Super-Resolution Imaging of Peroxisomal Proteins Using STED Nanoscopy

de Lange, Eline M F; Vlijm, Rifka

Published in:
Peroxisomes

DOI:
10.1007/978-1-0716-3048-8_5

Chapter 5

Super-Resolution Imaging of Peroxisomal Proteins Using STED Nanoscopy

Eline M. F. de Lange and Rifka Vlijm

Abstract

Peroxisomes are crucial organelles that occur in almost all eukaryotes. Well known are their roles in various metabolic processes, such as hydrogen peroxide detoxification and lipid metabolism. Recent studies indicated that peroxisomes also have several non-metabolic functions, for instance, in stress response, signaling, and cellular ageing. In mammalian cells, the small size of peroxisomes (~200 nm, near the diffraction limit) hinders unveiling peroxisomal structures by conventional light microscopy. However, in the yeast *Hansenula polymorpha*, they can reach up to 1.5 μm in diameter, depending on the carbon source. To study the localization of peroxisomal proteins in cells in more detail, super-resolution imaging techniques such as stimulated emission depletion (STED) microscopy can be used. STED enables fast (live-cell) imaging well beyond the diffraction limit of light (30–40 nm in cells), without further data processing. Here, we present optimized protocols for the fluorescent labeling of specific peroxisomal proteins in fixed and living cells. Moreover, detailed measurement protocols for successful STED imaging of human and yeast peroxisomes (using antibodies or genetic tags labeled with dyes) are described, extended with suggestions for individual optimizations.

Key words STED nanoscopy, Peroxisome, Yeast, Human, Cell culture, Immunofluorescence, Live-cell imaging, Super-resolution microscopy, Stimulated emission depletion microscopy

1 Introduction

Peroxisomes are single-membrane-enclosed organelles that are present in almost all eukaryotic cells. These organelles rapidly adapt in size, number, and composition upon changes in environmental conditions. Peroxisomes are involved in a large variety of metabolic processes, depending on the organism, tissue, and developmental stage. In humans, they are among others involved in β-oxidation of very-long-chain fatty acids, hydrogen peroxide degradation, and the biosynthesis of ether lipids and bile acids [1]. Peroxisomes are known as key metabolic organelles but have a much wider range of cellular functions. Recent studies indicated roles in aging, cancer, and neurodegenerative disorders, as well as

Michael Schrader (ed.), *Peroxisomes: Methods and Protocols*, Methods in Molecular Biology, vol. 2643,
https://doi.org/10.1007/978-1-0716-3048-8_5,

protective functions within the innate immune response [2]. These observations illustrate that the significance of peroxisomes in human health goes far beyond the relatively rare inherited, incurable, peroxisomal biogenesis disorders, caused by mutations in PEX genes. Such mutations result in severe diseases, which are generally lethal. In addition, mutations in genes encoding peroxisomal enzymes can lead to the absence of functional peroxisomes [3, 4].

During the last decades, the application of novel microscopy techniques invariably resulted in important new insights in peroxisome biology. The novel developments in the super-resolution microscopy technique STED (stimulated emission depletion microscopy) now enable the application of this method to both live- and fixed-cell studies of even the small mammalian peroxisome structures. STED is thus expected to elucidate important insights into peroxisome biology [5].

STED has been developed to bypass the diffraction limit of light microscopy, by stimulating excited fluorophores to emit, using a doughnut-shaped laser beam [6]. This effectively restricts the fluorescent emission to the small center-point of the donut [7], allowing multicolor images to be taken at a resolution of 30–40 nm. Using a single STED depletion beam in combination with different fluorescent dyes results in multicolor images with perfect (color-error free) protein (co)localization. The stable fluorescent dyes are either specifically bound to genetically fused tags for nontoxic live-cell labeling [8] or positioned through secondary antibody labeling in fixed samples. STED imaging does not need further extensive data processing and has a short (as fast as <1 s; *see* **Note 1**) imaging time when applied to peroxisomes. It is therefore very suitable for live-cell imaging. So far, two studies have been reported in which STED nanoscopy was used in peroxisome research with human cell lines, revealing a heterogeneous distribution of specific peroxisomal proteins [9, 10]. Other studies applied STED in living budding yeast [11] for determining the size of the organelle using a peroxisome-targeted GFP variant (EYFP-SKL) [12]. However, to our knowledge, STED has not been used for protein localization studies in yeast peroxisome research yet.

This chapter describes the use of both yeast and human cells in STED nanoscopy of peroxisomal structures. Both organisms are broadly used in peroxisome research but harbor some distinct differences. Mammalian peroxisomes are relatively small organelles (~0.2 μm in diameter) [13]. However, in the methylotrophic yeast *Hansenula polymorpha*, they drastically increase in size during growth on methanol and can reach up to 1.5 μm in diameter [14]. This makes these yeast species a suitable model organism for microscopy studies on protein distributions at peroxisomal membranes, especially because these cells have a cellular organization similar to human cells, and multiple peroxisomal proteins are highly conserved [15].

This chapter describes the detailed methods for the visualization of endogenous peroxisomal proteins by STED nanoscopy of human and yeast cells. Our protocols give guidelines for both live- (through genetic tags with externally added dyes) and fixed-cell labeling (through genetic tags or immunofluorescence). We also elaborate on the choice of different commercial dyes and tags and the order of labeling. Furthermore, we discuss the main imaging parameters and how to optimize these for individual samples.

2 Materials

When working with living cells, the solutions and materials used need to be sterile. Follow the biosafety and genetically modified organism (GMO) guidelines of your institution.

