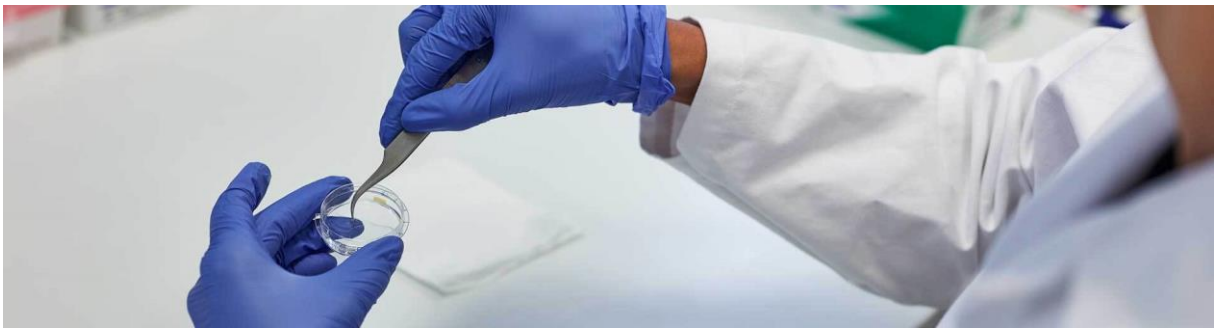
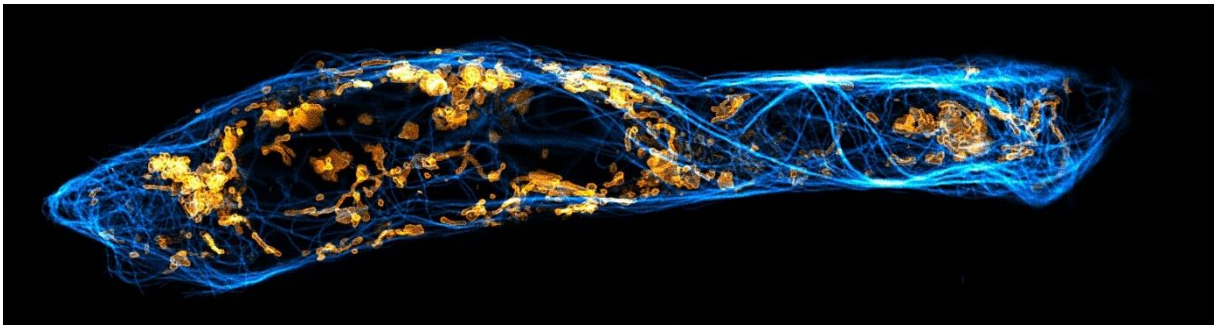
STED microscopy sample preparation





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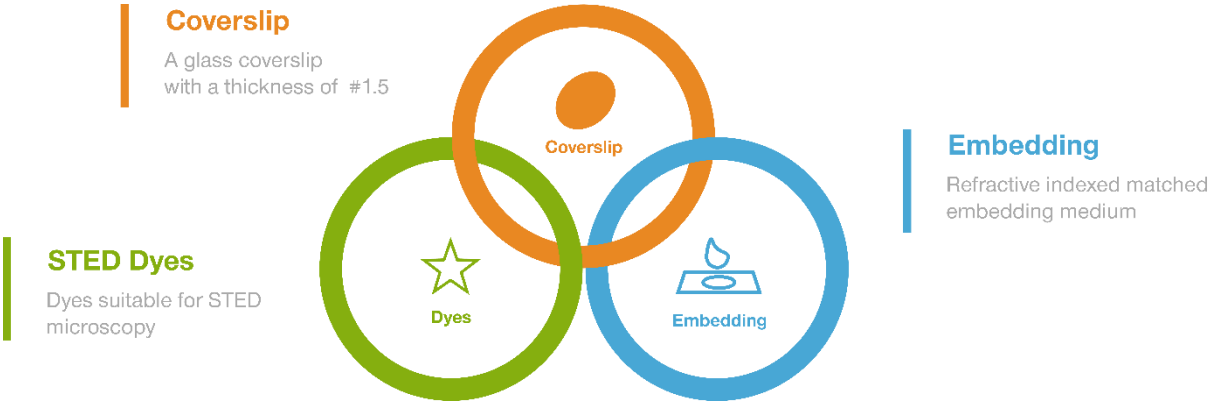


Figure 1: Three basic rules to adapt your confocal labeling protocol to STED sample preparation.

This document provides sample preparation and labeling strategies to facilitate successful STED experiments. These guidelines provide tips for selecting the most suitable fixatives and fluorescent probes, optimizing the labeling strategy, and ensuring proper sample mounting for optimal image quality. By carefully planning and implementing the guidelines described in this text, you can improve the quality and resolution of both your STED and confocal microscopy images.

Effective sample preparation is crucial for obtaining high-quality super-resolution fluorescence microscopy images. Factors such as the type of sample, the fixation method, and labeling technique used can all significantly impact the final image quality. While sample preparation for STED microscopy shares many similarities with standard confocal microscopy, there are also notable differences to consider. For instance, the choice of **fluorescent dyes suitable for STED**, the use of **1.5# glass coverslips** (thickness of 170 µm), and the choice of **embedding** medium with a refractive index that matches the refractive index of the objective lens immersion medium (**Figure 1**).

Note: While sample preparation for confocal microscopy can serve as a starting point, it is essential to recognize that STED microscopy has unique requirements that may necessitate adjustments to the preparation process. Therefore, it is advisable to carefully evaluate the sample preparation protocols in the context of the specific STED microscopy experiment's needs and optimize the process accordingly to achieve the best possible results.

Note: Our recommended abberior immuno-labeling protocol can be found here. (QR code or *download*).

0. A sample preparation quick start guide

Step 1: The basis for an excellent STED sample is a **good confocal sample**. We suggest optimizing your staining protocol (e.g. antibody concentrations, incubation times, etc.) to achieve good (i.e. bright with good signal-to-noise) confocal images first. The next step is adaptation for STED imaging.

Step 2: Are your **secondary antibodies conjugated to dyes that are suited for STED**? If not, exchange them for antibodies that are couple to STED-optimized dyes such as the abberior STAR dyes. As the obtainable resolution is highly dependent on the dye, please select *abberior* STAR RED or STAR 635 (P) (emission: far red, ex 640nm) for your protein of interest. The second most relevant protein of interest should be labelled with *abberior* STAR ORANGE (em: red/orange, ex 561nm). For the third, we suggest *abberior* STAR 460L (em: red/orange, ex 440/488 nm) or for STED with the 595nm STED laser abberior STAR GREEN (em: orange, ex 488 nm). Use the dyes in a dilution of 1:200. When using DAPI, please use it in low concentration (<0.5 µg/ml) and not in the embedding medium.

Step 3: Use an embedding **medium with a refractive index that matches the refractive index of the sample** and the objective lens immersion medium. for example, *abberior* **Mount Solid** which is the **preferred mounting medium** is suitable for STED imaging using the 60x oil objective on *abberior* STED systems. Allow sufficient time for the mounting medium to cure and reach its specified refractive index. If the 3D structure is of interest, please consider embedding your sample in *abberior* Mount Liquid. Please note that abberior mount liquid needs to be sealed to prevent the sample from drying out and to secure the coverslip to the slide. For further information, please see the section “embedding media”.

Step 4: Use **#1.5 coverslips, with a thickness of 170µm.** This is the coverslip thickness optimized for most high NA objectives. Using a #1 coverslip can greatly reduce image quality. Please mount samples in the center of the slide to allow high NA objectives to reach every part of your sample

Step 5: Image samples on a confocal or STED microscope.

Troubleshooting

Is the fluorescent **signal bright and clear**? If not, you can increase the primary antibody concentration and/or the incubation times.

Is the sample **low in background**? If not, please add more washing steps and check the fixation and blocking steps.

Note: For samples with high background, it can be helpful to include a washing step with a low concentration of detergent (e.g. PBS + 0.1% Tween) between the primary and secondary antibody incubation steps.
Are the structures on the **sample well preserved**? If not, check fixation and permeabilization protocol. Use specialized protocols for structures like Tubulin or Actin to achieve optimal structure preservation.

We also recommend the following methods articles:

C. A. Wurm, D. Neumann, R. Schmidt, A. Egner, S. Jakobs (2009) 'Sample Preparation for STED Microscopy' Methods Mol. Biol. 591, 185–199.

Tubulin fixation and staining protocol
A. Jimenez, K. Friedl, C. Leterrier. About samples, giving examples: Optimized Single Molecule Localization Microscopy. Methods, 174 (2020), p. 100-114, 10.1016/j.ymeth.2019.05.008

1. Cell preparation

1.1. Glass coverslips

Grow cells on **glass coverslips**. Do not use plastic coverslips. The choice of coverslip material is an essential consideration for obtaining high-quality images in STED microscopy. Glass coverslips are preferred to plastic coverslips as they have a smoother surface and greater transparency, resulting in reduced scattering and background noise, and enhanced resolution and contrast. Moreover, plastic coverslips can alter the polarization of the light, which can interfere with and, in some cases, abolish the depletion effect, leading to no improvement in resolution.

