

Living up to Life



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Quick Guide to STED Sample Preparation

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The guide's focus is set on the Leica TCS SP8 STED 3X with 592 and 660 nm STED laser

Stimulated emission depletion (STED) microscopy is a super-resolution method based on fluorescence confocal imaging, in which images are acquired by scanning a focused light spot over a region of interest and collecting the fluorescence sequentially pixel by pixel. The main strengths of this technology are:

1. Lateral resolution without any additional post-processing below 50 nm with the vortex donut
2. Intrinsic confocal optical sectioning, enabling the acquisition of planes of roughly 500 nm, 3 dimensional structures, even several tens of microns deep inside the tissue when using the vortex donut
3. Axial resolution below 130 nm by using the z donut
4. The ability to individually match the level and spatial distribution of resolution increase to the sample/application of interest
5. Fast image acquisition of several images per second
6. Live-imaging capabilities by using either fluorescent proteins or other fluorescent tags
7. Ability to choose fluorophores over a wide spectral range enabled by several different STED lasers at one instrument

Step 1: Choice of samples

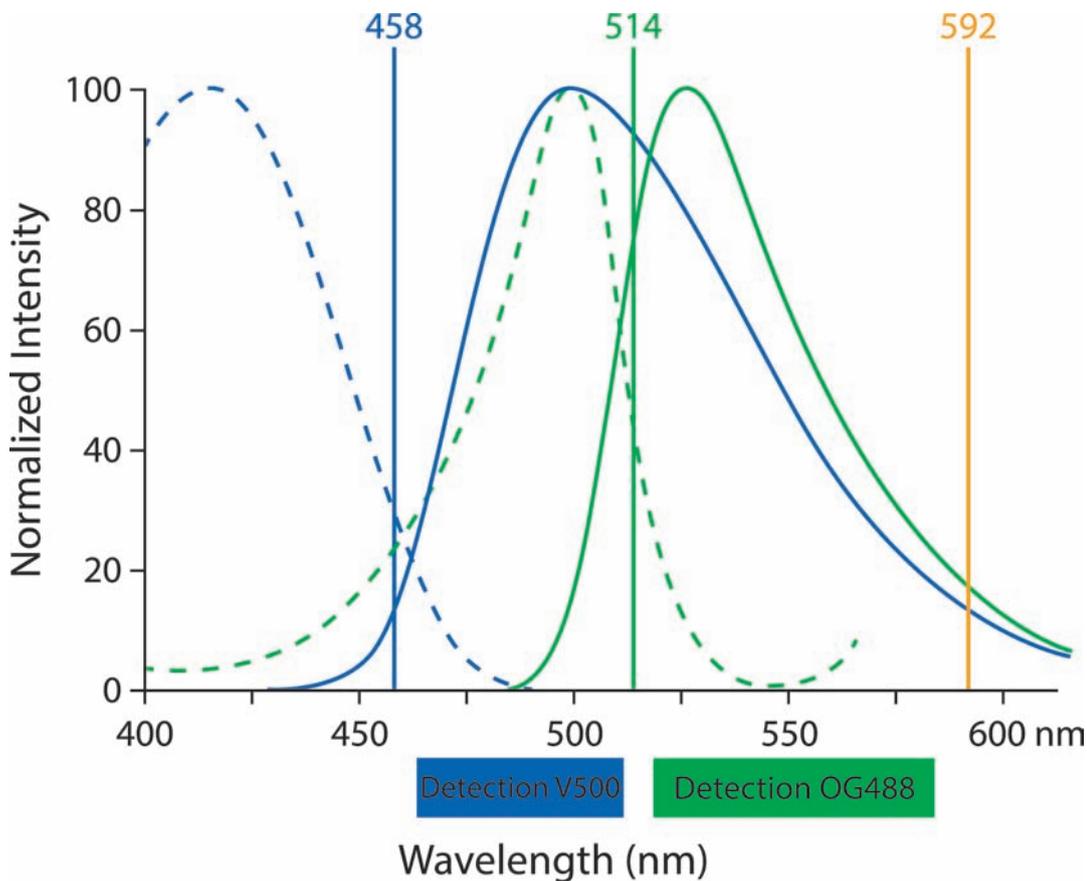
- STED can be applied on a big variety of samples, ranging from single cultured flat cells, tissue slices to whole animals, e.g. nematodes (*C. elegans*) and insects (*D. melanogaster*). Nonetheless some points should be considered. STED applies a specially developed STED 100x/1.4 oil objective, which has a working distance of 90 μm . Thus, the observed structure should be at most 80 μm away from the cover glass, but preferably within a 20 μm range for optimal performance. Additionally, in order to achieve the best results, the refractive index of the mounting medium should match the index of the immersion used (immersion liquid = 1.518, also see Step 5). There are currently no dip-in objectives in our portfolio.
- The structure has to be optically accessible. Auto-fluorescence, sudden and unpredicted changes of the refractive index (e.g. tissues containing air, myelin and fat) may influence the shape of the focal spot and consequently the performance of the microscope. If experience with clearing solutions is available, it might be worth testing.
- During STED imaging, samples are irradiated with strong light at a wavelength of 592 nm or 660 nm. It is of crucial importance that the sample is not absorbing light at these wavelengths.