2.1 Mammalian Cells and Culturing

1. Mammalian cell line of interest, here: HEK293 (Human embryonic kidney 293 cells).
2. Optional plasmids containing a HaloTag® (Promega Corporation) or SNAP-tag® (New England Biolabs) fusion protein (can be stably expressed after incorporation using CRISPR-Cas9 for endogenous expression or can be used through transient transfection; *see* **Note 2**).

2.1.1 Media and Buffers

1. Complete growth medium: Dulbecco's modified Eagle's medium (DMEM 1×), high glucose, GlutaMAX™ Supplement, pyruvate, supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin.
2. Live-cell-imaging medium: FluoroBrite™ DMEM supplemented with 10% FBS and Pen-Strep.
3. Phosphate-buffered saline (1× PBS): 2.7 mM KCl, 137 mM NaCl, pH 7.4, at 25 °C.
4. 2.5% Trypsin (10× stock, dilute in 1× PBS).
5. Poly-L-lysine (PLL) solution, 0.01% in H_2O.
6. 70% ethanol.
7. Trypan Blue (0.4% in 0.85% NaCl).
8. 100% Goat serum.

2.1.2 Equipment

1. Tissue culture hood with vacuum aspiration system.
2. Humidified CO_2 incubator (95% air, 5% CO_2, 37 °C).
3. Inverted light microscope (phase contrast).
4. 37 °C water bath.
5. Cell counter.

6. Clean and sterile borosilicate glass coverslips (18 mm ∅, No. 1.5H; *see* **Notes 3** and **4**).
7. Glass microscope slides.
8. Chamlide magnetic imaging chamber for 18 mm round coverslips, to control temperature, CO_2 concentration, and humidity (CM-B18-1, Live Cell Instrument Co., Ltd.).
9. Metal tweezers.

2.2 Yeast Cells and Cultivation

1. *H. polymorpha* yeast cells, here: NCYC495, *YKU80::URA3*; leu1.1 [16].
2. Plasmids containing the HaloTag® or SNAP-tag® fused to the protein of interest, here: pHIPZ Pex14-SNAP linearized and integrated in yeast cells for endogenous expression [17].

2.2.1 Media and Buffers

1. Growth and selection of positive transformants on YPD plates (1% yeast extract, 1% peptone, 1% dextrose, and 2% agar, heat-sterilized) containing 100 μg/mL zeocin and/or nourseothricin (filter-sterilized, added after sterilization and cooling down of the medium to ~60 °C).
2. For microscopy purposes, mineral medium (MM) [18] containing per liter: $(NH_4)_2SO_4$, 2.5 g; $MgSO_4$ * 7 H_20, 0.2 g; K_2HPO_4, 0.7 g; NaH_2PO_4, 3 g; yeast extract, 0.5 g and trace elements according to Vishniac and Santer (1957), 1 mL. After heat sterilization, a final concentration of heat-sterilized 0.5% glucose or filter-sterilized 0.5% methanol, 60 μg/mL leucine, and 1 mL filter-sterilized vitamin solution (1000× stock solution per liter: biotin, 0.1 g; thiamine, 0.2 g; riboflavin, 0.1 g; nicotinic acid, 5 g; p-aminobenzoic acid, 0.3 g; pyridoxal hydrochloride, 0.1 g; Ca-panthothenate, 2 g; inositol, 10 g) are added.

2.2.2 Equipment

1. Class II biological safety cabinet or a flame.
2. Shaking incubator at 37 °C, 200 rpm.
3. Tabletop centrifuge (Eppendorf centrifuge 5425).
4. Clean and sterile borosilicate glass coverslips (18 mm ∅, No. 1.5H; *see* **Notes 3** and **4**).
5. Glass microscope slides.
6. Chamlide magnetic imaging chamber for 18 mm round coverslips (CM-B18-1, Live Cell Instrument Co., Ltd.).
7. Metal tweezers.

2.3 (Live-Cell) Dyes and Antibodies (See Notes 5 and 6)

1. SNAP-Cell® 647-SiR (New England Biolabs, S9102S).
2. STAR RED, goat anti-rabbit IgG (Abberior, STRED-1002-500UG).

3. STAR 580, goat anti-mouse IgG (Abberior, ST580-1001-500UG).
4. STAR RED, goat anti-mouse IgG (Abberior, STRED-1001-500UG).
5. STAR 580, goat anti-rabbit IgG (Abberior, ST580-1002-500UG).
6. STAR 635P, donkey anti-goat IgG (Abberior, ST635P-1055-500UG).
7. STAR 460L, goat anti-mouse IgG (Abberior, ST460L-1001-500UG).
8. PEX14 polyclonal, rabbit IgG (Proteintech, 10594-1-AP).
9. Catalase polyclonal, goat IgG (Invitrogen PA5-18531).
10. TOMM20 monoclonal, mouse IgG (Abcam ab56783).
11. Alexa Fluor™ 488 Phalloidin (Invitrogen, A12379).
12. DAPI (diamidino-2-phenylindole), 5 mg/mL in sterile dH_2O (GeneCopoeia).

