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Sample Preparation & Labelling for STED Microscopy

Labelling Protocols

A good starting point is a protocol that you have confirmed works well for confocal microscopy, as it is likely to work well for STED microscopy too, as long as the dyes used are (replaced with) STED compatible dyes (see Table 1).

When staining tissue, increase the incubation times typically used when staining cells (actual times will vary depending on tissue type, thickness, etc.).

For detailed protocols, see https://abberior-instruments.com/knowledge/protocols/.

We also recommend the following methods article:
C. A. Wurm, D. Neumann, R. Schmidt, A. Egner, S. Jakobs (2009) 'Sample Preparation for STED Microscopy' Methods Mol. Biol. 591, 185–199.

Coverslips

Use **glass cover slips with a specified thickness of 170 µm (#1.5 or #1.5H)**. Most objective lenses on Abberior systems are corrected for this thickness of glass.

Do NOT use plastic cover slips or chambers with a plastic cover slip base. Plastic can cause aberrations and polarization changes in the STED beam, which will compromise imaging quality.

Do NOT use cover slips with grids, gratings or similar structures on the surface, as these will interfere with the shapes of the excitation and depletion foci, which in turn will compromise imaging resolution.

When growing cells, do so on the cover slip surface (not on the slide surface) to minimize the amount of mounting medium between the cover slip and tissue, and to minimize the distance between the cells and the objective lens.

When mounting tissue sections, mount the tissue on the cover slip (instead of onto the slide) to minimize the amount of mounting medium between the coverslip and tissue, and to minimize the distance between the tissue and the objective lens. Using large (e.g., 22 × 40 mm) and/or charged cover slips may facilitate this mounting procedure.

Embedding Media

The following embedding media are recommended for 2D STED microscopy:

- ✓ Abberior Mount Solid (Abberior GmbH, Göttingen, Germany)
- ✓ Abberior Mount Solid Antifade (Abberior GmbH, Göttingen, Germany)
- ✓ Prolong Glass/Gold/Diamond (use DAPI-free version)
- ✓ Mowiol/DABCO
- ✓ Fluormount-G (use DAPI-free version)

The following embedding media are recommended for 2D and 3D STED microscopy:

- ✓ Abberior Mount Liquid (Abberior GmbH, Göttingen, Germany)
- ✓ Abberior Mount Liquid Antifade (Abberior GmbH, Göttingen, Germany)
- ✓ Slowfade Glass/Gold/Diamond (use DAPI-free version)
- ✓ TDE

Prior to mounting, confirm that an objective lens with immersion medium that closely matches the refractive index of your mounting medium is available on the microscope you will be using. This is especially important for 3D-STED microscopy.

Do NOT use Vectashield, Vectashield HardSET, or any embedding medium that contains p-phenylenediamine as an antifade reagent.

When using ProLong Glass, note that some orange dyes may become red-shifted, causing cross-talk with the red channel.

Do NOT include DAPI, Hoechst, Propidium Iodide or Ethidium Bromide in your embedding medium, as these dyes may be excited by the STED laser, leading to high background and blurred images. If a nuclear counter stain is required, you may use DAPI or HOESCHT in VERY LOW amounts. Our recommended DAPI labelling protocol is included later in this document.

When using an embedding medium that cures, allow sufficient time between mounting and imaging for the medium to cure and reach the specified refractive index. DO NOT SEAL the cover slip, especially prior to curing as this will hinder the curing process.

Liquid media (e.g., Abberior Mount Liquid) do not require curing. Therefore, samples can be imaged immediately after mounting. However, the cover slip must be sealed (e.g., with nail polish or glue) to secure it to the slide.

Dyes for STED Microscopy

See Table 1 below for some dyes which can be used for STED Microscopy.

Table 1

Depletion		
775 Depletion	**Red dyes (640 nm excitation line)**	
775 Depletion	Abberior Dyes: ***STAR RED**, ***STAR 635P**, ***STAR 635** Alternatives: ***ATTO647N**, ATTO633, *Alexa 647*	Live-cell Organic Dyes: ***LIVE 610**, ***Silicon Rhodamine (SiR)**, ***JF646**
775 Depletion	**Orange dyes (561 nm excitation line)**	
775 Depletion	Abberior Dyes: ***STAR ORANGE**, STAR 580 Alternatives: ***AF594**, ***ATTO594**, AF568	Live-cell Organic Dyes: ***LIVE 550**, ***LIVE 560**, ***LIVE 590**, ***ATTO590**, JF549
775 Depletion	**Long Stokes-Shift (LS) dyes (485/488 nm excitation line)**	
775 Depletion	Abberior Dyes: ***STAR 460L** Alternatives: ***ATTO490LS**	Live-cell Organic Dyes: ***LIVE 460L**
595 Depletion	**Green dyes (485/488 nm excitation line)**	
595 Depletion	Abberior Dyes: *STAR GREEN*, *STAR 488* Alternatives: *AF PLUS 488*, *ATTO488*	Live-cell Organic Dyes: *LIVE 510* Fluorescent Proteins: *mNeon Green*, *GFP*, *YFP*, *etc.*
Confocal	**Blue Dyes (405 nm excitation line)**	
Confocal	DAPI, Hoescht (please use very low amounts!), AF405	

Legend

Highly recommended for STED

Increased photobleaching with STED

The concentration of Abberior STAR antibodies is always 1 mg/ml. For staining, prepare a 1:200 dilution of the antibody in blocking solution.