Step 2: Choice of fluorophores

There is a wide range of fluorophores performing well with Leica STED microscopes (e.g. see Appendix A). In order to achieve satisfying results within the few hours, in which a system demonstration is normally realized, it is advisable to stay with the repertoire of fluorophores suggested by Leica.

If this is not possible, fluorophores should be chosen, which have similar excitation and emission spectra as the suggested dyes. It is also beneficial to start with single color

stainings and, as soon as these are approved, move on to multicolor experiments. Please refer to Appendix B and C for the recommended fluorophor combinations for multicolor experiments, which do not require additional spectral unmixing. For STED to work efficiently, the emission spectra of both fluorophores need to show significant emission at the STED wavelength (see example of STAR 440SX and Oregon Green 488 in figure below).



In order to allow spectral separation of dyes with similar emissions different excitations are required. For STED with 592 nm this is frequently realized by using large Stoke's shift dyes (BD V500, STAR 440SX, STAR 470SX) with absorption spectra located farther to the left side of the spectrum, than the absorption spectrum of regular dyes. It is principally possible to include additional dyes for more colors. The gated STED technology allows a significantly

less stringent choice of dyes with respectable resolutions (significantly below 80 nm) for the same STED wavelength. This ultimately enables imaging of triple color labeling with the 592 STED line (e.g. STAR 440SX, Oregon Green 488 and Alexa Fluor 532). Gated STED triple color imaging with three standard dyes is achieved with the 660 STED laser (e.g. Alexa 488, Alexa Fluor 532 and TMR).

STED microscopy delivers the most reliable super-resolved co-localization data. The STED donut/line determines where fluorescence can be emitted and therefore the channels can be considered intrinsically aligned. One can of course also work sequentially with several STED lines to achieve the best possible resolution for the given fluorophores, but for perfect co-localization data this might require corrections as they are essential for other super-resolution technologies as well.

Additionally other fluorescent marker can be used as counterstainings with confocal resolution. The emission of these dyes, however, should reside outside the range of the STED detection, that could otherwise interfere with STED image quality. Furthermore it is likely that these dyes will absorb the strong STED depletion light and get bleached. Thus all reference images should be acquired before the STED images.

Note that the use of DAPI and Hoechst might have a negative influence on image quality (background), especially with the 592 nm STED depletion laser.

Step 3: Choice of primary antibodies / labeled structures

In order to analyze the performance of STED in a given sample, the experiment is optimally divided into two parts. Part A and B might be performed in parallel, with A being the control experiment for B.

A) Evaluation of the STED performance in the according environment

Fluorescent dyes and antibodies are sensible to the surrounding environment (pH, salt concentrations, redox agents). In order to check the performance of dyes from the given sample, in a first step, a well-established primary antibody should be chosen that gives a specific, bright staining. Even with no real biological relevance, this step helps to ensure that the dye is behaving as expected in the surrounding environment.

B) Working with the structure of interest

In a second step, technically more demanding stainings can be imaged that now address the actual structures of interest.

The labeling density plays an important role in super-resolution, but it cannot be properly checked with conventional microscopes. Often, increasing the antibody concentration during the staining procedure already helps in enhancing the sample quality. It is therefore advisable to work with higher antibody concentrations (2 to 5-fold) for STED imaging to ensure optimal labeling density.

It is advisable to test different primary and secondary dye concentrations in step A and B. The quality of the staining (brightness/background) should be checked and potentially optimized using conventional microscopy prior to the STED imaging.

