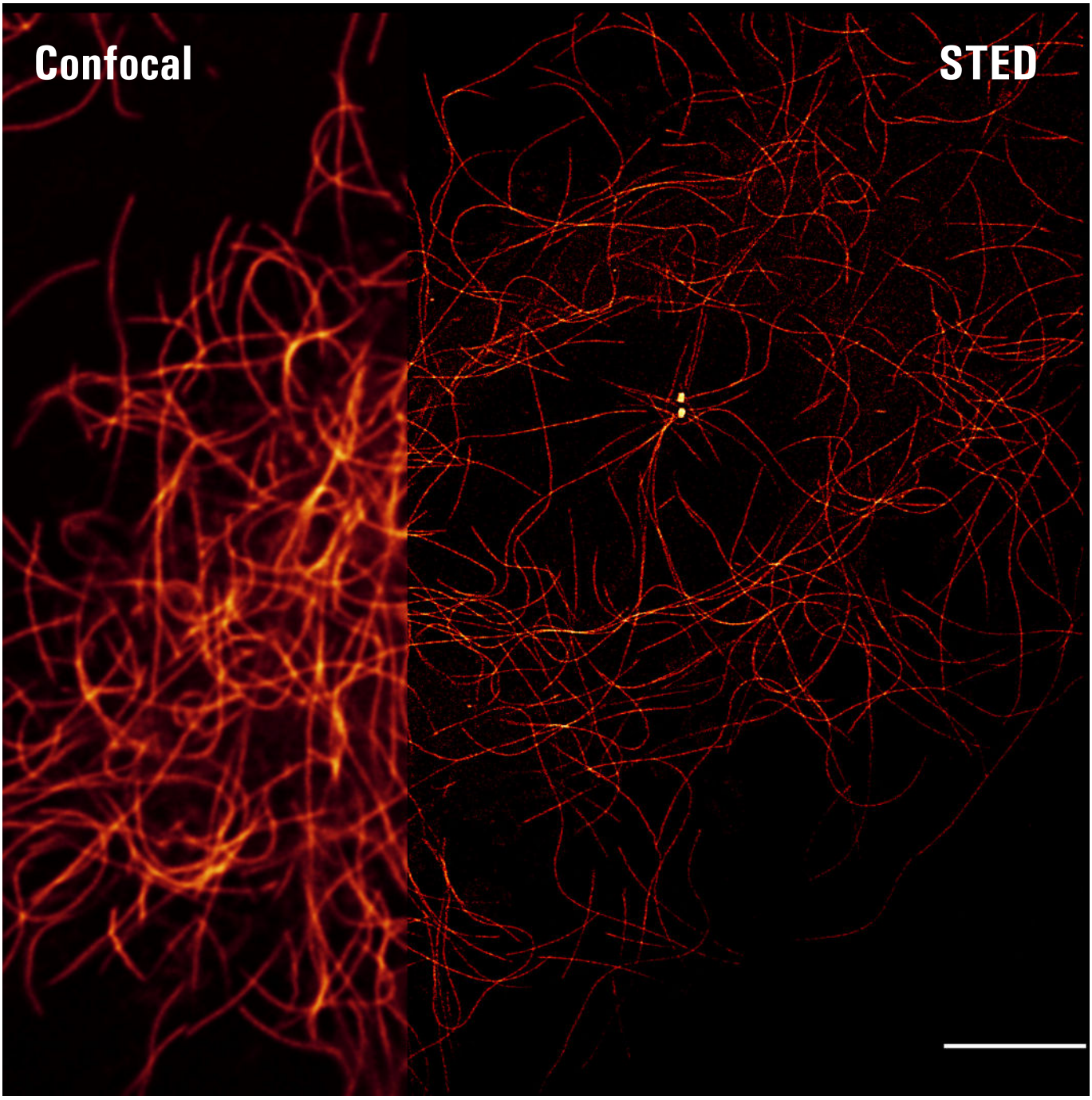
Name	Excitation (nm)	STED (nm)	Performance*
Tubulin Tracker Green	488	592	excellent
OG 488 BAPTA	494	592	excellent
TMR, Methyl Ester, Perchlorate (TMRM)	540	660	excellent
ATTO590–BG (for SNAP–tag[3])	590	775	excellent
SiR–based probes (SiR–tubulin, SiR–actin, SiR–CA)	635	775	excellent
Nile Red	561	775	excellent
Fluorogen Activated Proteins / Malachite Green	640	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.

[2]commmercially available from Spirochrome

[3]commercially available from New England Biolabs Inc.



2D STED live–cell imaging of microtubules stained with SiR–tubulin. Scale bar 2 µm. SiR–tubulin probe available from Spirochrome

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