STED Super-Resolution Microscopy

Background. Stimulated emission depletion (STED) microscopy is a super-resolution method based on the laser scanning confocal imaging of a sample labeled with fluorescent dyes. STED microscopy improves the resolution by reducing the area of an individual light-emitting spot, and thereby allowing the detection of structural features less than 200 nm apart. This is accomplished by superimposing a doughnut-shaped laser beam from a depletion laser (also called a STED laser) upon the focused excitation beam from an excitation laser. The wavelength of the depletion beam should target the red-shifted tail of the fluorophore emission. Because the STED beam has zero intensity in the center, it inhibits fluorescence emission only at the periphery of the excited region. This reduces the size of the region containing the molecules that fluoresce, as if the ‘‘focal spot’’ of the microscope is sharpened. Scanning the co-aligned excitation and STED beams across the sample yields the final super-resolution image, whereby the resolution can be adjusted by the intensity of the STED beam [15,17].
The Leica SP8 STED microscope at the MCC is equipped with 592, 660, and 775 nm depletion lasers. The 592 and 660 nm lasers are constant wave (CW) lasers and can achieve resolutions below 50 nm. The 775 nm laser is a pulsed laser and can achieve lateral resolutions below 30 nm. To date, the highest reported lateral resolution for biological samples using STED imaging was 20 nm when utilizing organic dyes and 50–70 nm when utilizing fluorescent proteins [8].

3D STED. By combining a ring-shaped depletion pattern in the x-y plane with another ring-shaped pattern along the z axis it is possible to improve resolution in both the lateral and axial directions simultaneously, allowing three-dimensional super-resolution imaging with a resolution of 110 nm in the axial (z) plane.

Gated STED. In STED imaging, the resolution is dependent on the lifetime of the excited state of the fluorophore. A STED laser silences long-living excited states of the fluorophore more efficiently than short-living ones. Within the exited spot, long-living states are found in the center while short-living states tend to be at the periphery in the proximity of the STED beam [17]. However, the efficiency of the STED laser to silence the emission is lower at the beam’s edges. By applying the time-gated detection and observing only the long-living states the size of the focal spot can be reduced further. By using a WLL laser and gated STED one can achieve more than 50% improvement in resolution than with STED CW.

Samples. STED can be used with a wide variety of samples, including cultured cells, tissue slices, and small organisms (e.g., nematodes and insects). The observed structure should be at most 80 µm away from the coverglass but preferably within 20 µm from it for optimal performance. To achieve the best results, the refractive index of the mounting medium should match that of the immersion liquid (oil, refractive index = 1.518). Autofluorescence and variations in the refractive index (e.g., tissues containing air, myelin and fat) may influence the performance of the microscope. During STED imaging, samples are irradiated with strong light at a wavelength of 592, 660 or 775 nm. Therefore, it is critical that the sample does not absorb light at these wavelengths.

Sample Preparation. When using immunolabeling, it is advisable to work with somewhat higher primary antibody concentrations. The labeling conditions (primary and secondary antibody concentrations and incubation times) and the quality of the stain (brightness, background) should be verified and optimized using conventional microscopy prior to STED imaging. Usually, the immunofluorescence protocols established and optimized in labs will work in STED microscopy with only minimal modifications. Smaller probes, e.g., nanobodies [13] or aptamers [11], can access more target molecules and produce more densely labeled images. It is advisable to start with single-color staining and, once it is optimized, to proceed to multicolor experiments. It is especially important to

add additional rinsing steps after the incubation with primary and secondary antibodies to remove unbound antibody and thereby reduce background. A sample preparation protocol suggested by Leica can be found at the end of this document. Please also note that DAPI and Hoechst may have a negative impact on image quality when present in samples illuminated with the 592 nm STED laser, and therefore their use should be avoided. From our experience, the use of 660 and 775 nm STED lasers on samples lightly stained with DAPI does not induce any artifacts in super-resolution images.

Sample Mounting. Use only #1.5 (170 µm-thick) coverslips. Leica recommends using Prolong Diamond and Prolong Gold mounting media. Good results are also obtained by using Mowiol with added 2.5% DABCO antifade [14,18].