Step 4: E.g. Antibody staining**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 an antibody staining procedure has a distinct influence on the sample quality. Each step will be briefly explained by means of a standard immunofluorescence protocol for cell culture, as an example to assist the user during the optimization steps for his/her own protocol:

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, comments are in brackets.

1. **Rinse 3x with PBS** (Cells should be washed, culture medium removed by rinsing the culture 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, which could otherwise lead to premature death and decomposition)
2. **Fix with 2% PFA in PBS for 15 min** (Fixation of samples is a critical step in the sample preparation, as it defines how well the structure will be preserved. With increasing resolution this step becomes more critical and should be addressed with care. PFA is a common fixative, but it is not always the best performing one. Some research in literature and optimization might be required here. Alternatively, a 5 min incubation with ice-cold (-20°C) 100% methanol can be used. The methanol fixation does not require additional permeabilization steps. Thus, steps 5 and 6 can be ignored with methanol fixation, also see below.)
3. **Rinse 3x with PBS** (Remove higher concentrations of fixatives for following steps.)
4. **Wash 3x with PBS for 5 min** (Remove the rest of fixatives for 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 better preserve the structure, 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 1 h** (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 the serum helps in preserving the cellular structure. Thicker tissues might require longer incubation times.)
8. **Incubate with primary antibody for 1h** (Use of higher antibody concentrations might be helpful for STED experiments, longer incubation times frequently give better results, but be aware of potentially increased background. In thicker samples (e.g. whole mounts) incubation may take up to days. Alternatively the incubation can be done at 4°C over night.)
9. **Wash 3x with PBS for 5 min** (Washing steps are important, especially when using high concentration of antibodies. 5 minutes is the absolute minimum for washing steps here. Otherwise move to 10 or 20 minutes incubations and more times for better results. Previous rinsing steps might speed up the process.)
10. **Incubate with secondary antibody for 1h** (You might need to adopt/optimize the antibody concentration for your application. Incubation with secondary antibodies should be performed similar to primary antibody incubation. For secondary antibodies a good starting point are dilutions of 1:100, when bought from commercially available sources, otherwise 5x higher than the recommended dilution. For Becton and Dickinson V500 stainings use the biotinylated antibody from Jackson ImmunoResearch Laboratories at dilutions of 1:100 at this step. Incubations can also be done overnight. Thicker tissues need longer incubation times.)
11. **Wash 3x with PBS for 5 min** (Remove unbound antibodies from sample. Longer and more washing steps will increase the quality and specificity of fluorescent label. Previous rinsing steps might speed up the process.)

12. Additional steps only needed when staining with BD V500:

– Incubate with Streptavidin-V500 for 30 min (Additional step when BD V500 is used for fluorescent labeling. Dilutions of V500 should be of 1:50. Longer incubations might be required for ticker tissues.)

– Wash 3x with PBS for 5 min (Remove unbound Streptavidin-V500 from sample. Longer and more washing steps will increase the quality and specificity of fluorescent label. Previous rinsing steps might quicken the process.)

13. Mount (See Step 5 below)

14. Store at 4°C

Finally, the staining should look crisp and bright, when observed through the ocular (e.g. for 592 STED: GFP settings for single color, or CFP/YFP settings for dual color with standard and large Stokes shift dye) and yield good signal to noise in confocal or widefield fluorescence microscopes.

Step 5: Mounting

The mounting medium should have a refractive index matching the immersion required by the objective to enable highest penetration depth without unwanted aberrations. Furthermore, no auto-fluorescence should be observed when irradiating it with 592 nm, or 660 nm laser light, nor should it contain DAPI or Hoechst (for 592 nm STED). Alternatively, if DNA/nucleus stainings are required, Picogreen (Invitrogen) was found to perform well with both 592 nm and 660 nm STED.

In some cases mounting media affected the fluorescence yield of large Stoke's shift dyes (e.g. VectaShield), or of fluorescent proteins and some green dyes (e.g. TDE – For a list of dyes working in TDE please refer to Staudt et al., 2007). Thus, we do not recommend the utilization of VectaShield together with large Stoke's shift dyes. Prolong Gold (Invitrogen) has performed well in our hands and is recommended by Leica.

