

Chapter 11

Sample Preparation for STED Microscopy

**Christian A. Wurm, Daniel Neumann, Roman Schmidt,
Alexander Egner, and Stefan Jakobs**

Abstract

Since the discovery of the diffraction barrier in the late nineteenth century, it has been commonly accepted that with far-field optical microscopy it is not possible to resolve structural details considerably finer than half the wavelength of light. The emergence of STED microscopy showed that, at least for fluorescence imaging, these limits can be overcome. Since STED microscopy is a far-field technique, in principle, the same sample preparation as for conventional confocal microscopy may be utilized. The increased resolution, however, requires additional precautions to ensure the structural preservation of the specimen. We present robust protocols to generate test samples for STED microscopy. These protocols for bead samples and immunolabeled mammalian cells may be used as starting points to adapt existing labeling strategies for the requirements of sub-diffraction resolution microscopy.

Key words: Fluorescence microscopy, Stimulated emission depletion microscopy, Nanoscopy, Superresolution, Immunofluorescence, Sample preparation.

1. Introduction

Stimulated emission depletion (STED) microscopy (1) and the related approaches to fundamentally break the diffraction barrier in far-field optical microscopy have been covered by several comprehensive reviews (2–5) and will not be discussed here in detail. In brief, STED microscopy typically uses a focused laser beam for excitation, which is overlapped with a second laser beam, the so-called “STED beam” that exhibits no light intensity at its focal center but strong intensities at the periphery. When excited, fluorophores exposed to the “STED beam” are almost instantly transferred back to their ground state by means of stimulated

emission. As a result, only molecules that are close to the zero at the center of the “STED beam” are allowed to fluoresce and contribute to the fluorescence signal. Confining the signal to such a subdiffraction-sized spot results in higher resolution imaging when the spot is scanned through the specimen (1, 6–8). Experiments using single molecules as test objects already demonstrated a spot size as small as 16 nm in the focal plane (9, 10).

Since its first utilization for the imaging of yeast cells (7), STED microscopy has been used for a number of applications in cell biology, including the visualization of proteins at the synapse (11), cytoskeletal elements (8), mitochondrial proteins (12), the analysis of lipid rafts (13), and others (14–17). It has also been successfully used for live cell applications (7, 18, 19). As until now, most applications relied on chemically fixed cells, we present protocols that describe the preparation of immunolabeled fixed cells. In addition, we provide protocols for bead samples that may be used as standard samples to determine the performance of the instrument.

The increased resolution of STED microscopy, like any subdiffraction microscopy, may disclose shortcomings of the sample preservation which are concealed by the lower resolution of conventional (confocal) microscopy. Also, special care has to be taken to avoid spherical aberrations induced by the refractive index mismatch between the immersion system and the embedding medium of the sample, which would immediately deteriorate the obtainable optical resolution. The protocols presented here are intended as starting points for the preparation of other samples; likewise they may be used as benchmarks to test the performance of a microscope.

2. Materials

We have successfully used the reagents described below. In many cases other commercially available equivalents may also be used. If not specified otherwise, all chemicals used were of analytical grade. Standard consumables and equipment as present in most molecular biology labs is required. For imaging of the samples a custom-built STED microscope (11, 13, 14, 19) or a commercial STED microscope (Leica Microsystems, Wetzlar, Germany) can be used.