Do NOT label with DAPI or Hoescht, if it can be avoided. These dyes may be excited by the STED laser, leading to high background and blurred images. If a nuclear counter stain is required, you may use DAPI or HOESCHT in VERY LOW amounts (see recommended DAPI staining protocol below).

Recommended DAPI Staining Protocol

1. Prepare a stock solution of 1 mg/ml in PBS.
2. For staining, dilute the stock solution further by 1:5000 in PBS.
3. Incubate the cells in the staining solution for 2 to 5 minutes at room temperature.
4. Wash (~3 times is recommended) any unbound DAPI thoroughly.

Recommended Phalloidin Staining Protocol

We recommend using a phalloidin concentration of 200 U/ml, which equates to 6.6 nmol/ml.

The molar mass of phalloidin probes differs and depends on the dye's molecular mass. For Abberior dyes, the molecular mass is written on the pouch and on the vial that the dye is provided in.

Example calculation using STAR 580 Phalloidin:

molar mass = 1474 µg/µmol

amount = 20 µg

20 µg/ 1474 µg/µmol = 0.0136 µmol = 13.6 nmol

13.6 nmol/ 6.6 nmol/ml = 2.06 ml

Therefore, to obtain a stock solution of 200 U/ml, dissolve the STAR 580 phalloidin in 2.06 ml of water-free DMF (alternative options include DMSO or MeOH).

For staining, dilute the stock solution 1:200 in blocking solution (e.g., BSA).

Fluorescent Proteins

For STED imaging, use organic dyes whenever possible. Organic dyes are superior to fluorescent proteins (FPs), especially with respect to brightness and photostability.

As an alternative to FPs in live-cell experiments, cell-permeable organic dyes (e.g., Abberior LIVE dyes) may be used in conjunction with self-labelling protein tags e.g., SNAP-tag. See "Live-cell STED imaging" section below.

Choosing Colors

Single-color STED Samples

- ✓ For the best results (especially with respect to resolution), choose a red organic dye e.g., Abberior STAR RED or Abberior STAR635P.
- ✓ Second best option is an orange dye e.g., Abberior STAR ORANGE or AF594.

Two-color STED Samples

- ✓ Choose a red + orange dye combination e.g., STAR RED + STAR ORANGE. This combination is especially ideal for colocalization experiments, as the images can be acquired in line sequential mode.
- ✓ Use the red dye to label the structure for which you require the best resolution.

Three-color STED Samples

- ✓ Choose a red + orange + long Stokes-shift combination e.g., STAR RED + STAR ORANGE + STAR460L. This combination is ideal for colocalization experiments, as the images can be acquired in line sequential mode.
- ✓ A red + orange + green combination is also possible (note that the 595 nm depletion line is a feature of the FACILITY and INFINITY STED systems only).
- ✓ Red and orange will give the best resolution, so use these for the structures that require the best resolution.
- ✓ If a red + orange + long Stokes-shift combination is used, you may acquire all three channels in a single acquisition using line sequential mode.
- ✓ If a red + orange + green combination is used, the green channel must be acquired in a separate frame acquisition only AFTER you are done acquiring the red and orange channels. This is because the 595-depletion laser used for the green channel will bleach the red and orange dyes.

Live-cell Imaging

For live-cell STED imaging, cell-permeable organic dyes (e.g., Abberior LIVE dyes) are preferred over fluorescent proteins (FPs), as they are brighter and more photostable. Abberior LIVE dyes may be used for the direct labelling of tubulin, actin, DNA, etc. (see https://abberior.shop/abberior-LIVE) or in conjunction with self-labelling protein tags (e.g., SNAP-tag) for more flexible labelling options.

Recommendations for choosing colors are the same as described above. For example,

1-Color 775 nm STED: use LIVE 610 (red channel, first choice) or LIVE 550/560 (orange channel, second choice)

2-Color 775 nm STED: use LIVE 610 (red channel) + LIVE 550/560 (orange channel) or SiR (red channel) + LIVE 590 (orange channel)

3-color 775 nm STED: use LIVE 610 (red channel) + LIVE 550/560 (orange channel) + LIVE 460L (long-Stokes' shift channel)

595 nm STED (not recommended): use LIVE 510 (green channel)

Recommended Live-Cell Imaging Media

In general, imaging media for STED microscopy must be non-absorbing in the excitation channels and non-fluorescent in the detection channels.

For mammalian cells, HDMEM (Dulbecco's Modified Eagle's Medium) buffered with HEPES (Invitrogen, USA) or DMEMgfp-2 (Evrogen, Moscow, Russia) may be used.

For yeast cells, many Synthetic Complete media (without yeast extract and peptone) may be used.

Contact

If you have any further questions, do not hesitate to contact us at info@abberior-ia.com.