Very good results were also obtained with rather simple self-made glycerol based mounting as described below:

A) Glycerol

By combining different amounts of water (or PBS) and glycerol (or even just pure glycerol) the refractive index (RI) can be precisely adjusted between 1.33 and 1.47. Additionally, it is easy to prepare and suitable for longer sample storage at – 20°C.

B) Mowiol

Take 6 g of glycerol (analytical grade) and add 2,4 g of Mowiol powder (Calbiochem # 475904), 6 ml of Aqua dest., 12 ml of 0.2 M TRIS buffer with pH 8 and stir the solution for approximately 4 hours. Subsequently let the solution rest for additional 2 hours. Incubate the Mowiol for 10 min at 50°C (water bath) and centrifuge the solution for 15 min at 5000 g. Finally, take the supernatant and freeze the medium at – 20°C for storage. Mowiol is so far the best suited medium for STED images and can be used for – 20°C storage of samples.

All Leica objectives with coverglass correction are corrected for #1.5 coverslips (optimal: 0.170±0.01 mm thick, Hecht-Assistent, cat. number 1014/2424), which should be used for mounting and drastically enhances image quality compared to #1 coverslips not only for STED but also for confocal imaging.

Common antifades, e.g. DABCO (2.5 %) or NPG (4 %), also may cause significant changes to the photo-physical properties of dyes and are sometimes used with STED.

Step 6: Live-imaging

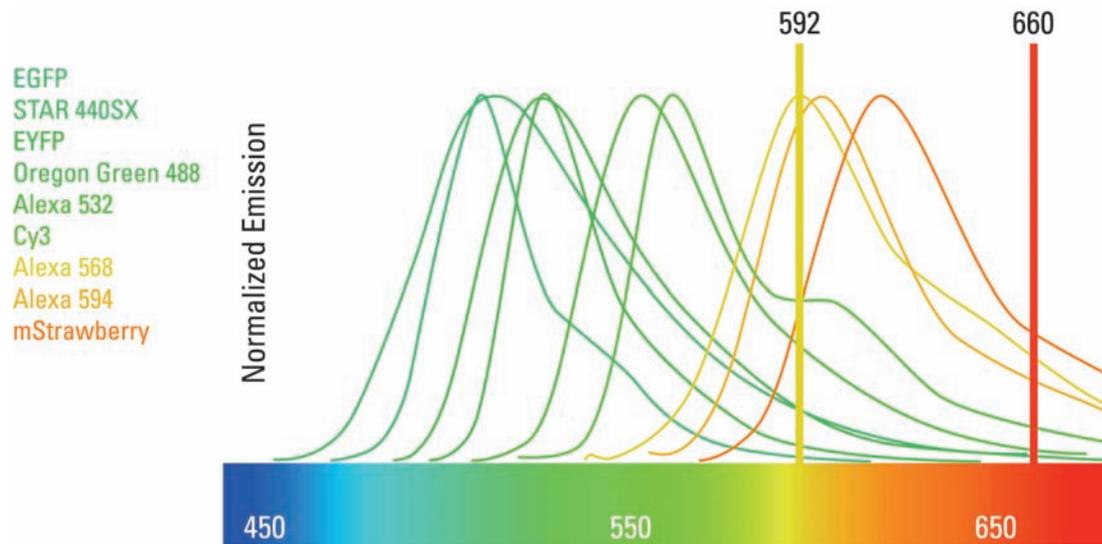
Leica TCS SP8 STED 3X module is currently only supported for inverted microscope stands and live-imaging procedures have to be adapted accordingly. Good results have been reported with a series of fluorescent proteins.

Fluorecent Proteins	Excitation (nm)	Depletion (nm)
mTurquoise2	434	592
eGFP	484	592
EmGFP (Emerald)	487	592
mNeonGreen	506	592
eYFP	514	592 / 660
Venus	515	592 / 660
mCitrine	516	592 / 660
dsRed	558	660
mStrawberry	574	660

But also organic dyes used for labeling living cells (e.g. Tubulin Tracker Green, Oregon Green BABTA) were convincing.

If live-imaging experiments are desired, it is advisable to contact the Leica personnel in order to clarify the experimental procedure and if the necessary equipment is present at site.