2.1. General Materials

1. Cover slips No. 1 (0.13–0.16 mm thick) (Menzel Gläser, Braunschweig, Germany) (*see Note 1*)

2. Microscopy slides (ISO Norm 8037/I, 26 × 76 mm, 1 mm thick (Menzel Gläser)
3. Phosphate-buffered saline (PBS, 137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4)

2.2. Materials for Embedding Media

1. Mowiol 4-88 (Calbiochem, Darmstadt, Germany)
2. Tris-HCl buffer (Tris(hydroxymethyl)aminomethane, 0.2 M, pH 8.5)
3. Glycerol
4. 1,4-Diazabicyclo[2.2.2]octan (DABCO) (Sigma-Aldrich, Saint Louis, MO)
5. 2,2'-thiodiethanol (TDE), highest purity (Sigma-Aldrich)

2.3. Materials for Bead Sample

1. Fluorescent microspheres (FluoSpheres, crimson fluorescent (625/645), 10⁶ beads/ml) diameter 0.02 μm, 0.04 μm (custom-made), 0.1 μm (custom-made) or 0.2 μm (Molecular Probes/Invitrogen, Eugene, OR)
2. Absolute ethanol
3. Poly-L-lysine solution (0.1% (w/v) in H₂O (Sigma-Aldrich)
4. Embedding medium (*see* **Section 3.1**)
5. Nail polish

2.4. Materials for Immunofluorescence Labeling

1. Paraformaldehyde (PFA) (powder)
2. Absolute methanol
3. Triton X-100 solution (0.5% (v/v) in PBS)
4. Blocking solution (5% (w/v) BSA in PBS)
5. Primary antibodies (anti-Tom20, FL-145, rabbit polyclonal, Santa Cruz Biotech., Santa Cruz, CA; anti-β-tubulin, MAB3408, mouse monoclonal, Sigma-Aldrich)
6. Fluorophore-labeled secondary antibodies

3. Methods

3.1. Embedding Media

The use of immersion lenses with high numerical apertures is compromised by spherical aberrations induced by the refractive index mismatch between the immersion and the embedding medium (20, 21). A solution to this problem is to adapt the index of the embedding medium to that of the immersion medium. This is crucial for high-resolution imaging. As a beneficial secondary effect, several of the utilized mounting media reduce photobleaching (22–24).

3.1.1. Mowiol

Mowiol is widely used as embedding medium for fluorescence microscopy. For imaging of Mowiol embedded samples, oil-immersion lenses may be used. Mowiol hardens over time, alleviating the need to seal the sample. Mowiol, although convenient and sufficient for many STED applications, may be not the best choice for the most demanding applications (*see Note 2*).

1. Mix 6 g glycerol with 2.4 g Mowiol 4-88 in a 50 ml centrifuge tube and stir 1 h on magnetic stirrer.
2. Add 6 ml water and stir for further 2 h.
3. Add 12 ml Tris-HCl buffer (0.2 M, pH 8.4) and heat up the solution to 50°C (in a water-bath) for more than 2 h under constant agitation. Extend this step, until the Mowiol 4-88 is completely dissolved.
4. Optional: add antifading agents (e.g., 25 mg/ml DABCO); stir for more than 4 h (*see Note 3*).
5. Centrifuge at 7500g for 30 min to remove any undissolved solids.
6. Aliquot in eppendorf tubes and store at -20°C. Each aliquot can be used for several weeks if stored at 4°C.

3.1.2. TDE (2,2'-thiodiethanol)

TDE is a mounting medium allowing for the adjustment of the refractive index ranging from that of water (1.33) to that of immersion oil (1.518). It is miscible with water at any ratio (25). Because TDE does not harden, the sample has to be sealed with nail polish (*see Notes 4 and 5*).

1. Mix 97 ml 2,2'-thiodiethanol (TDE) with 3 ml PBS (*see Note 6*).
2. Stir for at least 15 min.
3. Adjust pH to 7.5 with HCl/NaOH (*see Note 7*).
5. Check refractive index with refractometer and adjust refractive index to 1.518 by addition of 100% TDE or PBS, if necessary (*see Note 8*).

3.2. Preparation of Bead Samples

One possibility to determine the resolution of a fluorescence microscope is to image fluorescent microspheres that are distinctly smaller than the resolution of the instrument. The actual physical size of the beads may vary, which could complicate the analysis of the images (*see Note 9*). A typical STED image of a bead sample is shown in **Fig. 11.1**.