Using #1.5 glass coverslips with a **thickness** of 170um is recommended for optimal STED imaging. The use of high precision coverslips (e.g. 1.5H) is optional.

For live-cell imaging, it is crucial that the bottom of the dish, well or chamber is also #1.5 glass. Examples are shown in **Figure 2**.

Note: Gridded coverslips should be used with caution as they may cause aberrations and diffraction of the STED beam. When using gridded coverslips, it is best to avoid imaging the cell or area of interest on the grid itself.

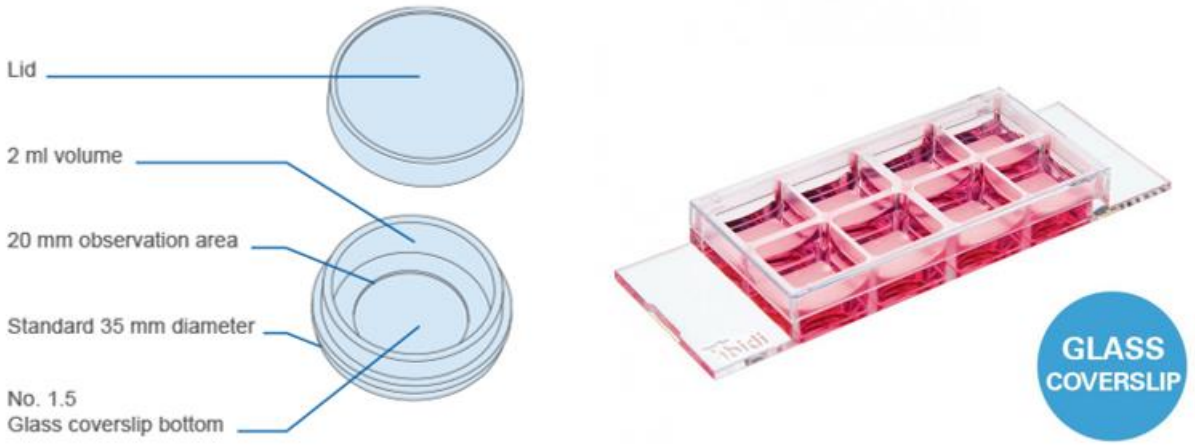


Figure 2: Examples of glass coverslips well-suited for live-cell STED microscopy. Cells can be seeded in standard 35 mm (left) or 8-well glass bottom dishes (right). Source: https://ibidi.com

1.2. Coverslip Coating

Coating the coverslip is not required for super-resolution microscopy but can sometimes help cells adhere to the glass. It can also allow cells to attach, spread and flatten on the coverslip, which enhances final imaging quality. Typical coating agents include but are not limited to Matrigel, fibronectin and poly L-lysine. Please note that some coatings can increase background signal as dye or antibodies may bind to them.

1.3. Growth conditions

Samples should be grown under **optimal conditions** for the best results. When working with cultured cells, this includes allowing the cells to recover after treatment (e.g. at least 12 hours after passaging), and to settle, spread, and reach a suitable confluency. The final confluency should be high enough to avoid searching for a needle in a haystack during the imaging session, but low enough to avoid overgrowth on the coverslip. This range depends heavily on the cell line. For example, a final confluency of around 70 % is recommended when working with HeLa cells.

Please note that overexpression of proteins can cause artefacts, so it may be important to consider endogenous knock-in methods instead. The number of proteins can also greatly affect imaging, as low levels of protein can result in low labeling density and therefore low signal.

2. Fixation methods and primary antibody labeling

2.1. Fixation

Chemical fixation of the sample can preserve the structure and morphology of cells and tissues by cross-linking proteins. Imaging of fixed cells tends to obtain a higher final resolution as fixation helps to stabilize the sample and prevents degradation. Common fixatives include formaldehyde, glutaraldehyde, methanol, and acetone. Various protocols for different fixations methods can be found here (*abberior* labeling for STED microscopy, QR code).

Note: Antigen sub- and ultrastructure can be damaged or altered by most chemical fixation methods, thereby masking antibody epitopes. The resulting decreased primary antibody binding efficiency might be retrieved by **heat induced antigen retrieval**.

Paraformaldehyde (PFA) fixation is a common fixation protocol for most cell types and tissues. Cells or tissues are fixed with 4% PFA for 10-20 minutes (cells) to hours (tissue sections) at room temperature, followed by washing with phosphate-buffered saline (PBS). PFA fixation crosslinks proteins, preserving cell morphology and antigenicity, and enabling detection of both cytoplasmic and membrane-bound proteins.

Glutaraldehyde (GA) is a widely used fixative for electron microscopy and for light microscopy. It effectively preserves the fluorescent emission of chlorophyll, allowing post-fixation imaging, and exhibits excellent structural preservation for many proteins. To achieve fixation, 25% electron microscopy grade glutaraldehyde is usually adjusted to a final concentration of 0.25-1% depending on the cell density of the

sample and incubated along with PFA. While GA provides stronger fixation compared to PFA, it may alter the antigenicity of epitopes in the sample and prevent the binding of primary antibodies, making it incompatible with certain antibodies.

Aldehyde fixation, especially with glutaraldehyde, can produce autofluorescence by binding amines and proteins to generate fluorescent products. This autofluorescence can be quenched by incubating the sample with 100 mM NH_4Cl and 100 mM glycine. This process neutralizes the remaining reactive aldehyde groups, reducing the formation of fluorescent adducts and subsequent background fluorescence.

Methanol fixation: This is a common fixation protocol for detecting cytoplasmic proteins, especially for dedicated tubulin fixation protocols as well as for cells grown in suspension, such as blood cells or lymphocytes. Cells are fixed with cold methanol (-20°C) for 5-10 minutes, followed by washing with PBS. Methanol fixation permeabilizes the cell membrane, allowing antibodies to access intracellular proteins.

Artefacts can arise during the preparation, fixation, or staining of a sample, causing misleading effects. To avoid artefacts, it is important to consider the specific structure that you want to localize and optimize the **fixation protocol** to minimize the disruption of those structures. For example, some fixation methods, such as PFA fixation, cause fragmentation of the Golgi or tubular transport intermediates, which results in a distorted final image. Moreover, chemical fixatives like PFA can also lead to high auto-fluorescent background signal.

Many staining protocols include a **blocking step** prior to primary antibody labeling, which involves applying a substance to the sample to reduce non-specific binding of antibodies or other molecules. Blocking can help improve the specificity and sensitivity of the assay and reduce the risk of artefacts. Common blocking solutions include 1-5% bovine serum albumin (BSA) or 1-5% normal serum from the same species as the secondary antibody. BSA is a commonly used protein blocking agent . Normal serum contains IgG that can bind non-specifically to the sample, thereby blocking non-specific binding of the secondary antibody.

2.2. Tissue preparation

Sample preparation for tissues plays a critical role in determining the final quality of the sample and often deviates from standard preparation of cells due to cell density and volume of the samples. While some samples can be mounted whole, others must be sectioned first as their thickness prevents optimal antibody labeling or the working distance of the objective is limited. Usual section thicknesses range between 5 µm and 100 µm.

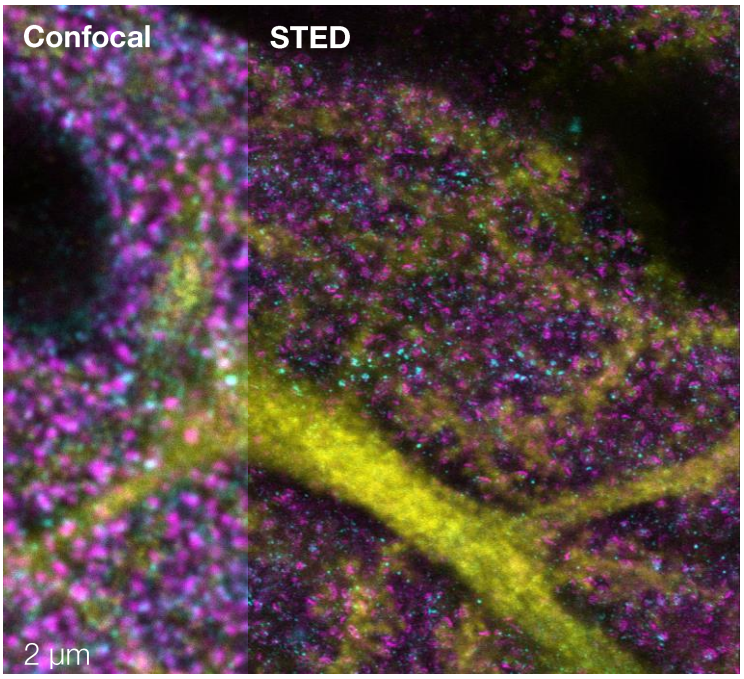


Figure 3: Synaptic proteins labelled in a cerebellar brain slice kindly provided by Ali Jalil and imaged at MIFOBIO2021

Note: Chemical fixation in tissues typically takes longer than in monolayer cell cultures. The required incubation times depend on the volume and thickness of the sample.