2.4 Fixed Sample Preparation for Confocal or STED Nanoscopy

1. 4% paraformaldehyde (PFA): dilute a formaldehyde solution (for molecular biology, ≥36% in H_2O) in 1× PBS.
2. Permeabilization solution: 0.2% Triton X-100 in 1× PBS. Invert solution until Triton X-100 is completely dissolved, avoid generating foam.
3. Blocking solution: 10% goat serum in 1× PBS. Store solution at 4 °C.
4. Mowiol mounting medium: Dissolve 2.4 g of Mowiol 4-88 in 4.75 mL of glycerol (>99.5%) by slowly stirring on a magnetic plate for 1 h at RT. When dissolved, add 6 mL of MilliQ® water, and stir for 1 h at RT. Leave the solution without stirring at room temperature (RT) for at least 2 h. Add 12 mL of 100 mM tris, pH 8.5, and stir for at least 24 h at 50 °C until it is dissolved (*see* **Note 7**). Let the mixture cool down to RT, and centrifuge for 30 min at 3000× *g* (Eppendorf centrifuge 5702 RH). Aliquot the supernatant containing the completely dissolved Mowiol (the precipitate in the pellet can be discarded). Store at 4 °C for short-term storage, and use (working solution), or at −20 °C for long-term storage. It is stable for at least a year when stored at −20 °C.

2.5 STED Nanoscopy

1. STED microscope: we used a commercial microscope manufactured by Abberior Instruments GmbH. The setup contains a STED laser (775 nm) and four excitation lasers (640, 561, 488, and 405 nm). The microscope is also equipped with a CoolLED pE-2 excitation system and a 100× oil immersion objective (Olympus UPLSAPO/1.40).

2. Immersion oil: type F30CC (Olympus) for 23 °C [room temperature (RT) measurements] and type 37LDF code 387 (Cargille Laboratories) for measurements at 37 °C.
3. Imspector Image Acquisition & Analysis Software v16.3 from Abberior Instruments (by Andreas Schönle, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany, available through Max-Planck-Innovation GmbH, Munich, Germany).
4. Raw images do not need further processing. However, Fiji software (ImageJ v1.8.0) [19] can be used for data visualization and background subtraction.
5. For live-cell STED measurements, the microscope is equipped with a cage incubator containing temperature, gas, and humidity control (Okolab). The microscope is further equipped with an autofocus system (Olympus).

3 Methods

STED nanoscopy offers the possibility to visualize peroxisomes in much greater detail (30 nm resolution) than other light-microscopy techniques often applied, e.g., confocal laser scanning microscopy (250 nm resolution). The sample preparation for STED nanoscopy is fairly similar to that of confocal microscopy. To illustrate the importance of the improved resolution, we show a comparison of confocal and STED imaging of peroxisomal proteins in Fig. 1. Here, PEX14 (localized on the peroxisomal membrane) and catalase (positioned inside the organelle) can only be discriminated using STED nanoscopy, as in confocal microscopy, these proteins seem to colocalize. STED nanoscopy allows proteins of interest to be stained through antibody labeling or the binding of cell-permeable fluorescent dyes to a fusion tag. When immunofluorescence is applied, only the choice of the secondary antibody has to be adjusted to include a STED suitable dye. As the main criterion for STED dyes is their stability, these dyes are simultaneously suitable for confocal microscopy as well (*see* Subheading 2.3 and **Note 6** for examples of STED suitable secondary antibodies). Important for STED nanoscopy is that the required labeling density (and thus the used antibody concentrations) in general should be significantly higher than custom in confocal microscopy, as the structures otherwise appear to be non-connected (sparse, under-labeled) with the increased resolution. Simply increasing the antibody concentration however is not sufficient, since too much over-labeling results in blurred images. The antibody concentration thus has to be optimized [20] for each sample.

An alternative to immunofluorescence is the use of genetic tags, which covalently bind small, stable dyes. This method allows a much more specific, endogenous labeling of proteins in both