1. Dilute the beads in ethanol. The typical dilution factor is 1:10³ for 100 nm beads and 1:10⁵ for 20 nm beads.
2. Sonicate the beads at least for 5 min (in ultrasonic bath) (*see Note 10*).

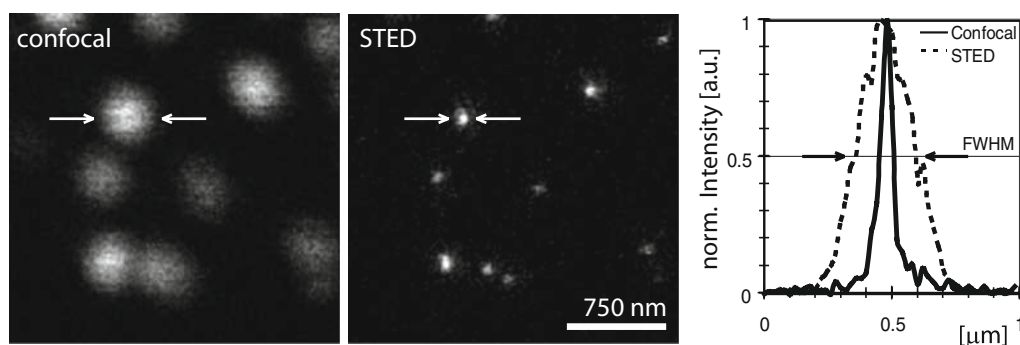


Fig. 11.1. Determination of the microscope's resolution. One way to determine the resolution of a microscope is to image fluorescent beads whose size is considerably smaller than the optical resolution of the instrument (21). Shown are dispersed fluorescent microspheres (crimson fluorescent, specified diameter: 40 nm (for details see **Section 2.3**)). Left: confocal image. Right: corresponding STED image. The right panel shows normalized intensity profiles through the bead marked by the arrows. The full width at half maximum (FWHM) is a measure for the optical resolution. The obtained resolution was about 250 nm (confocal) and about 60 nm (STED).

3. Clean the cover slip with ethanol.
4. Apply the bead suspension to cover slip (Use 10 μ l bead suspension for a 30 mm cover slip) and allow to dry on air (see **Notes 10 and 11**).
5. Mount with the required embedding medium (see **Section 3.1**).

3.3. Immunofluorescence Labeling

Indirect immunofluorescence labeling is a widely used method to visualize specific structures in fixed cells. The same methods can be applied for STED microscopy. However, due to the higher resolution of STED microscopy, the requirements for sample preparation tend to be stricter than for conventional optical microscopy.

Most notably, inadequate sample preparation and labeling efficiencies may not be noticed with confocal microscopy, but is clearly visible with STED microscopy. Thus care has to be taken to utilize optimal fixation conditions to ensure structural preservation (see **Note 12**). Simultaneously, extraction conditions need to be chosen so that optimal accessibility for the antibodies to the structures is possible. The antibody concentrations, incubation times, and temperatures should be optimized to ensure optimal brightness and signal-to-noise ratios in the images (see **Note 13**). It may be necessary to optimize the labeling procedures for any new structure or specimen.

We present here labeling protocols for two standard samples (microtubule cytoskeleton and TOM complexes on the mitochondrial surface) which proved to be robust. Furthermore, these samples can be routinely used to determine the performance of a STED microscope. The staining procedures may be used as a starting point to optimize different samples.

3.3.1. Labeling of the
Microtubule
Cytoskeleton in
Methanol-Fixed
Mammalian Cells

Microtubules form a complex and interweaved network. Because the diameter of antibody decorated microtubules is below 70 nm (8), they are convenient test objects to monitor the performance of a STED microscope (**Fig. 11.2a**). The microtubule cytoskeleton can be fixed with ice-cold methanol (*see Note 12*).