A widespread sectioning method is the embedding of the sample in paraffin. The sample needs to be dehydrated first before it is permeated with paraffin. Once the paraffin blocks are hardened, they can be sliced into thin (5-8 µm) sections with a microtome.

Vibratomes use horizontally vibrating blades to obtain 40-400 µm thin sample slices. If the sample is not chemically fixed prior to slicing, the sections themselves should undergo fixation.

Cryo-fixation is a method that can keep the sample in a more native state than chemically fixed ones. In cryo-fixation, samples are vitrified, i.e. rapidly frozen down in cryogenic solvents, which allows for the preparation of ultra-thin slices.

Note: One way to improve transparency and refractive index matching in tissue samples is tissue clearing. Physical or chemical based approaches, such as water-based solutions or organic solvents (e.g., *abberior TDE*), can be used to match the refractive index of the sample, allowing improved contrast and visualization of deeper structures. However, tissue clearing can be harmful for some fluorophores, resulting in lower brightness levels. TDE can be used for STED imaging while other clearing reagents might require testing and adaption for optimal super-resolution imaging.

Note: When **mounting tissue sections**, **mount the tissue on the cover slip** (instead of onto the slide) to minimize the amount of mounting medium between 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.

2.3. Antibody labeling

Antibody labeling is a widely used technique for visualizing specific proteins or structures in a sample. Specificity of the antibody is crucial for accurate results. It is also important to consider the epitopes of the protein being targeted and how they may be affected by chemical fixation, especially with glutaraldehyde as it can alter the conformation of the target protein and thereby masking original antibody epitopes.

Note: Please use mild and gentle permeabilization or extraction protocols to minimize the disruption of cellular structures and the risks of introducing artefacts.

Note: Blocking free epitopes is an important step before applying primary antibodies as it decreases unspecific antibody binding. Common blocking solutions include bovine serum albumin and milk powder, but the optimal solution may vary depending on the sample and the specific antibodies being used. In some cases, it may also be helpful to include very low concentrations of detergents such as Tween-20® or Triton X-100®.

Note: Often, antibody labeling is performed in low volumes to use the least amount of antibodies. Please make sure your sample does not dry out in the process, e.g. by using a humid chamber during the labeling process.

Cross-reactivity of the antibody species should also be considered (**Figure 4**). Labeling one after the other can enhance the overall labeling Additionally, the labelling time is depending on the thickness of the sample as antibodies need more time to penetrate and diffuse through thicker samples like tissues.

Species	*Primary / Secondary*	*Monoclonal / polyclonal*	*Isotype*	*Commercially available*	*Quality*
Mouse	++ / (+)	+ / Rare	IgG / IgM	+	++
Rabbit	++ / (+)	Rare / +	IgG	+	++
Goat	++ / ++	+ / +	IgG	+	(++)
Guinea Pig	+ /	+ / +	IgG	+	+
Chicken	+ /	+ / +	IgY	(+)	+
Donkey	/ ++	+ / +	IgG	+	++
Sheep	/ +	+ / +	All	+	+

Figure 4: Example of common primary and secondary antibody species. Antibodies raised in mouse and rabbit are a good choice as primaries together with secondaries raised in goat or donkey.

Note: There are different **isotypes of immunoglobulins** (**Figure 4**), such as IgG or IgM, that are structurally different to each other. Please choose appropriate secondary antibodies that specifically bind the corresponding immunoglobulins of the primary antibody, as it might lead to low signal intensities otherwise.

Note: When choosing secondary antibodies, it is important to consider the host species. If the host species of one of the primary antibodies is the same as the secondary antibody, it can lead to unwanted clustering and crosstalk.

For example, suppose you want to visualize two proteins, A and B, using antibody labeling. Antibody A is raised in goat, and antibody B is raised in rabbit. To label Protein A, you would use a secondary antibody raised in a species other than goat or rabbit. If this is not possible, you can label the primary antibodies sequentially. First, incubate the donkey anti-goat secondary antibody, followed by washing, and then the goat anti-rabbit secondary antibody.

By doing so, you avoid crosstalk. If you label the secondary antibodies together without sequential labeling, the donkey anti-goat antibody will bind not only to the goat primary antibody (which is wanted) but also to the goat anti-rabbit secondary antibody (which is unwanted).

Note: Antibody labeling of extracellular targets can be a useful technique for live-cell microscopy as it eliminates the need for permeabilization. While this approach can provide additional information, it is important to be aware of potential artefacts that may arise, such as downstream signaling triggered by the binding of antibodies to certain receptors.

Another way to label your target is the use of **nanobodies** that are conjugated with fluorophores. These nanobodies directly target and bind to their epitopes and are much **smaller than antibodies**, which may offer some advantages in certain situations. For example, the small size of nanobodies (**Figure 5**) may allow them to access small or difficult-to-reach targets that might be inaccessible to larger probes. However, nanobodies have less binding sites for fluorophores compared to standard primary secondary antibody labeling, thus making them appear less bright.

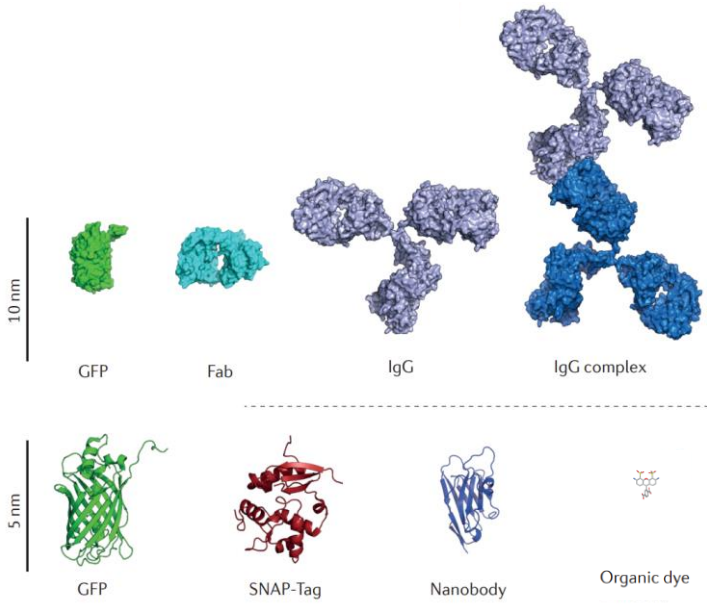


Figure 5: Comparison of the different labeling strategies and their sizes. Conjugated primary secondary antibody complexes are much larger than nanobodies or the SNAP-tag for example. (Image from Sahl, S., Hell, S. W., Jakobs, S., Nat Rev Mol Cell Biol. 2017 Nov;18(11):685-701)

3. Choosing the right Dyes

While many dyes can be used for confocal microscopy, it is important to note that not all of them are suitable for STED. Although some dyes may perform adequately in STED, using STED optimized dyes can greatly improve resolution and image quality.

Note: Here are some recommended **dye combinations** that work well for **immuno-fluorescence STED microscopy**:

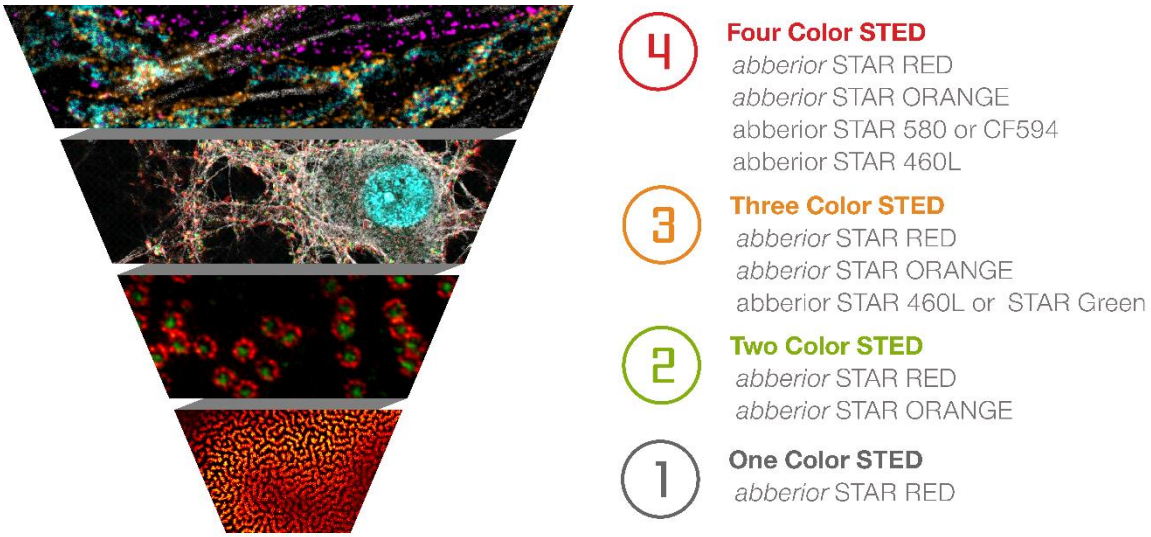


Figure 6: Suggested combination for multicolor STED imaging

3.1. STED optimized dye properties

abberior offers a wide range of STED compatible dyes such as *abberior* ***STAR RED,*** *abberior* ***STAR ORANGE***, *abberior* ***STAR GREEN,*** *abberior* ***STAR 460L***. However, STED microscopy allows for a variety of dyes from different sources, feel free to **try out** the dyes that are already available to your lab.