Appendix A: List of conjugates for staining



Fluorophore	Excitation (nm)	Depletion (nm)	Provider	Cat. Number
Biotinylated Antibody	-	-	Jackson Immuno-research	115-065-003 (mouse) 111-065-003 (rabbit)
BD Horizon V500**	458 / 470	592	Beckton & Dickinson	561419 (Streptavidin)
Abberior STAR 440SX**	458 / 470	592	Abberior	2-0002-003-7 (mouse) 2-0012-003-4 (rabbit)
ATTO 488	488	592	Sigma-Aldrich	62197 (mouse) 18772 (rabbit)
Abberior STAR 488*	488	592	Abberior	2-0002-006-8 (mouse) 2-0012-006-5 (rabbit)
Alexa Fluor 488*	488	592	life technologies	A11001 (mouse) A11008 (rabbit)
Chromo 488*	488	592	Active Motif	15051 (mouse) 15061 (rabbit)
FITC	488	592	Sigma-Aldrich	F0257 (mouse) F0382 (rabbit)
DyLight 488*	488	592	Thermo Scientific	35502 (mouse) 35552 (rabbit)
Chromo 505**	488 / 514	592	Active Motif	15050 (mouse) 15060 (rabbit)
Oregon Green 488**	488 / 514	592	life technologies	O6380 (mouse) O6381 (rabbit)
Abberior STAR 470SX**	470	660	Abberior	2-0002-004-4 (mouse) 2-0012-004-1 (rabbit)
Alexa Fluor 514**#	514	592/660	life technologies	A31555 (mouse) A31558 (rabbit)
Alexa Fluor 532**#	532	592/660	life technologies	A11002 (mouse) A11009 (rabbit)
Alexa Fluor 546**	546	660	life technologies	A11030 (mouse) A11035 (rabbit)
Cy3**	550	660	life technologies	A10521 (mouse) A10520 (rabbit)
Tetramethylrhodamine/TRITC**	554	660	life technologies	A16071 (mouse) T2769 (rabbit)

Fluorophore	Excitation (nm)	Depletion (nm)	Provider	Cat. Number
Alexa Fluor 555**	555	660	life technologies	A21424 (mouse) A21429 (rabbit)
CF 555*	550	660	Biotium Inc.	20031 (mouse) 20232 (rabbit)
Alexa Fluor 568**+	568	660	life technologies	A11004 (mouse) A11011 (rabbit)
Alexa Fluor 594**#	594	660	life technologies	A11032 (mouse) A11037 (rabbit)

Appendix A: List of some conjugates reported to work with Leica STED microscopes.

* recommended dyes for single color experiments;

** recommended dyes for single, dual and triple color experiments

might require advanced imaging parameter settings due to higher anti Stroke's excitation, e.g. small pinhole, low STED laser power and high gate start values.

Appendix B: Recommended dual color dye combinations for single STED laser lines*

STED: 592 nm, 660 nm					
Dye1			Dye2		
Name	Excitation	Emission	Name	Excitation	Emission: e.g.
BD Horizon V500	458/470	475 – 510	Oregon Green 488/Chromeo 505	514/520	523 – 580
Abberior STAR 440SX	458/470	475 – 515	Oregon Green 488/Chromeo 505	514/520	523 – 580
Alexa Fluor 532	514	520 – 565	TMR/ TRITC/ Alexa Fluor 568	580	590 - 650
Chromeo 505	505	515 – 565	TMR/ TRITC/ Alexa Fluor 568	580	590 - 650

* Dye spectra might shift, due to environment conditions, conjugation type and sample age. Slight adjustments of excitation lines and detections ranges might be required for optimal spectral separation. In principal, the suggested dyes were tested and found to have no, or only minimal cross-talks between channels.

Appendix C: Recommended triple color dye combinations for single and multiple STED laser lines*

STED: 592 nm, 660 nm								
Dye1			Dye2			Dye3		
Name	Excitation	Emission	Name	Excitation	Emission	Name	Excitation	Emission
STAR 440 SX**	470	475 – 505	Oregon Green 488	510	515 – 530	Alexa Fluor 532	540	550 – 585
Oregon Green 488**	470	475 – 525	Alexa Fluor 532	532	538 – 550	TMR/ TRITC	580	590 – 650
Alexa Fluor 514**	480	490 – 535	Alexa Fluor 546	540	545 – 580	Alexa Fluor 594	590	600 – 650

* Dye spectra might shift, due to environment conditions, conjugation type and sample age. Slight adjustments of excitation lines and detections ranges might be required for optimal spectral separation. In principal, the suggested dyes were tested and found to have no, or only minimal cross-talks between channels.

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

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