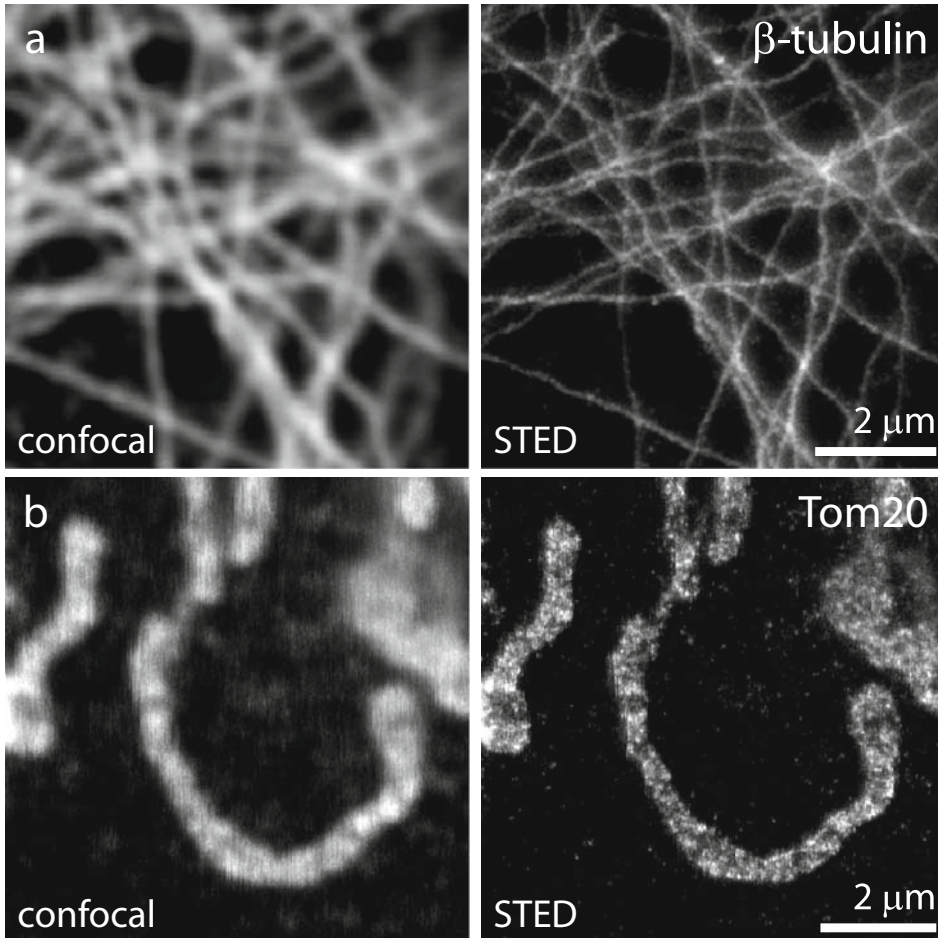


Fig. 11.2. Confocal and STED images of immunolabeled sub-cellular structures. **(a)** Microtubule cytoskeleton. PtK2 cells were fixed with ice-cold methanol and labeled with primary antibodies specific for β -tubulin. The secondary antibody was labeled with Atto647N. *Left*: confocal image. *Right*: corresponding STED image taken with the microscope as described in (28). **(b)** TOM (translocase of the outer mitochondrial membrane) complex. PtK2 cells were fixed with formaldehyde and labeled with primary antibodies specific for Tom20 (a subunit of the TOM complex). The secondary antibody was labeled with Atto647N. *Left*: confocal image. *Right*: corresponding STED image. In the STED image individual TOM clusters, which are concealed in the confocal image, are clearly discernible. The lateral resolution in both STED images was in the order of 50 nm.

1. Grow mammalian cells on cover slips (typically to a confluency of 50–80%) (*see Note 14*).
2. Fix and permeabilize the cells by incubating the cover slips in ice-cold methanol (-20°C) for 5 min (*see Note 12*). All subsequent steps are performed at room temperature.