Note: Whether a dye is suited for STED microscopy depends on several factors:

1) The **emission spectrum** of a STED dye has to overlap with the wavelength of the STED laser (e.g. 775 nm or 595 nm). Only if the dye is able to absorb the energy of the STED laser it can be depleted by it.

2) The **quantum yield** of the dye measures the efficiency of energy absorption and conversion into emitted photons. Dyes that have a higher quantum yield are generally more efficient for STED microscopy as more light usually allows for higher resolution.

3) The **stability** of a dye is also an important factor. Dyes that are sensitive to photobleaching should not be used for STED, as the achievable resolution will be limited.

Note: For reliable high-quality STED images, it is recommended to choose dyes that emit in the far-red to orange spectrum.

abberior offers a wide range of STED optimized dyes. For immunofluorescence STED microscopy we suggest a combination of ***abberior STAR RED, STAR ORANGE, STAR-460L*** and ***STAR GREEN*** as

they offer high brightness and stability for many applications. Far-red dyes are particularly favorable, as they have the highest overlap with the 775 nm STED laser and the highest quantum efficiency. They also tend to be the brightest and most stable dyes and usually give the best resolution.

Adding an **orange dye** (excited by the 561 nm laser) is recommended **for dual-color imaging,** as they are usually also depleted by the same 775 nm STED laser. The combination of red and orange dyes is a good choice, as the emitted wavelengths can be separated by spectral detection, thus also making near-simultaneous line-by-line STED image acquisition of both channels possible.

Using a **long stokes shift dye** (e.g. *abberior* STAR-460L) can be a viable alternative to green dyes as these can also be depleted by the 775 nm STED laser, thus enabling acquisition of three STED channels with just one STED laser with optimal overlay for colocalization studies.

Another way to obtain three color STED images is the use of a **green dye** such as *abberior* STAR Green which emits in the **green spectra** and is depletable by the **595 nm STED laser**.

Note: Green dyes tend to be more **sensitive to photobleaching**, hence shorter exposure times and lower STED laser powers are favorable (e.g. shorter dwell times, larger pixel sizes, less line accumulations). In the end, red and orange dyes will usually result in better resolution than green ones.

Note: The STED image of the green dye should be acquired as an **additional frame step, AFTER the red/orange** image was obtained, since the 595 nm laser bleaches the orange and red fluorophores.

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

3.2. DAPI

DAPI or Hoechst are commonly used to stain nucleic acids in many samples, particularly chromosomal DNA. Although fluorogenic, DAPI/Hoechst can lead to additional high background signal. Moreover, potential **two-photon excitation** of DAPI/Hoechst by the STED laser can result in **cross-talk** in the corresponding channels.

- DAPI/Hoechst should be used in **low concentrations (<0.5 µg/ml)**.
- Please refrain from adding DAPI/Hoechst to your mounting media to avoid background.

Try to AVOID labeling with DAPI or Hoechst if possible. 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 Hoechst in very low amounts.

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.

3.3. Phalloidin

Phalloidin is commonly used to stain F-Actin. Directly coupled to a fluorophore such as *abberior* STAR 580 it gives a bright stain that visualizes the actin cytoskeleton in cells as well as tissue.

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

The molar masses of phalloidin probes differ and depend 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 *abberior* 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 *abberior* 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).

Note: Phalloidin stains are less stable than antibody labeling. Phalloidin samples should be imaged within approximately 2 weeks of preparation and samples should be stored in the fridge continuously to maintain a strong signal and to avoid the dissociation of Phalloidin from F-actin.

Recommended dyes for fixed samples for multi-color STED microscopy (Figure 7):

Depleted by Pulsed STED	775 nm			595 nm
Excitation laser	640 nm	561 nm/ 594 nm	440/485 nm	485 nm
Always recommended	**Abberior STAR RED** Down to 25nm resolution	**Abberior STAR ORANGE** Abberior STAR 580 Alexa 594	**Abberior STAR 460L**	Abberior STAR GREEN Abberior STAR 488 Oregon Green 488
Sample dependent recommended	ATTO 647N ATTO 643 ATTO 633 AlexaFluor 647 Cy5	Abberior STAR 600 ATTO 594 CF 594 Cy3		AlexaFluor 488 ATTO 488 FITC

Figure 7: Dye recommendation for standard immunofluorescence STED microscopy in fixed cells. The use of bold and underlined dyes is highly recommended.

3.4. Cross-Talk

The term **cross-talk** refers to the phenomenon in fluorescence microscopy where fluorescence from one fluorophore is detected in the emission channel of another fluorophore, leading to false or misleading fluorescence signals. In case you deviate from our suggested dye combinations for STED microscopy or use new dye combinations, we recommend **checking for cross-talk** (Figure 7) as their excitation and emission spectra might overlap. Cross-talk can be reduced by, for example, lowering laser excitation powers and adjusting/narrowing the detection window. Additionally, switching the dyes of affected channels, for example putting the **brightest dye on the dimmest structure**, or using another dye combination altogether, might help to reduce cross-talk. Furthermore, you might want to play with antibody concentration to level out significant differences in brightness.

3.5. Autofluorescence

Autofluorescence is a common phenomenon that can significantly impact image quality in plant and animal tissues. It arises from native components like flavins, porphyrins, and chlorophyll in plants, as well as fixatives like PFA or Glutaraldehyde. These structures have distinct lifetimes compared to the fluorophores used for labeling, making them challenging to distinguish from labeled structures. However, adjusting detector gating can help reduce or eliminate autofluorescence, especially by **shortening the detection window** at the start for short-lived autofluorescence and at the end for long-lived chloroplast autofluorescence. Another approach is to use TIMEBOW or FLIM analysis to isolate fluorescence with a specific lifetime, which can serve as a reference space for the autofluorescent signal. By carefully controlling autofluorescence, you can improve the image quality and obtain more accurate information from your sample.

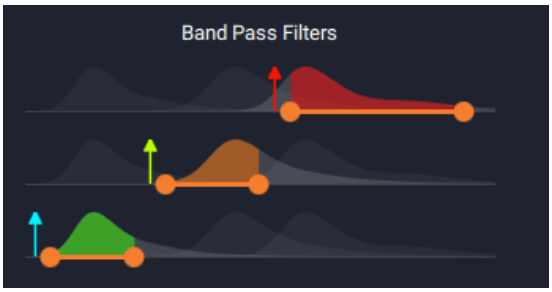


Figure 8: Example of spectral detection windows of three different channels; abberior STAR RED, STAR ORANGE and STAR GREEN. Detection windows are chosen automatically by the Lightbox software upon dye selection and can be freely adjusted during imaging. The dyes are acquired in line-sequential mode to minimize excitation-induced cross-talk.

Fluorescent lifetime imaging (termed ***TIMEBOW*** in the Lightbox software) is another technique to distinguish two dyes that cannot be separated by spectral detection due to their spectral similarities. TIMEBOW separates spectrally similar signals via differences in their fluorescence lifetime. Furthermore, TIMEBOW can be applied to detect and subtract autofluorescence and background from, for example, chlorophyll or chemical fixatives (e.g. PFA/GA) induced autofluorescence.

4. The right embedding medium and mounting

STED microscopy requires no dedicated imaging buffer as, for example, dSTORM does. However, the choice of embedding medium does affect the quality of STED images. The type of embedding media should be chosen based on the specific needs of the sample and the desired outcome of the imaging experiment. There is a variety of commonly used embedding media, including hardening, non-hardening (liquid), and aqueous media, which all have different advantages and downsides.

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
- Fluoromount-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

Embedding media	*RI matching*	*3D STED depth*	*Effect on sample*	*Miscellaneous*
abberior Mount Solid AF or Thermo Fisher ProLong Gold AF	++	< 20 µm	• Shrinkage during curing process. • Structures appear flatter	• Go-to embedding medium
Thermo Fisher ProLong Glass	+++	> 20 µm		• Spectral changes in some orange dyes
abberior Mount Liquid AF	+(+)	< 10 µm	• No sample shrinkage	
abberior TDE	+++++	Very deep	• Tissue clearing for deeper imaging	• Complex protocol • Incompatible with fluorescent proteins or phalloidin
Methyl salicylate	+++++	Very deep		• Complex protocol

Figure 9: *Different embedding media and their refractive index (RI) matching, possible 3D STED depth and effects on the sample.*

4.1. Matching the refractive index

It is important to consider the available **objective lenses and their immersion media** in relation to the mounting media used to embed your sample (**Figure 10**). The refractive index of your embedding medium should match that of your objective immersion medium as closely as possible. (**Figure 11**). For example, **oil objectives** should be used for imaging samples embedded in hardening media, **water objectives for aqueous media (e.g. PBS), and silicon or glycerol objectives** for non-hardening media such as *abberior MOUNT LIQUID*. Keep in mind that the refractive index of immersion oils is dependent on the temperature and can change along with it. There are immersion oils optimized for different temperatures, including for room temperature and 37 °C such as Cargill Immersion Oil Type 37.