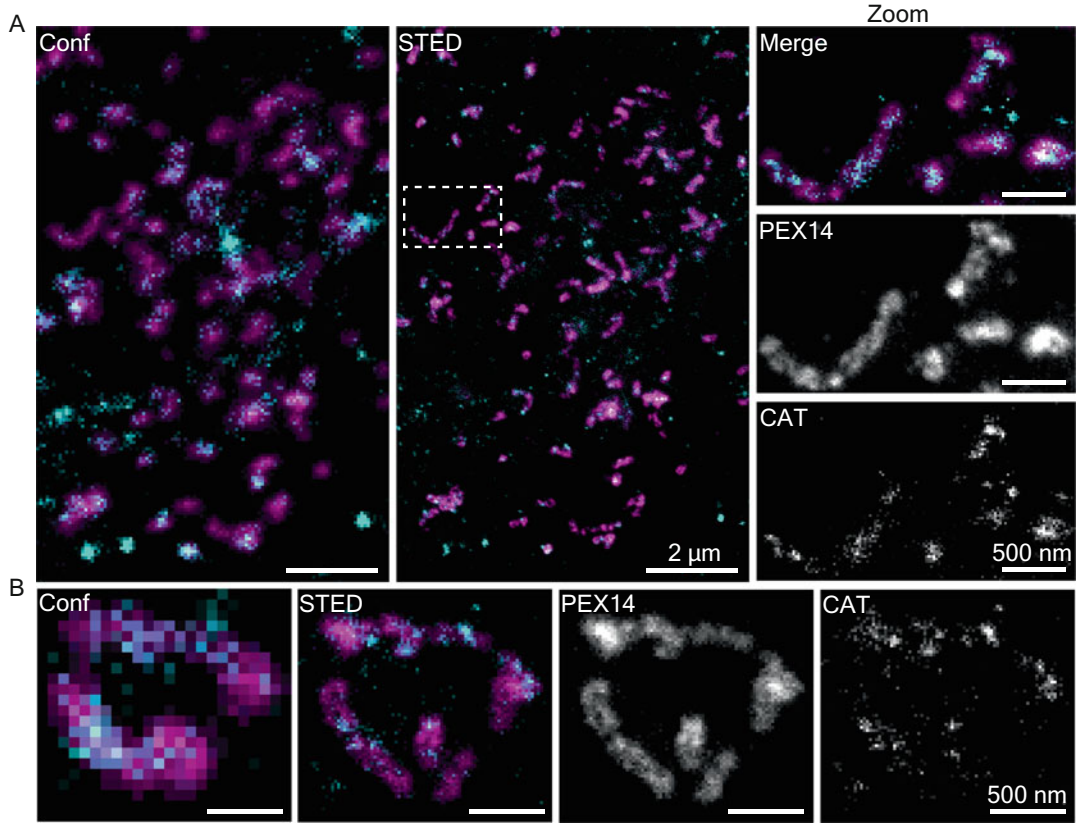

Fig. 1 Confocal versus STED imaging of the peroxisomal membrane protein PEX14 and the matrix protein catalase in HEK293 cells. (**a**) A confocal and corresponding STED image of several peroxisomes in a single cell are shown, immunostained using PEX14 (*magenta*) and catalase (*cyan*) antibodies. The images on the right show a zoom of the boxed area. Only STED nanoscopy clearly localizes the catalase inside the peroxisomes, whereas in confocal microscopy, these labels appear to colocalize. (**b**) Another example of confocal versus STED nanoscopy (pixel size 70 and 20 nm respectively) of the merged (left) and individual (right) images showing the immunostained PEX14 (*magenta*) and catalase (*cyan*) of human peroxisomes

fixed and living cells. These tags (e.g., HaloTag® or SNAP-tag®; for the construction of these strains, *see* **Note 2**) are fused to the protein of interest. The nontoxic, cell-permeable dyes that bind to these tags are very suitable for live-cell imaging but also survive fixation (before or after the labeling). When the samples are labeled before instead of after fixation, it could result in lower background signal, since the excessive (non-bound) dye can actively be removed by the cells. To prevent the cell form pumping out the dyes too efficiently (resulting in a lower labeling density), the drug verapamil can be used in mammalian cell culture. Additionally, higher dye concentrations are often necessary to obtain sufficient labeling in living cells. Fortunately, over-labeling is not an issue here, because only one dye can bind to the genetic tag (unlike antibodies).

When performing the labeling after fixation, lower dye concentrations are required for a good signal. Nevertheless, it could lead to higher background noise. However, many commonly used dyes are fluorogenic to some extent, which means that they only fluoresce when specifically bound. Therefore, the background signal from non-specific binding is extremely low in these cases, especially when compared to immunofluorescence. Next to this, permeabilization is not needed after the fixation, when only using (cell-permeable) dyes instead of antibodies. The tags are also smaller in size, possibly leading to a better resolution. However, because there is no amplification of the fluorescent signal, unlike secondary antibody labeling, the intensity will be lower. Additionally, immunofluorescence has the advantage that no genetic modifications are required.

The protocols for each of these different labeling approaches are described in detail in the following paragraphs. All our protocols are optimized for a 775 nm STED laser. A 595 nm STED laser is also commercially available, but this wavelength is more invasive for live-cell imaging (as cells have a higher absorbance at 595 nm).

3.1 Seeding HEK Cells for Immunofluorescence

For a T75 flasks with mammalian cells:

1. Warm up the complete DMEM (*see* Subheading 2.1.1, **item 1**), trypsin, and PBS.
2. Use an ~80% confluent T75 flask containing HEK cells from the CO_2 incubator.
3. Remove the growth media.
4. Add 1× PBS to the bottom of the flask, swirl the flask around, and remove PBS.
5. Add 1 mL of 1× Trypsin to the cells.
6. Incubate the flask at 37 °C, 5% CO_2 for several minutes, until cells are detached.
7. Take 8 mL complete DMEM (*see* Subheading 2.1.1, **item 1**), and resuspend the cells.
8. Mix the cell suspension 1:1 with Trypan Blue (0.4% in 0.85% NaCl) in a tube. Make sure that the cells are pipetted up and down right before taking a sample.
9. Pipet the cell mixture in a counting chamber and count the cells.
10. Dilute the cells to the correct volume for 10^5 cells per 1 mL medium.
11. Seed the cells (final volume 1 mL/well) on clean, PLL-coated coverslips in a 12-well plate, for fixation (or transfection) the next day (≥ 24 h; *see* **Note 2**).
12. Place the plate in the CO_2 incubator at 37 °C.