3. Wash samples twice in PBS.
4. Incubate for 5 min in blocking solution (*see* **Note 15**).
5. Dilute primary antibody specific for β -tubulin to a final concentration of $\sim 5 \mu\text{g}/\text{ml}$ in blocking solution.
6. Centrifuge antibody solution for at least 1 min at 15,000*g* (*see* **Note 16**).
7. Incubate sample for 1 h in antibody solution (*see* **Note 17**).
8. Wash cells twice in PBS (*see* **Note 18**).
9. Dilute labeled secondary antibody (anti mouse) to a final concentration of $\sim 1\text{--}20 \mu\text{g}/\text{ml}$ in blocking solution. Optimal dilution depends on the quality of the antibody (*see* **Note 19**).
10. Centrifuge antibody solution for at least 1 min at 15,000*g*.
11. Incubate sample for 1 h in secondary antibody solution (*see* **Note 20**).
12. Wash cells in PBS for at least 5 min.
13. Mount sample on slide (*see* **Section 3.3.3.** and **Notes 21 and 22**).

3.3.2. Labeling of the TOM Complex in Formaldehyde-Fixed Mammalian Cells

The TOM (translocase of the outer membrane of mitochondria) complex is the major import pore for mitochondrial precursor proteins (26). Using conventional (confocal) microscopy the TOM complexes appear to be evenly distributed on the mitochondria. Only sub-diffraction resolution microscopy reveals that they are located in small clusters at the surface of the mitochondrial tubules (12, 14) (**Fig. 11.2b**). Due to the three-dimensional arrangement of the TOM complexes on the mitochondria and their inhomogeneous size distribution, this is a more challenging sample for high-resolution microscopy than the microtubule network. For this sample formaldehyde fixation and membrane permeabilization with detergent are required.

Preparation of 8 % (w/v) Formaldehyde Solution

1. Mix 80 ml of water and 8 g of paraformaldehyde powder.
2. Stir the suspension to disperse the powder.
3. Add 1 ml of 1 M NaOH.
4. Heat the solution to 60°C under continuous stirring until the suspension becomes clear (~ 15 min) (*see* **Note 23**).
5. Add 10 ml concentrated PBS (10 \times concentrated, pH 7.4).
6. Adjust pH to 7.4 with HCl.
7. Add water to 100 ml.
8. Store at 4°C for 1 week or at -20°C for longer time periods (*see* **Note 23**).

Immunofluorescence
Labeling

1. Grow mammalian cells on cover slips (typically to a confluency of 50–80%).
2. Chemically fix cells by incubating the cover slips for 5–10 min in formaldehyde solution prewarmed to 37°C.
4. Wash cells twice in PBS.
5. Extract by incubating in 0.5 % (v/v) Triton X-100 in PBS for 5 min.
6. Incubate for 5 min in blocking solution (*see Note 15*).
7. Dilute primary Tom20 specific antibody to a final concentration of ~2 µg/ml in blocking solution.
8. Centrifuge antibody solution for at least 1 min at 15,000*g* (*see Note 16*).
9. Incubate sample for 1 h in antibody solution (*see Note 17*).
10. Wash cells twice in PBS (*see Note 18*).
11. Dilute labeled secondary antibody (anti-rabbit) to a final concentration of ~1–20 µg/ml in blocking solution. Optimal dilution depends on the quality of the antibody.
12. Centrifuge antibody solution for at least 1 min at 15,000*g*.
13. Incubate sample for 1 h in diluted secondary antibody.
14. Wash cells in PBS for more than 5 min.
15. Mount sample on slide (*see Section 3.3.3. and Notes 21 and 22*).

3.3.3. *Embedding of Cell Samples*

Embedding with Mowiol

1. Pipette a small drop of Mowiol (prewarmed to room temperature) onto the slide.
2. Mount cover slip with sample on slide and remove excess Mowiol with tissue paper.
3. Leave slides for several hours in the dark to allow the Mowiol to harden (*see Notes 2 and 3*).