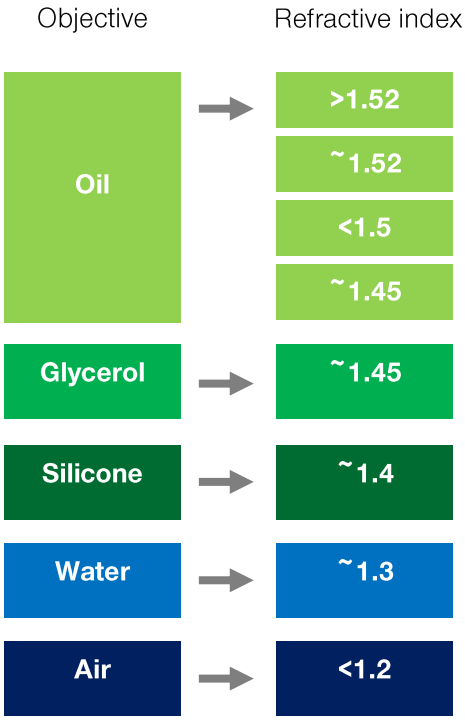


Figure 10: Exemplary objectives and their refractive indices. They should be matched to your samples to reduce aberrations

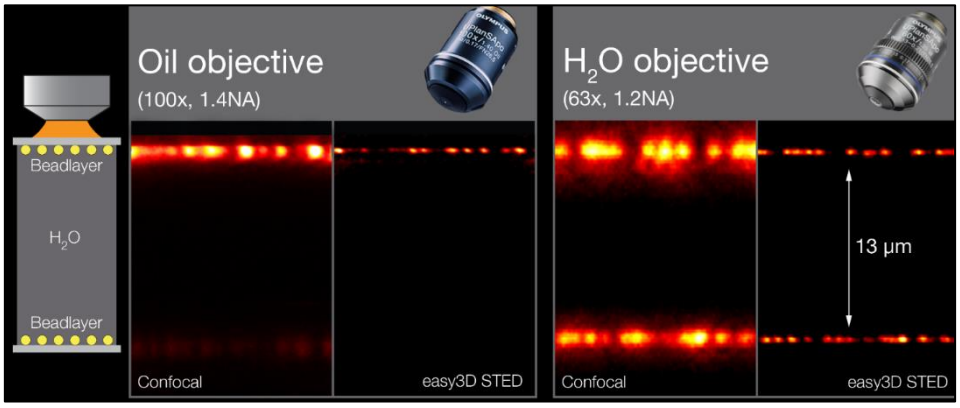


Figure 11: Example of a mismatch between the sample and objective immersion liquid (left, oil) with 3D STED, which is especially sensitive to aberrations. In this case, using a water immersion objective (right) allows for better resolution in the more distant bead layer as the medium between the two layers is also water

4.2. Hardening media

For most cases, it is recommended to **use hardening mounting media** (e.g. the **preferred *abberior* Mount Solid Antifade**) as they provide the highest sample **stability** and reduce movement of the sample. They also often contain **antifade agents**, which improve the brightness and stability of fluorophores. However, the polymerization of hardening mounting agents **flattens** the sample (**Figure 12**), which is **advantageous for 2D imaging** but distorts the shape of 3D structures. Samples mounted with hardening media are typically used with immersion oil objectives as their refractive index usually matches that of glass. They should be left overnight at room temperature in the dark to fully polymerize. The sample can often last for months, even years, when **stored** properly at **4 °C** in the dark.

Other suitable hardening media include Mowiol, ProLong™ Glass, ProLong™ Gold.

Note: Hardening media are the **go-to embedding** media for highest resolution and highest sample stability.

Note: When your *abberior* Mount Solid is hard or gelatinous, please **heat it** in a water bath at 70 °C until it turns liquid again and let it cool down before usage.

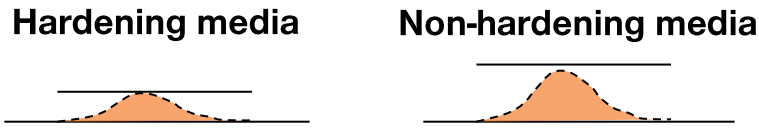


Figure 12: Hardening media polymerize, thereby stabilizing the sample and fluorophores for high resolution and brightness in final images and sample durability. This also flattens the sample in the process. Non-hardening media do not contain polymerization agents, retaining the shapes of cellular structures, which might be advantageous for volumetric 3D STED acquisitions.

4.3. Non-hardening media

Non-hardening mounting media, such as *abberior* Mount Liquid, are a good choice for preserving the native state of the sample and maintaining subcellular localization when imaging whole organelles with 3D STED microscopy (**Figure 11**). Although these media are usually matched to silicon oil and may contain antifading agents, they tend to exhibit higher photobleaching and lower image quality due to sample movement and the presence of floating particles. Samples embedded in non-hardening media also have a shorter viable lifespan compared to hardening media, even with proper storage at 4°C in the dark. When using non-hardening media with a refractive index similar to water, water immersion objectives may be advisable for optimal imaging. It is important to seal non-hardening mounting media to prevent evaporation and changes in refractive index. Common sealing agents include transparent nail varnish, two-component silicon-based glue or two-component epoxy resins.

Note: Non-hardening media are used for volumetric 3D STED acquisitions for volumetric rendering. Other suitable non-hardening media are for example buffered Glycerol with DABCO, TDE or PBS.

Note: Embedding media based on glycerol or aqueous buffer often show superior autofocus performance compared to hardening media.

4.4. Aqueous buffer

Aqueous buffer as mounting media, such as PBS, are used to keep the native state of the sample and are typically used with water immersion objectives. However, aqueous buffer media often do not contain antifading agents, which results in increased photobleaching compared to other mounting media. Moreover, they can contain high concentrations of salts and do not provide sample stabilization or inhibit particle movement. Taken together, samples mounted in aqueous buffer will degrade rapidly and, importantly, decrease the quality and resolution of final images.

Note: Embedding medium (e.g., *abberior* TDE) matching the refractive index to the employed objective and immersion also minimizes scattering and spherical aberrations, which results in enhanced contrast and benefits deeper tissue imaging (**Figure 10**).

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 HOECHST in VERY LOW amounts.

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.

4.5. Mounting

It is recommended to **mount only one**, maximum two **coverslips per slide.** The objectives usually have a high numerical aperture and are quite close to the coverslips. Hence, the objective could bump into the slide holder or might not reach the coverslip itself if the coverslips are mounted at the edges of the slide.

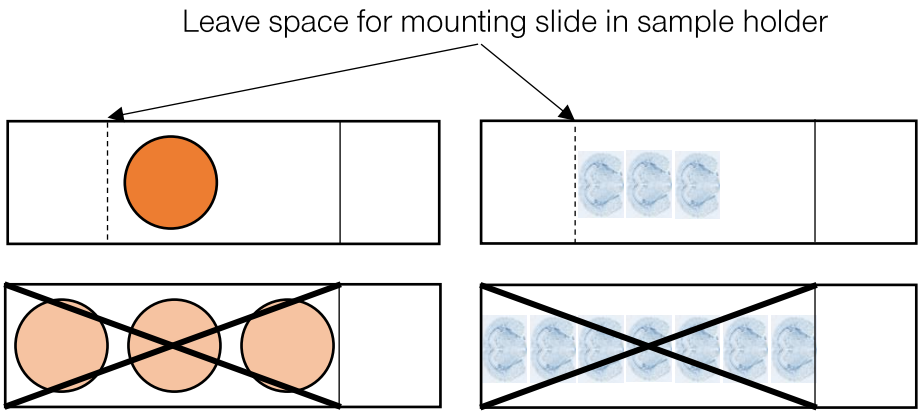


Figure 13: Please mount **one coverslip per slide** and **position it in the center** (see below) Coverslips might also be mounted on slides containing a small cavity to encompass a reservoir for various buffers, for example for live-cell microscopy or MINFLUX.

4.6. Storage

There are several factors that affect the durability of mounted samples, including the temperature and lighting conditions in which they are stored, the type of mounting media used, and the specific properties of the sample itself. It is generally recommended to store mounted samples at **4°C in the dark** to preserve their quality and prevent degradation. **Hardening mounting media such as abberior Mount Solid Antifade** tend to provide the highest sample stability and are the easiest to store and ship. Non-hardening and aqueous media degrade rapidly. Specific properties of the sample also affect its durability. Phalloidin, for example, will degrade faster than an antibody labeling and lasts only for 1-2 weeks, even if stored properly. Samples should be shipped in durable slide holders and protected from light (**Figure 14**).