3.2 Immunofluorescence of Fixed HEK Cells for STED Nanoscopy

1. Wash the seeded cells that are grown on the coverslips with 1× PBS (0.5 mL, in the 12-well plate).
2. Fix with 300 μL 4% PFA for 15 min at RT.
3. Discard the PFA, and wash 3× with PBS, by exchanging the liquid in the 12-well plate.
4. Permeabilize with 1 mL of 0.1% Triton X-100 freshly diluted in PBS for 15 min (*see* **Note 8**).
5. Wash 3× with PBS: 30 s, 1 min, and 10 min.
6. Block in 1 mL 10% goat serum in PBS for 1–2 h at RT (*see* **Note 9**).
7. Dilute the primary antibodies in 10% goat serum. Put a droplet (30 μL) of antibody mixture on Parafilm® in a petri dish. Place the coverslips cell down on the mixture. Create a humid chamber by adding a damp tissue on the side of the dish so the antibody mixture will not evaporate, but prevent contact between the tissue and the samples. Incubate for 1 h at RT (*see* **Note 10**).
8. Wash 4× with PBS: 30 s, 30 s, 10 min, 50 min by placing the coverslips back in the 12-well plate (*see* **Note 11**).
9. Dilute the secondary antibodies 1:80 in 10% goat serum in PBS (30 μL/coverslip).
10. Incubate 45 min at RT on Parafilm® in a humid chamber, in the dark.
11. Wash 4× with PBS: 30 s, 30 s, 1 min, and 2 min.
12. Add DAPI 1:50.000 (in PBS) for 4 min, 1 mL per well (*see* **Note 12**).
13. Wash 4× with PBS: 30 s, 30 s, 10 min, and 45 min (*see* **Notes 11** and **13**).
14. Take the coverslip out of the 12-well plate using tweezers, and briefly tap the edge on a tissue to remove excess water.
15. Mount with Mowiol by inverting the coverslip on a glass microscope slide containing 10 μL mounting medium. Dry overnight at RT, in the dark (*see* **Note 14**).
16. Transfer to a microscope and capture images, or store the slides at 4 °C for imaging at a later timepoint (*see* **Note 15**).

3.3 HaloTag® or SNAP-Tag® Labeling of Mammalian Cells for STED Nanoscopy

Live-cell compatible dyes can be covalently bound to a protein of interest by fusing a HaloTag® or SNAP-tag® to this protein through genetic engineering. These dyes are available in a range of different colors (excitation/emission wavelengths) and properties, also allowing pulse-chase experiments. These organic dyes are very photostable (other than fluorescent proteins like GFP) and

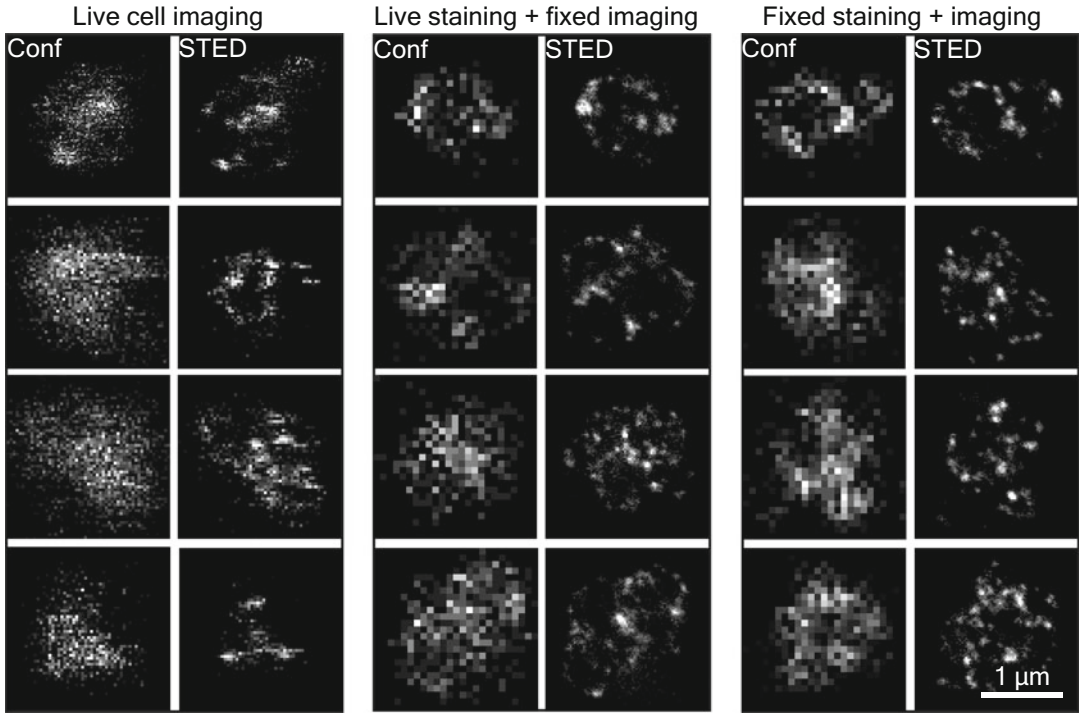

Fig. 2 Sample preparation methods: Living and fixed-cell labeling and imaging of peroxisomes in the yeast *H. polymorpha*. This figure compares live-cell labeling and imaging (left panels), with live-cell labeling and fixed-cell imaging (middle panels), and with fixed-cell labeling and imaging (right panels). Each of these methods are valid, and the spotted pattern of Pex14 on the peroxisomes is in all preparations as expected from literature [21], but the method might impact the final resolution or level of background. Live-cell STED imaging shows a significant increase in resolution compared to confocal microscopy (left panels) yet still offers the great possibility to follow cellular processes in real time. In our examples of fixed cell imaging (compare middle and right panels), the order of labeling does not matter as no clear difference between the live or fixed staining is visible in these images. However, it is worth investigating the order of labeling to maximize the labeling density and minimize the background signal. In each sample, Pex14 is genetically fused to the SNAP-tag®, to enable both live- and fixed-cell labeling with STED suitable dyes. The strain is endogenously labeled using the SNAP-Cell® 647-SiR dye after growth for 12 h in mineral medium supplied with methanol. Every panel shows the confocal and corresponding STED image of a single cell with one or multiple peroxisomes (four examples of each condition shown). All images have the same scale

thus suitable for STED nanoscopy. As mentioned before, they can be used to stain proteins for live-cell imaging or for fixed sample preparations (*see* Fig. 2). After fixation, the samples can be stored at 4 °C to be imaged at a later timepoint.