Embedding with 97 %
(v/v) TDE

To prevent cellular structure from distortion by osmotic shock during embedding in TDE, the exchange of water with TDE must be slow. Therefore a dilution series is required.

1. Prepare TDE dilution series with PBS: 10% (v/v) TDE, 25% (v/v) TDE, 50% (v/v) TDE, and 97% (v/v) TDE.
2. Incubate labeled sample in 10% (v/v) TDE for 10 min.
3. Incubate labeled sample in 25% (v/v) and 50% (v/v) TDE for 5 min each.
4. Incubate labeled sample in 97% (v/v) TDE twice for 5 min each.
5. Mount sample with 97% (v/v) TDE on slide.
6. Remove excess TDE.
7. Seal with nail polish (*see Note 5*).

4. Notes

1. Each objective lens is designed and assembled to achieve optimum performance with specific cover slips. Using a cover slip of, for example, the wrong thickness can introduce (spherical) aberrations into the imaging process which degrade resolution. Consult the manufacturer of your objective lens about which cover slip to use.
2. Mowiol is a solution of polyvinyl alcohol. It hardens over some days and can be molten again by heating the samples in buffer. The refractive index of Mowiol solutions may vary between batches. During hardening the refractive index changes from close to glycerol (~ 1.45) to close to immersion oil (~ 1.518). Some groups report shrinkage of the samples upon hardening.
3. Embedding media for fluorescence microscopy often contain chemicals that are supposed to reduce bleaching of the fluorescent probes. Prominent examples include 1,4-diazabicyclo[2.2.2]octan (DABCO, 25 mg/ml), *N*-propyl gallate (NPG, 0.5% (w/v)) and *p*-phenylene diamine (PPD, 1 mg/ml) (22–24). In rare cases these reagents reduce the dyes fluorescence intensity. The effectiveness of an antifading reagent has to be evaluated for any given fluorophore. We found that Mowiol (with DABCO) is frequently a good option. However, other (commercially available) embedding media may also be considered. Currently, TDE is, to our knowledge, the only embedding medium that allows a precise tuning of the refractive index to match that of immersion oil.
4. TDE is an inexpensive, non-toxic glycol derivative. Commercially available TDE may contain impurities. It can be further purified by distillation (boiling temperature 164–166°C at 27 hPa).

Due to its high refractive index (~ 1.522) and full miscibility with water, it is suited as mounting medium with precisely adjusted refractive index (also *see* **Note 8**). Unlike Mowiol, TDE does not harden. Samples need to be sealed with nail polish.

The properties of some fluorophores are changed in TDE: Especially the absorption and emission spectra, but also the quantum efficiency and bleaching properties may be altered when the samples are mounted in TDE.

5. Nail polish is often used to seal microscopy samples. Colored or glittering nail polish should be avoided. Certain solvents in the nail polish may quench the fluorescence.
6. Typically TDE is used in combination with a phosphate buffer, but other aqueous buffers also work.
7. Due to the viscosity of TDE, the measurement of its pH is difficult. Either a special pH electrode has to be used or, if standard electrodes are used, one has to wait for a long time until equilibrium has been reached.
8. TDE is hygroscopic. The refractive index of undiluted TDE (~ 1.522) may vary due to its water content.
9. Due to the production process of the beads, the variability of their diameter tends to become large for small beads (**Fig. 11.3**). If uniformly stained beads are substantially smaller than the microscope resolution, the measured brightness is proportional to the bead volume. Hence polydispersity leads to broad brightness distributions.