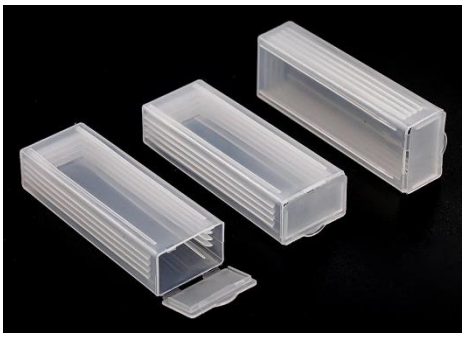


Figure 14: Example of storage boxes for microscopy slides. They protect your sample from light and physical damage. Please store and send samples in appropriate containers.

4.7. Sample description and slide labeling

To ensure successful imaging of your samples, please include the following information when shipping your samples:

1. Type of sample (cells, tissue, in vitro sample)
2. Type of section (cryostat, vibratome, ultramicrotome) and thickness of the section (typical 5 µm, 20 µm, or 100 µm)
3. Embedding medium (*abberior* Mount Solid, *abberior* Mount Liquid, Other)
4. Confocal images of the sample to ensure sufficient labeling and structural preservation before shipping (if possible)
5. Detailed information on the target structure and its location within the sample
6. Expected localization of the structure to be imaged and your general expectations for the imaging experiment
7. Dyes used for labeling (including the species of the secondary antibody)
8. Any specific modules you are interested in (adaptive optics, adaptive illumination, MATRIX, TIMEBOW, etc.)
9. Type of imaging to be performed (2D image, volume stack, 2D STED, or 3D STED)

Please ensure that the labels on the slides are descriptive and legible. You can label your slides with a number or identifier and provide the necessary information on the attached sample description sheet.

By providing this information, we can ensure that your samples are imaged successfully and to your satisfaction.

Sample Information	***Options***
Type of sample	[ ] Cells, [ ] Tissue,] In vitro sample, [ ] Other: _____
Type of section (if applicable)	[ ] Cryostat, [ ] Vibratome,] Ultra-microtome, [ ] Other: _____
Mounting Medium	[ ] abberior Mount Solid, [ ] abberior Mount Liquid, [ ] Other: _____
Thickness of section	[ ] 5µm, [ ] 20µm,] 100µm, [ ] _____
Confocal images provided	[ ] Yes [ ] No
Target structure information	[ ] Provided [ ] Not provided
Expected localization of structure	[ ] Provided [ ] Not provided
Dyes used for labeling	[ ] Provided [ ] Not provided
Interested in specific modules	[ ] Adaptive Optics, [ ] Adaptive Illumination,] MATRIX, [TIMEBOW, [ ] Other: _____
Type of imaging	[ ] 2D image, [ ] Volume,] 2D STED, [3D STED,
Comments	

4.8. Labeling for live-cell STED microscopy

The overall parameters of live-cell STED microscopy differ significantly from fixed cells and thus do the requirements for the dyes. One of the main differences is that it is not possible to use antibodies to target intracellular proteins in living cells as the cell membranes are not being disrupted to allow large antibodies to enter. As a result, the dyes used for live-cell imaging must be cell permeable (usually < 500 Dalton) to freely enter cells without damaging them. There are multiple different strategies to label specific targets in living cells (Stockhammer & Bottanelli, 2020). A wide variety of live-cell imaging dyes are commercially available and *abberior* also offers a large portfolio of cell permeable dyes.

Note: **Fluorescent probes** are often used for live-cell imaging, as they can be selectively targeted to specific structures within the cell. Examples of commonly used fluorescent probes include jasplakinolide derivatives (target actin), MitoSOX Red (mitochondria) and Hoechst (DNA).

4.9. Self-labeling enzymes

Another way to visualize specific proteins in living cells involves the expression of fusion-proteins that consist of the protein of interest fused to self-labeling enzymes such as **SNAP and Halo (Figure 15)**. These enzymes rapidly form covalent bonds with highly specific ligands via click-chemistry. Common ligands are cell permeable and include benzylguanine (SNAP, for example *abberior* LIVE 610-BG) and chloroalkene (Halo, for example JFX650-CA) as their reactive group, which are coupled to organic dyes, allowing visualization of the fusion protein. These labels enable multi-color fluorescence microscopy and can also be used for MINFLUX and fixed cell labeling.

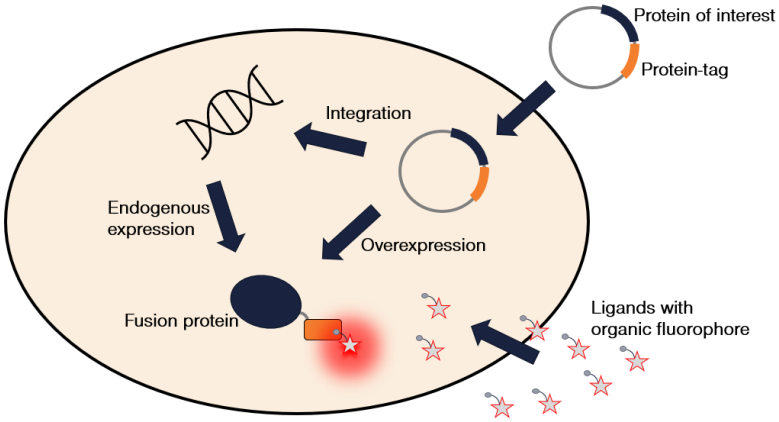


Figure 15: Live-cell labeling strategy with self-labelling enzymes and cell permeable ligands coupled to organic dyes.

4.10. Fluorescent proteins

There is a broad spectrum of different fluorescent proteins (**Figure 16**) which are still widely used to visualize targets of interest and in principle work in STED microscopy. However, these **fluorescent proteins** have only limited use in STED microscopy as they are usually **less bright** and **more susceptible to photo-bleaching** compared to the organic dyes we recommend for STED microscopy. recover, the excitation and emission spectra of fluorescent proteins are usually quite broad, which **increases the risk of cross-talk** in multi-color microscopy.

Note: We recommend the **use of organic dyes** rather than fluorescent proteins for STED microscopy, as they usually deliver higher resolution and image quality.

Green fluorescent proteins can generally achieve a better resolution than red fluorescent proteins due to their higher brightness.

A comprehensive **fluorescent protein database** can be found at www.fpbase.org

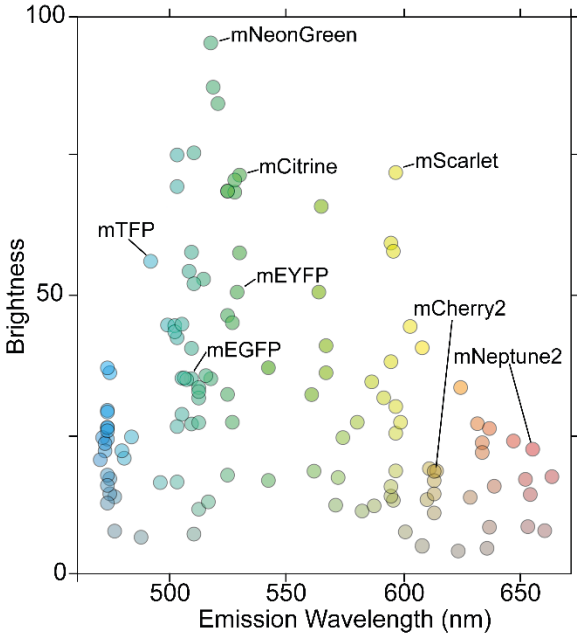
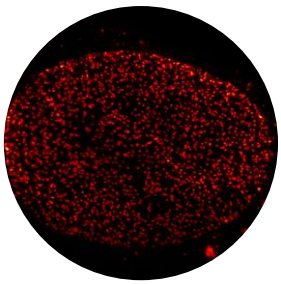


Figure 16: Emission wavelengths of various fluorescent proteins and their relative brightness. Fluorescent proteins in the green to yellow spectrum offer higher brightness compared to red ones

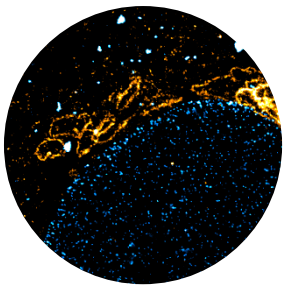
4.11. Recommended dyes for live-cell STED microscopy

For optimal live-cell multi-color STED microscopy, it is recommended to use far-red and orange dyes in combination with long stokes shift dyes as a third color. These dyes are preferred as they provide better penetration depth and lower phototoxicity, allowing for longer imaging times and better preservation of cell viability. Additionally, using long stokes shift dyes minimizes spectral overlap between the different channels, resulting in clearer and more distinguishable images.

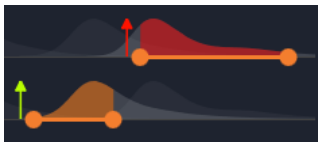


1 Color

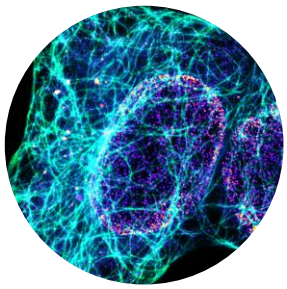
- *abberior* ***LIVE 610***[1,2]
- **JFX650**[2]



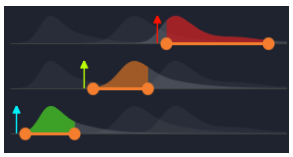
2 Colors



- *abberior* ***LIVE 550, LIVE 560*** or ***LIVE 590*** [1,2]
- **JF571** or **JF585** [2]



3 Colors



- *abberior* ***LIVE460L*** [1]
- Fluorescent protein (e.g. mNeonGreen)

[1] - Coupled to fluorescent probes

[2] - Coupled to self-labeling enzymes such as SNAP and Halo

For an exemplary workflow for live-cell staining and mounting, see **Figure 17**.