In general, living cells are vulnerable to light toxicity. Therefore, in live-cell imaging, the applied laser powers are often less intense compared to fixed-cell imaging (compare left to middle panels in Fig. 2), which leads to a somewhat lower resolution. Also, as living cells are dynamic, the total imaging time should be minimized to prevent image distortions due to movements. In the case of fixed cells (Fig. 2, middle and right panels) with, e.g.,

SNAP-tag® labeling, either the labeling could be performed before or after fixation. It is worth investigating the order of labeling to maximize the labeling density and minimize the background signal.

3.3.1 Live-Cell Labeling of HEK Cells

1. Follow all steps from Subheading 3.1, the seeding of cells.
2. Prepare a 5× working stock solution of Halo or SNAP dye in warm, complete DMEM (*see* Subheading 2.1.1, **item 1**), and mix thoroughly (*see* **Note 16**).
3. Label the cells expressing the HaloTag®/SNAP-tag® fusion protein(s) by replacing one-fifth of the medium with the 5× labeling solution, and mix gently. This results in a final concentration of 1 μM dye in 1 mL medium (*see* **Notes 10** and **17**).
4. Incubate the cells in a 37 °C, 5% CO_2 incubator for 30–60 min.
5. Wash the cells three times with 1 mL of warm, fresh medium.
6. Incubate the cells in complete DMEM at 37 °C, 5% CO_2 for 30 min. These wash **steps** (**5** and **6**) can be omitted if fluorogenic dyes are used.
7. Using sharp tweezers, transfer a coverslip from the 12-well plate to a Chamlide magnetic imaging chamber (CM-B18-1, Live Cell Instrument Co., Ltd.) that fits into the cage incubator of the microscope for temperature and CO_2 control.
8. Add 500 μL warm live-cell-imaging medium (*see* **Note 18**).
9. Transfer the sample to a microscope (at 37 °C), and capture images immediately.

3.3.2 Fixed-Cell Labeling of HEK Cells with Live Dyes

1. Follow **steps 1–3** from Subheading 3.2 to fix the seeded cells (*see* **Note 19**).
2. Dilute the Halo/SNAP dye(s) to a final concentration of 1 μM in PBS, and place a droplet (30 μL) on Parafilm® in a petri dish with a damp tissue on the side.
3. Label the cells expressing the HaloTag®/SNAP-tag® fusion protein by inverting the coverslip cell down on the mixture. Incubate 1 h at RT (*see* **Note 10**).
4. Follow **steps 13–16** from Subheading 3.2.

3.4 HaloTag® or SNAP-Tag® Labeling of Fixed Yeast Cells for STED Nanoscopy

1. Grow the yeast cells overnight (o/n) in 20 mL MM/glucose at 37 °C shaking (200 rpm).
2. Dilute the o/n culture to $OD_{600} = 0.1$, and grow until an OD >1.6. Dilute this culture again to $OD_{600} = 0.1$, and grow until an OD >1.6.
3. Dilute to OD = 0.1 in 20 mL MM/methanol, and grow cells for 6 h at 37 °C shaking.
4. Harvest 7.5 OD-units of cells by centrifugation at 1818× *g* for 5 min at RT.

5. Wash the cells with 1 mL PBS in a 1.5 mL conical tube, and centrifuge for 1 min at 1818× *g*.
6. Resuspend the pellet in 500 µL 2% PFA in PBS, and fix for 30 min at RT.
7. Coat ethanol-washed coverslips with 300 µL PLL (30 min at RT) in a 12-well plate, rinse 3× with PBS, and air-dry.
8. Wash the cells with 1 mL PBS, and centrifuge at 1818× *g* for 1 min.
9. Resuspend the pellet in 100 µL PBS + 1 µM dye(s), and incubate for 1 h at RT. Perform all steps from now on in the dark (*see* **Note 20**).
10. Wash the cells 3× with PBS (1 mL, last wash 20 min), and centrifuge at 1818× *g* for 1 min.
11. Place 400 µL of cell-suspension on a PLL-coated coverslip in a 12-well plate, and incubate for 15 min at RT.
12. Wash the coverslip containing the cells 3× carefully with PBS (*see* **Note 13**). Take the coverslip out of the 12-well plate using tweezers, and tap the edge briefly on a tissue to dry the sample.
13. Mount with 10 µL Mowiol on a glass microscope slide (invert the coverslip on it), and dry overnight at RT (*see* **Note 14**).
14. Image using STED nanoscopy, or store the slide(s) at 4 °C for imaging at a later timepoint (*see* **Note 15**).