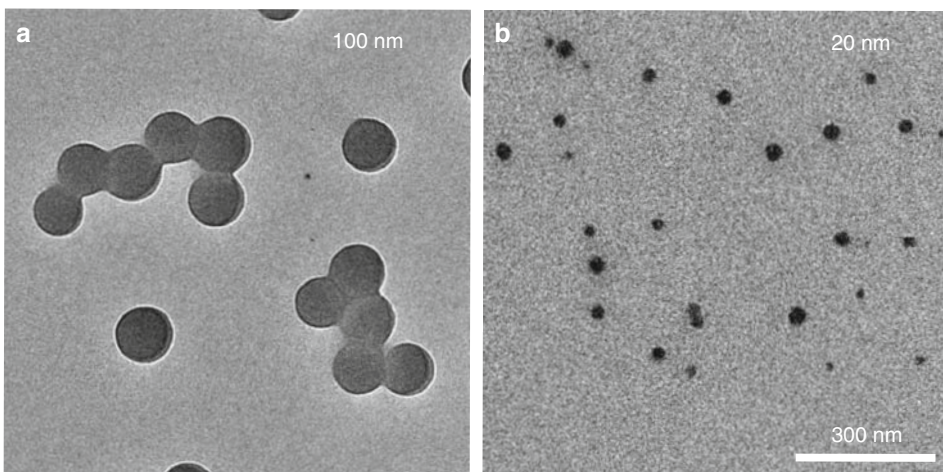


Fig. 11.3. Electron micrographs of crimson fluorescent microspheres. (a) 100 nm beads (see **Section 2.3**) exhibit a monodisperse size distribution ($100 \text{ nm} \pm 7.5\%$), whereas (b) 20 nm beads (see **Section 2.3**) are more polydisperse ($20 \text{ nm} \pm 50\%$). The variation in bead size has to be taken into account when determining the resolution of a microscope.

10. The concentration of purchased beads is typically very high. In suspension, they tend to aggregate, which can be alleviated by dilution and sonification before use. During drying, ethanol or surface contaminants on the cover slip may lead to droplet formation and thereby to an inhomogeneous bead distribution on the cover slip.
11. The STED beam exerts a force on particles which have a different refractive index than the surrounding medium

(optical tweezer effect). Beads may start to move or even detach from the cover slip. Increasing the stickiness of the surface by using Poly-L-lysine solution can avoid this.

12. Fixation is required to preserve cellular structures. Cross-linking fixatives like formaldehyde are advantageous for most structures. (The (microtubule) cytoskeleton may be an exception.) Due to this cross-linking activity, fixatives also may hinder antibodies from reaching their target structure. To overcome this, fixation is often combined with extraction (e.g., organic solvents like methanol, Lavdovsky's fixative, or Carnoy's fluid), which may also influence the preservation of the cellular structures. This may be a much more severe problem in STED microscopy than in conventional diffraction limited microscopy because in the latter case the fixation/extraction induced artifacts may be hidden by the lower resolution. Hence special care has to be taken to control proper preservation of cellular structures. Protocols sufficient for confocal microscopy may not be sufficient for STED microscopy.
13. Many antibody suppliers suggest performing antigen retrieval. To this end, the sample is heated (to up to 120°C) for a certain time (27). Sometimes heating is performed under high salt conditions and/or at extreme pH values. Often these procedures work astonishingly well; still special attention to the proper preservation of the analyzed structures is mandatory.
14. Immunofluorescence labeling described here was successfully performed with several mammalian cell lines, ranging from human cervix carcinoma cells like HeLa to rodent kidney cell lines like BHK-21. Information about proper cultivation conditions is available from the American Type Culture Collection (www.lgcstandards-atcc.org).
15. Several blocking agents can be used, including skimmed milk (coldwater fish) gelatin or BSA. A number of rather expensive blocking agents are on the market, including serum of the antibody producing host species or synthetic blocking agents. We recommend the use of BSA for routine labeling. For specialized applications the use of other blocking agents may be necessary.

The blocking solution should be freshly prepared. It can be stored for longer time periods at -20°C .