4.12. 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), FluoroBrite DMEM (Thermo Fisher, 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.

4.13. Live cell imaging

Imaging of **living cells** (**Figure 16**) requires some additional preparations to obtain high quality images that accurately represent the state of the cells. It is possible to image in normal growth media, however there are also **imaging solutions** that are optimized to both keep the cells happy and to acquire high quality fluorescence microscopy images. This **live-cell imaging media** can contain **antifading** reagents and should be **free from pH-indicators** (usually phenol-red) as these can increase background signal. Supplementing the live-cell imaging solution with amino acids and other **nutrients** (e.g., FBS) decreases the risk of starvation. For imaging samples longer than 30-60 min, it is recommended to **buffer** the live-cell imaging solution (for example with HEPES) and perform imaging at **37 °C**.

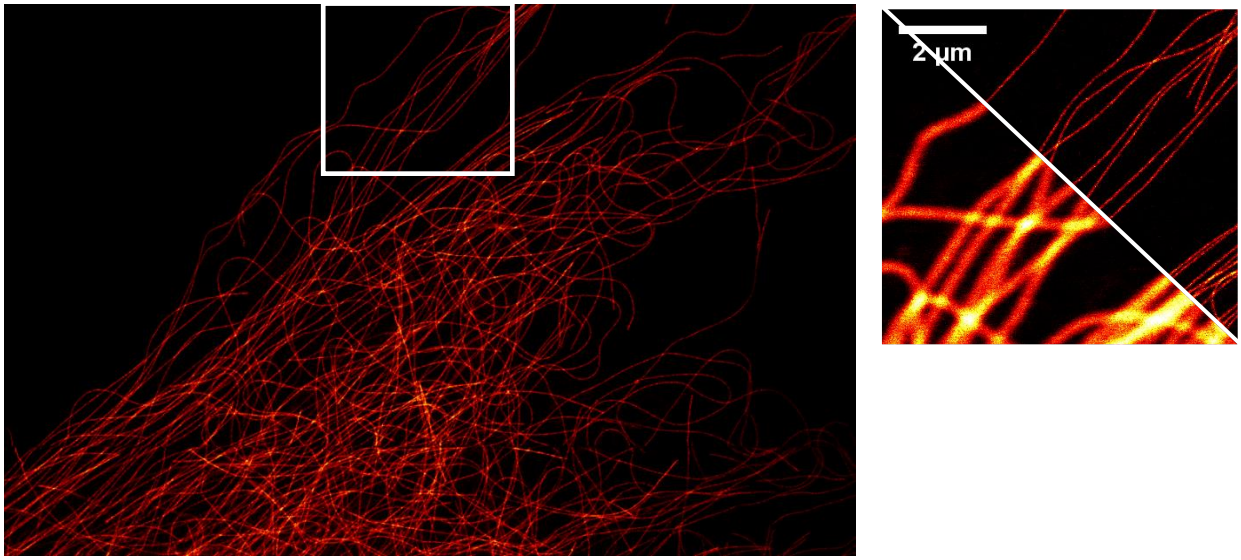


Figure 17: STED image of HeLa cells stained with abberior LIVE 610 tubulin.

Note: In some cases, efflux pumps can exclude dyes from the cells, which can be prevented by using inhibitory drugs such as Verapamil.

Photobleaching is a consequence of strong illumination and is affected by the photophysical parameters of the dye and its available photon budget. To minimize photobleaching, lower STED powers and the use of *abberior* **Adaptive Illumination** can be beneficial.

Note: It might help to supplement the imaging media with low concentrations of dyes that replenish bleached fluorophores and pro-long imaging conditions.

4.14. Workflow for staining and mounting for a live-cell microscopy experiment

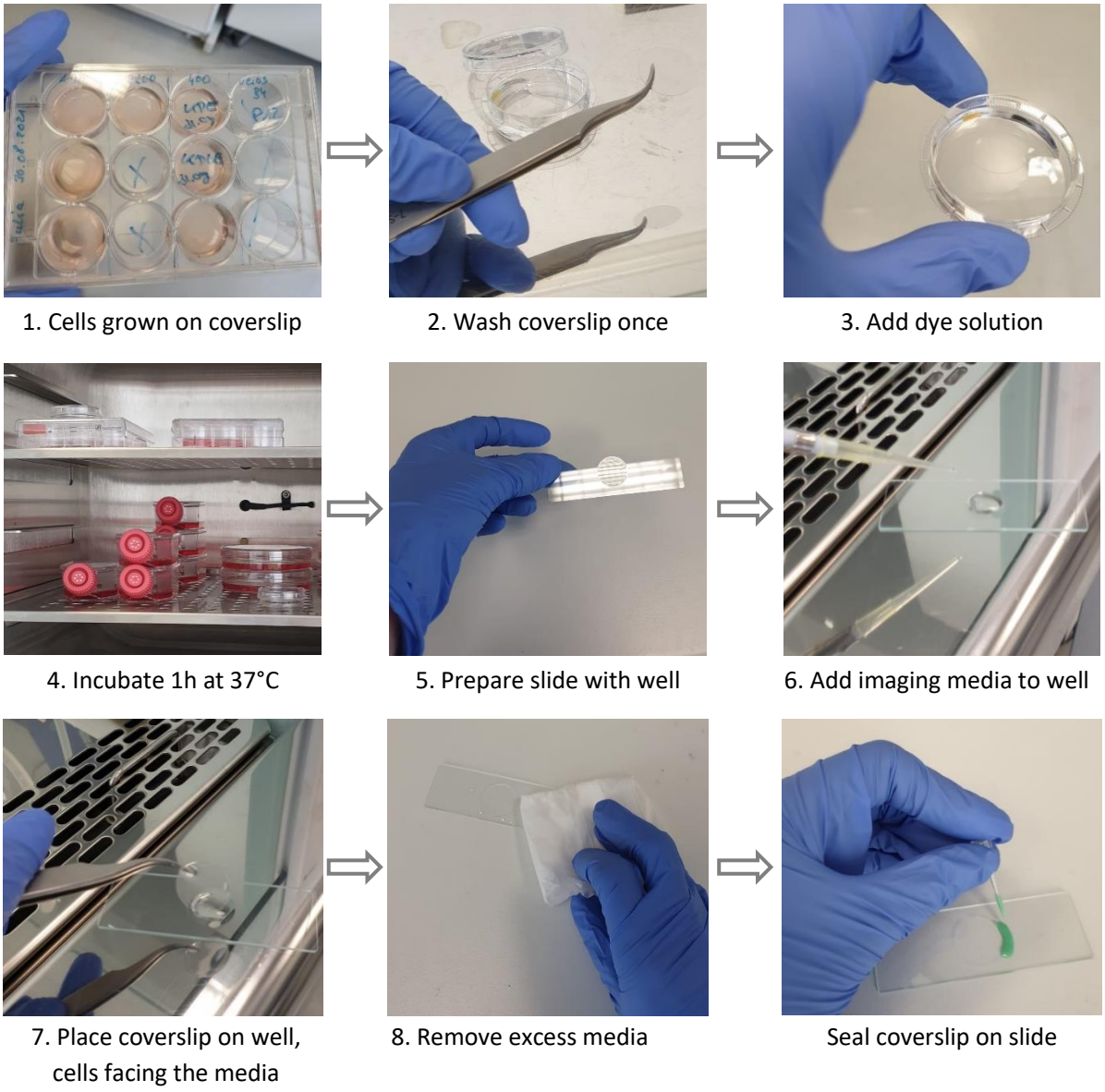


Figure 18: Workflow for sample preparation of living cells. Cells were grown on coverslips, stained, washed and mounted on microscopy slides containing a small cavity.

5. Imaging session

This section is designed to provide you with the necessary information to successfully image your STED optimized samples on your microscope.

The parameters in STED imaging are briefly described below:

Laser Excitation: It is important to choose the right laser excitation power to achieve bright signal, minimal bleaching, and a good signal-to-noise ratio in the final STED image. We recommend choosing the laser excitation power according to the raw photon counts of the confocal image, indicated in the dye chip menu. The laser excitation should be strong enough to generate a good signal, but not so strong that it causes excessive bleaching or increases background, which degrades overall image quality. The excitation in the STED channel should in general be 25% to 100% higher (depending on the used STED depletion power) compared to confocal to achieve optimal image quality and to counteract the minimal depletion occurring in the center of the STED donut.