Live-cell STED. The recent development of membrane-permeable silicon-rhodamine (SiR) dyes has made it possible to perform STED imaging in living cells [2,4,5,9-12]. These dyes are resistant to photobleaching and require a red-shifted 775 nm depletion laser, which appears to have lower photo-damaging effects on live cells than the 592 and 660 nm STED lasers. It is also possible to perform STED on live cells labelled with fluorescent proteins (eGFP, Citrine, eYFP) or with the SNAP-tag protein labeling system utilizing the cell-permeable substrate tetramethylrhodamine (TMR) [3,6].

Recommended dyes for various STED lasers
592 nm: Oregon Green 488, Alexa 488, Atto 488, Abberior STAR 488, Chromeo 488.
660 nm: Alexa 555, Atto 550, Alexa 568, Abberior STAR 580, Alexa 594, Atto 594.
775 nm: Alexa 594, Atto 594, Abberior STAR 520SXP, Abberior STAR 580, Abberior STAR Red, Abberior STAR 635P, Atto 647N, Alexa 660, Atto 665, CF680R.

Suggested dual color dye combinations for single STED laser lines:

STED laser	Dye 1			Dye 2		
	Name	Ex	Em	Name	Ex	Em
775	STAR 580	575	582-632	STAR 635P	640	655-720
775	STAR 580	575	582-632	Atto 647N	645	655-715
775	Alexa 594/Atto 594	594	600-650	Alexa 660	660	670-830
775	Alexa 594/Atto 594	594	600-650	CF680R	670	680-830
775	STAR 520SXP	515	600-650	STAR 635P	635	642-700
775/660	Atto 550/Alexa 555	550	558-600	Alexa 594/Atto 594	600	608-658
660	Oregon Green 488	492	500-550	Alexa 568	580	590-640
660	Oregon Green 488	492	500-550	Alexa 594/Atto 594	594	600-650
592	BD Horizon V500	458	465-505	Oregon Green 488	510	517-557
592	STAR 440SX	458	465-505	Oregon Green 488/STAR488	510	517-557

Suggested triple color dye combinations for single STED lines:

STED laser	Dye 1			Dye 2			Dye 3		
	Name	Ex	Em	Name	Ex	Em	Name	Ex	Em
775	STAR 580	570	577-620	STAR 635P	625	627-665	CF680R	670	677-730
775	STAR 520SXP	515	600-650	STAR 580	570	577-620	STAR 635P	640	655-720
775	Atto550/Alexa 555	550	557-600	Atto594/Alexa 594	600	608-650	Alexa660	660	670-730
775	Atto550/Alexa 555	550	557-600	Atto594/Alexa 594	600	608-650	Atto647N	650	660-720
660	Alexa514	480	490-535	Alexa555/Atto 550	550	558-600	Atto594/Alexa 594	600	608-650

It is recommended that you contact MCC staff to discuss specific combinations of dyes for your multicolor super-resolution experiments.

Deconvolution of STED Images. Deconvolution of STED images is highly recommended. At the MCC, the Leica LAS X confocal software is conveniently bridged with the Huygens Professional software for the additional deconvolution of confocal and STED images. The Huygens deconvolution algorithms reduce noise in STED images and improve contrast by up to 10 fold. They also increase image resolution by a factor of 2 in both the lateral (XY) and axial (Z) directions [19]. The software has a specific STED stabilization option built into the deconvolution wizard. This feature corrects for sub-pixel resolution shifts, making the image restoration process more optimal and further improving resolution.

Links

List of dyes used in STED
https://nanobiophotonics.mpibpc.mpg.de/dyes/
Atto dyes (Sigma)
http://www.sigmaaldrich.com/life-science/cell-biology/detection/learning-center/atto.html
Abberior dyes
http://www.abberior.com/shop/Labels-by-Application:::1.html
SiR probes for live cell STED imaging
http://www.cytoskeleton.com/live-cell-reagents/spirochrome
Nano-Boosters (Nanobodies)
https://www.chromotek.com/products/nano-boosters/

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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 immuno-fluorescence 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 overnight.)

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 Immunoreserach 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)

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.