Unspecific background labeling is a common problem. Often insufficient blocking of the cells results in (high) background. To overcome this problem, the concentration of the blocking agent and/or the time of blocking may be increased. Most other problems originate from the primary

and secondary antibodies. These may be solved by optimizing the antibody concentrations, incubation times, and washing conditions.

16. Many antibody-fluorophore conjugates tend to precipitate and should be centrifuged before use. This procedure will remove any aggregates that may have formed during storage.
17. After fixation, the samples must not become dry. Otherwise the specificity of the labeling is often lost. To keep the samples humid, labeling is best performed in a humidity chamber. Alternatively, the cover slips can be submersed in antibody solutions in a microtiter plate.
18. Some protocols suggest washing the sample in PBS-Triton X-100 and blocking anew with blocking solution, prior to application of the secondary antibody. In case of an unspecific background, this is recommended.
19. Several fluorophores were successfully employed for STED microscopy (*see Table 11.1*). The number of fluorophores suitable for STED microscopy is rapidly increasing. Currently, antibodies labeled with these fluorophores are not

Table 11.1
List of fluorophores used for STED microscopy

Dye name (manufacturer/distributor)	Excitation wavelength	STED wavelength	Reported spatial resolution (direction)	Reference(s)
ATTO532 (ATTO-Tec, Siegen, Germany)	470 nm	603–615 nm	25–72 nm (<i>xy</i>)	(11, 15, 17, 30, 31)
Chromeo 488 (Active Motif, Carlsbad, CA)	488 nm	602 nm	< 30 nm (<i>xy</i>)	(32)
YFP	490 nm	595 nm	<50 nm (<i>xy</i>)	(33)
GFP	490 nm	575 nm	~70 nm (<i>xy</i>)	(34)
ATTO565 (ATTO-Tec)	532 nm	640–660 nm	30–40 nm (<i>xy</i>)	(35)
MR 121 SE (Roche, Mannheim, Germany)	532 nm	793 nm	~50 nm (<i>z</i>)	(8)
NK51 (ATTO-Tec)	532 nm	647 nm	~50 nm (<i>xyz</i>)	(12)
RH 414 (Invitrogen Carlsbad, CA)	554 nm	745 nm	30 nm (<i>z</i>)	(36)
ATTO590 (ATTO-Tec)	570 nm	690–710 nm	30–40 nm (<i>xy</i>)	(35)
ATTO633 (ATTO-Tec)	630 nm	735–755 nm	30–40 nm (<i>xy</i>)	(35)
ATTO647N (ATTO-Tec)	635 nm	750–780 nm	~50 nm (<i>xy</i>)	(14, 19, 37)

Modified from www.nanoscopy.de (Dept. of NanoBiophotonics, Max Planck Institute for Biophysical Chemistry, February 2009). Note that the number of fluorophores that are demonstrated to be suitable for STED microscopy is steadily increasing.

always commercially available; custom labeling may be required or is advantageous to ensure a high quality of the labeled antibody conjugate. A robust protocol may be found at (29), www.probes.com or www.atto-tec.com.

20. Some antibody-fluorophore conjugates may result in an unwanted unspecific staining of cellular structures. For example, Atto647N is known to label mitochondrial membranes to some extent. Control experiments are required to determine the level of unspecific background for each secondary antibody.
21. A nuclear counterstain can be performed by mounting the samples in Mowiol or TDE containing DAPI (2 $\mu\text{g}/\text{ml}$).
22. After immunofluorescence labeling, the samples should be stored at 4°C. The samples should be imaged within the next few days.
23. Prolonged heating of the formaldehyde solution at 60°C, or too high temperatures, will induce the formation of formic acid, deteriorating the properties of the fixative. Formaldehyde is toxic. Preparation of the formaldehyde solution from solid PFA must be done under a fume hood.

For most applications, fresh formaldehyde solutions may be kept in the refrigerator for 1 week or stored at -20°C for longer periods. Occasionally it has been reported that the use of fresh formaldehyde is superior.

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