Pinhole: The right pinhole size is crucial for achieving high-quality images. It controls the amount of out-of-focus light that reaches the detector, resulting in better contrast and image quality, and it determines the thickness of the optical section. Therefore, it is important to select the optimal pinhole size to achieve high-quality STED microscopy images with good signal-to-background ratio, optical sectioning, and resolution in Z.

For 2D STED imaging of orange and red fluorophores a pinhole of 1AU usually yields optimal results.

Here are some use cases where changing pinhole size can be beneficial:

When acquiring 3D STED images and especially volumes, closing of the pinhole to 0.8-0.7AU can reduce the haze seen in 3D STED images that is stemming from excited fluorophores beneath and above the focal plane that are not depleted by the 3D STED donut. The smaller pinhole reduces this haze effect.

In case a sample is very bright and a further reduction of the laser excitation power is not possible, closing the pinhole is an option to reduce overall signal strength to avoid detector saturation.

For fluorophores excited by the 488nm and lower wavelength laser closing the pinhole to 0.8 to 0.7 AU is recommended to obtain optimal performance as the PSF here is smaller and the closing of the pinhole improves resolution in z.

Dwell-time: The dwell time as well as the line accumulations define how long each position/pixel in the image is illuminated and recorded. The length of the dwell time is defined by the image signal strength, the speed of movement of the object to be imaged and the resistance of the specific fluorophore to bleaching. Especially green fluorophores depleted by the 595nm STED laser show much better photostability and less bleaching when imaged at 1.5 to 2 µs dwell time. To achieve a good signal-to – noise ratio in this case the number of line accumulations is increased.

Pixel size: Adjusting the pixel size is a crucial factor for obtaining high-quality STED images. The optimal pixel size depends on the resolution required, the STED power used, and the sensitivity of the dye to depletion. A balance must be struck between optimal resolution and sufficient illumination of the sample. If the pixel size is too small, it can result in decreased signal and image quality due to bleaching. For standard STED samples, pixel sizes typically range from 15 to 50 nm. It is important to avoid using pixel sizes that are significantly smaller than necessary, as this can lead to an increased total applied laser energy per area, which can cause stress for the fluorophores and result in bleaching. Therefore, pixels should be as large as possible while still being small enough to achieve the desired resolution.

STED depletion: The resolution improvement achieved by STED microscopy is dependent on the power of the STED donut used. The central hole of the donut becomes smaller with higher depletion power, resulting in improved resolution. However, the relationship between STED power and resolution is not linear but follows a negative logarithmic curve. This means that a small increase in power can lead to a significant improvement in resolution, while higher powers may yield only marginal improvements and are suitable for the smallest structures or fluorophores most resistant to depletion.

To optimize STED imaging parameters, it is recommended to start imaging at a low STED power of 5-10% and gradually increase the power while monitoring resolution and sample bleaching. The optimal STED power depends on the sensitivity and stability of the dye, the size of the labelled structures, and the amount of fluorescent signal. High STED powers above 40% may lead to sample bleaching, which can be mitigated by using abberior Adaptive Illumination. By adjusting the STED power and monitoring the resolution, it is possible to optimize the imaging conditions.

5.1. Autofocus

Turning on the **autofocus** might help to stabilize the sample, keeping it in focus when you are experiencing z-drift.

Note: The autofocus uses a red shifted laser and is based on the total-internal-reflection (TIR) of this laser at the coverslip-immersion medium interface. Using a mounting medium with matching refractive index (e.g. Prolong Glass) can hinder TIR and the use of autofocus. In this case a non-hardening mounting medium like *abberior* Mount Liquid or PBS can give a stronger reflection for the autofocus to work. It helps to know where the labeled structures are located within the cell and what their known sizes are. Please keep in mind that STED cannot deliver higher resolutions than the actual sizes of the structures. Always stay in focus to reduce background and blurry images.

To find the optimal focus, focus through the structure in confocal mode. Choose the relevant focal plane for optimal STED imaging.

Test for different STED settings, imaging parameters, and rate of photobleaching in areas of the sample that are not necessarily the most interesting. Also, less STED laser power is sometimes more, as the best obtainable resolution is not always needed. Higher STED laser powers can also bleach more fluorophores, thereby reducing the overall image quality. Moreover, lower STED power may be more suitable for time-lapse imaging or volume, as lower amounts of photobleaching occurring with each cycle help to ensure longer imaging.

Note: If working with a new sample or dye please start with low STED powers, increasing them until there is no more gain in resolution.

Detecting low light intensities in fluorescence microscopy can have many reasons, such as low abundance of target molecules or sparse labeling density. By putting the brightest dye, usually the far-red one, on sparsely expressed proteins can help to boost dim signals. Increasing antibody or fluorescent probe concentrations or staining times might result in more densely labelled structures.

Note: Please adjust your pixel size depending on the imaging experiment. Confocal, 2D STED, and 3D STED achieve different lateral and axial resolution (**Figure 19**).

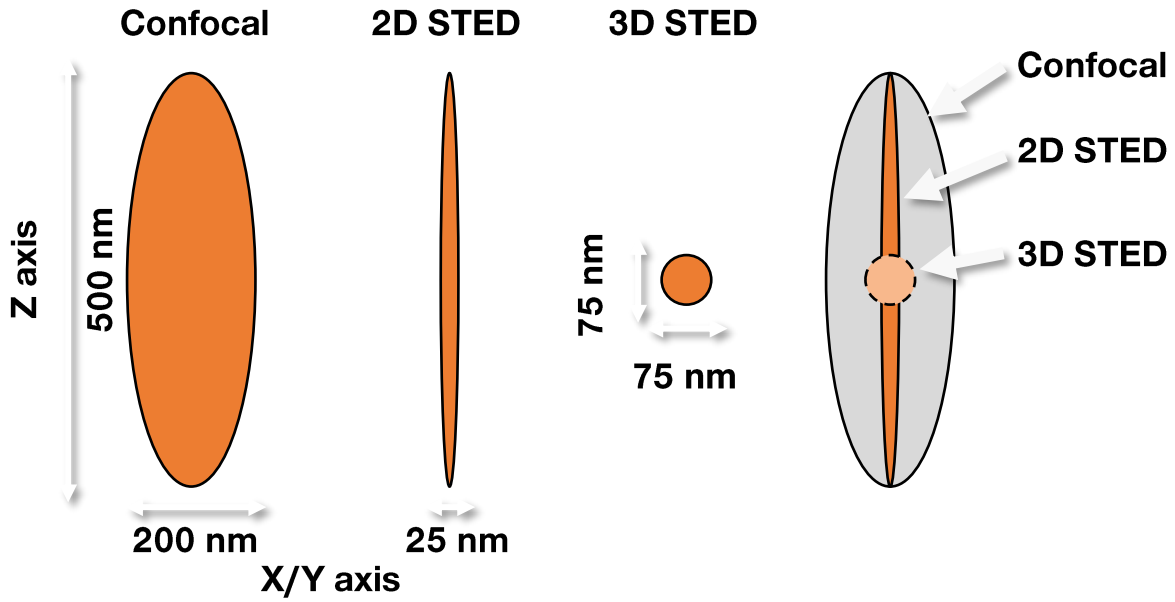


Figure 19: Comparison of the dimension of the resulting point spread functions in confocal, 2D and 3D STED images. 2D STED has the best lateral resolution compared to confocal and 3D STED, but does not increase axial resolution. 3D STED can achieve an isotropic resolution below the diffraction limit of light.

Photobleaching is a consequence of the photophysical parameters of a dye and its available photon budget. To minimize bleaching of fluorophores in fixed samples a smart selection of dyes, lower STED powers, and the use of adaptive illumination can be beneficial for sample stability.

Applying *abberior* **adaptive illumination** (**RESCUE** and **DYMIN**) will decrease the illumination and, therefore, photo-bleaching of the sample. Adaptive illumination can especially help to reduce photobleaching in volumetric 3D STED stacks.

- Whenever more than one label is present, **cross-talk / bleed-through** are always a risk and should be kept in mind. **Adjusting spectral detection** windows or **switching labels** might help to reduce cross-talk if present.
- It is a good idea to **save** the imaging session from time to time.

5.2. Improving signal to noise

Background signal is present in every image and has many reasons. Falsely labeled structures, such as unspecific bound antibodies, and high concentrations of fluorophores can contribute to unwanted background signal in fluorescence microscopy. To reduce these background contributions, it is important to **block and wash** the sample properly. Light from above and below the focal plane causes unwanted **photon background noise**. To reduce this type of background, it may be helpful to further decrease the size of the **pinhole** or to use the *abberior* **MATRIX detector**. Autofluorescence can also contribute to overall background noise and can be reduced by adjusting the time gating setting as autofluorescence is usually short-lived.

Note: STED microscopy delivers the highest image quality in focal planes close to the glass coverslip. In case your structure is more distant from the coverslip we suggest the use of an adaptive optics module.

As light travels through the sample it encounters various structures which cause refractive index mismatching, leading to spherical aberrations. Accumulating spherical aberrations are common in thicker samples and can result in blurred or distorted images with increasing distance to the coverslip.

Note: Spherical aberrations can be corrected by the adaptive optics module including the SLM and the deformable mirror, which adapts the beam front to compensate for these aberrations.