3.5 STED Nanoscopy

1. Clean the microscopy slide with a tissue containing ethanol, and add the appropriate oil to the objective (*see* "Methods," Subheading 2.5, **item 2**).
2. Invert the slide onto the aligned microscope, and focus the sample (*see* **Note 21**). Select the appropriate scan area and pixel size (~18 nm for fixed samples or ~25 nm for living samples). The image length along the scan direction, and therefore the drift, should be minimal. The optimal pixel size should be determined for each sample, to prevent under- or oversampling (*see* Fig. 3). Nyquist theorem [22] shows that reducing the pixel size until roughly 2.3 times smaller than the object that is being resolved (resolution) will lead to more information. Even smaller pixels will not improve the image quality as no more information is obtained. Importantly, smaller pixels will lead to both a higher light dose (causing bleaching and possibly light toxicity) and a longer scan time (causing drift). Undersampling on the other hand results in a lower final resolution. Thus, it is important to optimize the pixel size for each sample.
3. Optimal selection of laser powers and detection windows. Our (far-)red dyes (640 nm excitation) are here detected with a spectral window between 650 and 730 nm. The 640 nm laser

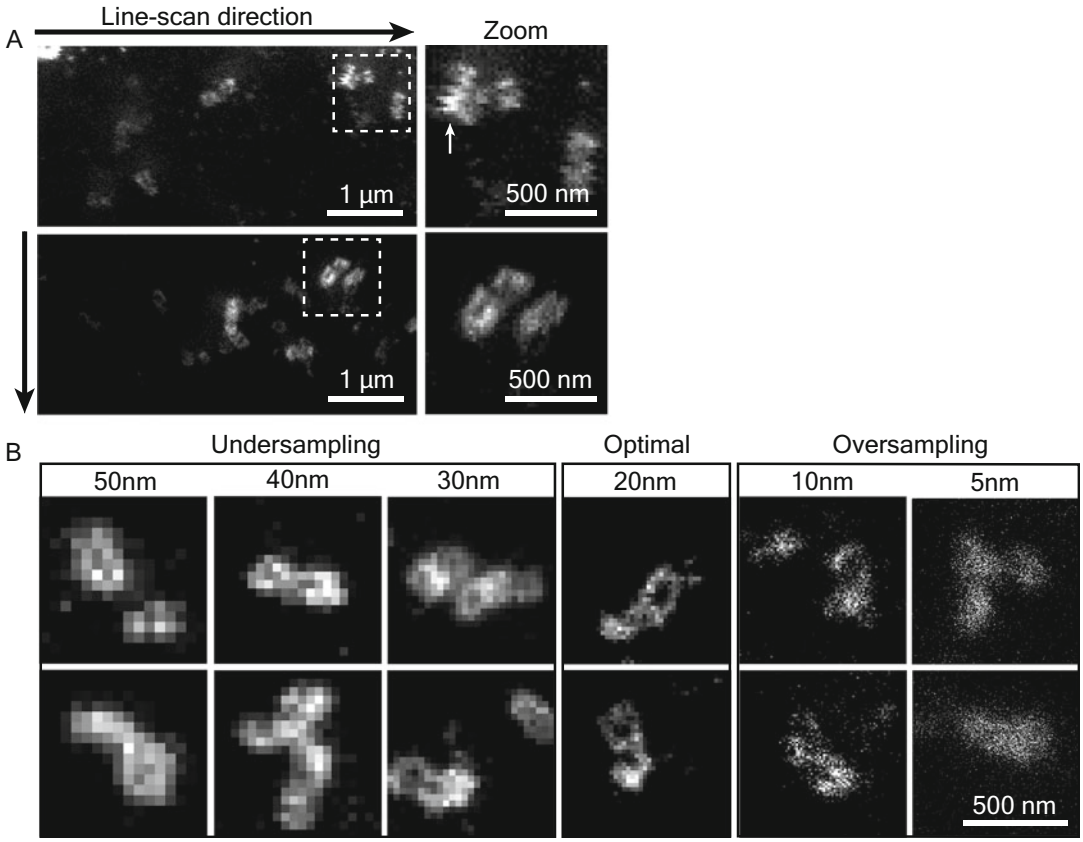

Fig. 3 The effect of scan settings on the image quality. (**a**) Scan direction. The image length along the scan direction should be minimized to diminish the effect of sample drift or cell movement (living samples) between consecutive lines. The two images shown are measured under the exact same scan conditions, with the exception that the top panel has a relatively long scan line (and fewer lines, 5.0 × 2.4 µm), compared to the bottom panel, which has shorter and more scan lines (2.4 × 5.0 µm). Therefore, the time between consecutive lines in the top panel is larger than in the bottom panel, which clearly results in drift between lines (*see* arrow top right inset) and thus a lower image quality. For best results, the image dimension along the scan direction should thus be chosen to be minimal. Insets show higher-magnifications of the boxed areas. (**b**) Pixel size. The ideal pixel size to achieve the best resolution depends on, e.g., the microscope, its settings, and the sample quality. Here, from left to right, we illustrate how undersampling (pixel size too large) and oversampling (pixel size too small) lead to lower resolutions. If a resolution of about 30 nm is expected, according to the Nyquist theorem [22], the optimal pixel size thus is about 20 nm (30×30 nm $= 900$ nm^2, $900/2.3 = 391$, $\sqrt{391} = 20$ nm). As the final resolution is sample specific, the pixel size should be optimized for each sample. All images have the same scale

is excited with 1% of its maximum power and the 775 nm STED laser with 20–40% of its maximum power. The orange dyes (580 nm excitation) are detected with a spectral window between 570 and 640 nm with 10% of its maximum excitation power, in combination with 60–80% of the 775 nm STED laser. For STED, gating is also applied. These settings are strongly dependent on the used setup and should thus be

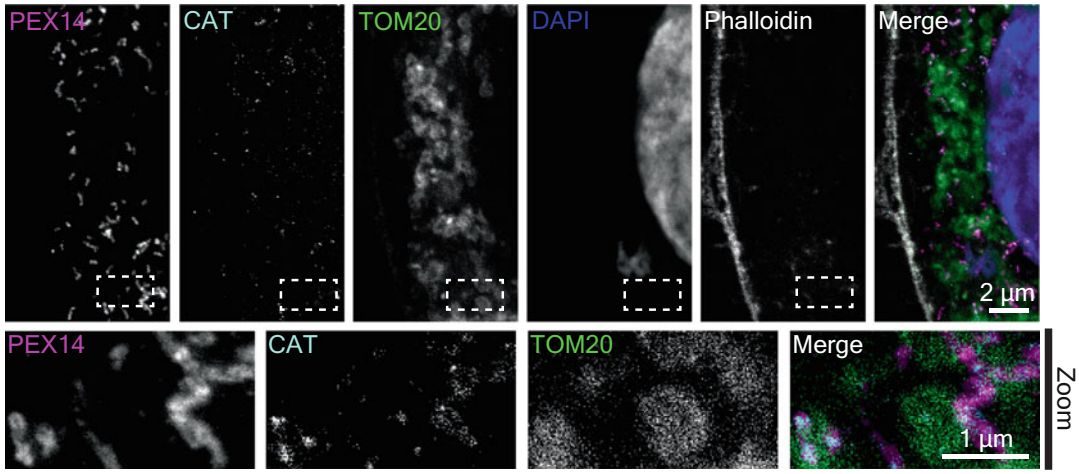

Fig. 4 Five-color fluorescence microscopy with three STED and two confocal channels of fixed HEK293 cells. The overlay (merge) on the top right shows clearly separated structures of the peroxisomal membrane (PEX14, *magenta*) and matrix (CAT, *cyan*) together with mitochondria (TOM20, *green*), DNA (DAPI, *blue*), and actin (phalloidin, *grey*). Color unmixing was performed for phalloidin as described in Fig. 6. The lower panels show a zoom of the boxed areas in the top panels. These zoomed images both show the color overlay and the individual channels

determined on individual basis. For multicolor images, the detector settings should be optimized depending on the fluorophore combination applied (*see* Fig. 4).

4. Adjust the exposure times and pinhole diameter according to the brightness of the specific labeling and the background signal of the sample. Here, a pinhole of 0.8 AU is used. The chosen dwell time is 50 µs for fixed samples, but this could be decreased to 10 or 20 µs for dynamic live-cell samples to reduce blurring. The reduction in signal due to the shorter dwell time can be partially compensated by a larger excitation power.
5. Apply adaptive illumination (e.g., RESCue or DyMIN) to reduce photobleaching and enable long-term measurements.
6. Collect multicolor STED images using microscopy software and visualize in ImageJ [19]. Possible post-processing (e.g., background subtraction, quantifications, or bleed-through correction; *see* **Note 22**) can also be performed when processing the raw data in ImageJ.

4 Notes

1. Similar to confocal imaging, STED nanoscopy is a scanning technique. Therefore, the time it takes to acquire an image scales with the scanned area and volume.

2. A stable cell line expressing a HaloTag® or SNAP-tag® fusion protein, e.g., constructed using CRISPR-Cas9 [23], can be used. If this is not available, one can also choose to transfect the cells with purified plasmid (e.g., pcDNA3.1+) 24 h after seeding and before fixation. A possible protocol includes the following: Dilute 2 μg of DNA in 150 mM NaCl to a final volume of 50 μL per well and 4 μL of jetPEI® in 150 mM NaCl to 50 μL. Vortex both solutions gently, and spin down briefly. Add the 50 μL jetPEI® solution to the 50 μL DNA solution, and vortex immediately. Incubate for 15–30 min at RT. Add the jetPEI®/DNA mixture dropwise onto the cells (100 μL in 1 mL of serum-containing medium), and gently swirl the plate. Return the plate to the incubator for 24 h.
3. It is important to use high-precision microscope cover glasses with a thickness of 170 μm (No. 1.5H, Cat. No. 0117580, Paul Marienfeld GmbH & Co. KG). The thickness and quality of the borosilicate glass have a crucial impact on the final resolution. Some brands have a large variety in thickness, which leads to spherical aberrations when used with high numerical aperture (NA) objectives optimized for 170 μm coverslips. When mounted on glass slides, the shape (not thickness) of the coverslip can be varied. However, for live-cell experiments, only the 18 mm coverslips fit in the medium-containing Chamlide magnetic imaging chamber (CM-B18-1, Live Cell Instrument Co., Ltd.) of the microscope.
4. Instead of the cells seeded on coverslips, which are placed in a Chamlide magnetic imaging chamber, other high-quality borosilicate glass (170 μm thickness) bottom dishes, such as the Nunc™ Lab-Tek™ II CC2™ Chamber Slide System (Cat. No. 154917), can be used as well.
5. It is advisable to consider the different labels when performing dual-color imaging using dye pairs which are depleted by the same STED depletion beam. Due to the increased STED depletion efficiency of more red dyes, these dyes often result in a higher STED resolution and are thus superior for STED imaging (*see* Fig. 5). Therefore, it is often advisable to label the most interesting or weakest expressed protein with a (far-)red dye (~640 nm excitation) and the other protein(s) with a more orange dye (~580 nm excitation). A second consideration concerns the design of the genetic construct for endogenous labeling as there is a potential difference in labeling efficiency between the SNAP-tag® and HaloTag®. Often the HaloTag® in combination with SiR fluorophores results in brighter labeling than SNAP® with SiR [24].
6. Additional suitable dyes are several "Janelia" dyes, e.g., Janelia Fluor® 635 and 585 HaloTag® (Cat. No. CS315103 and

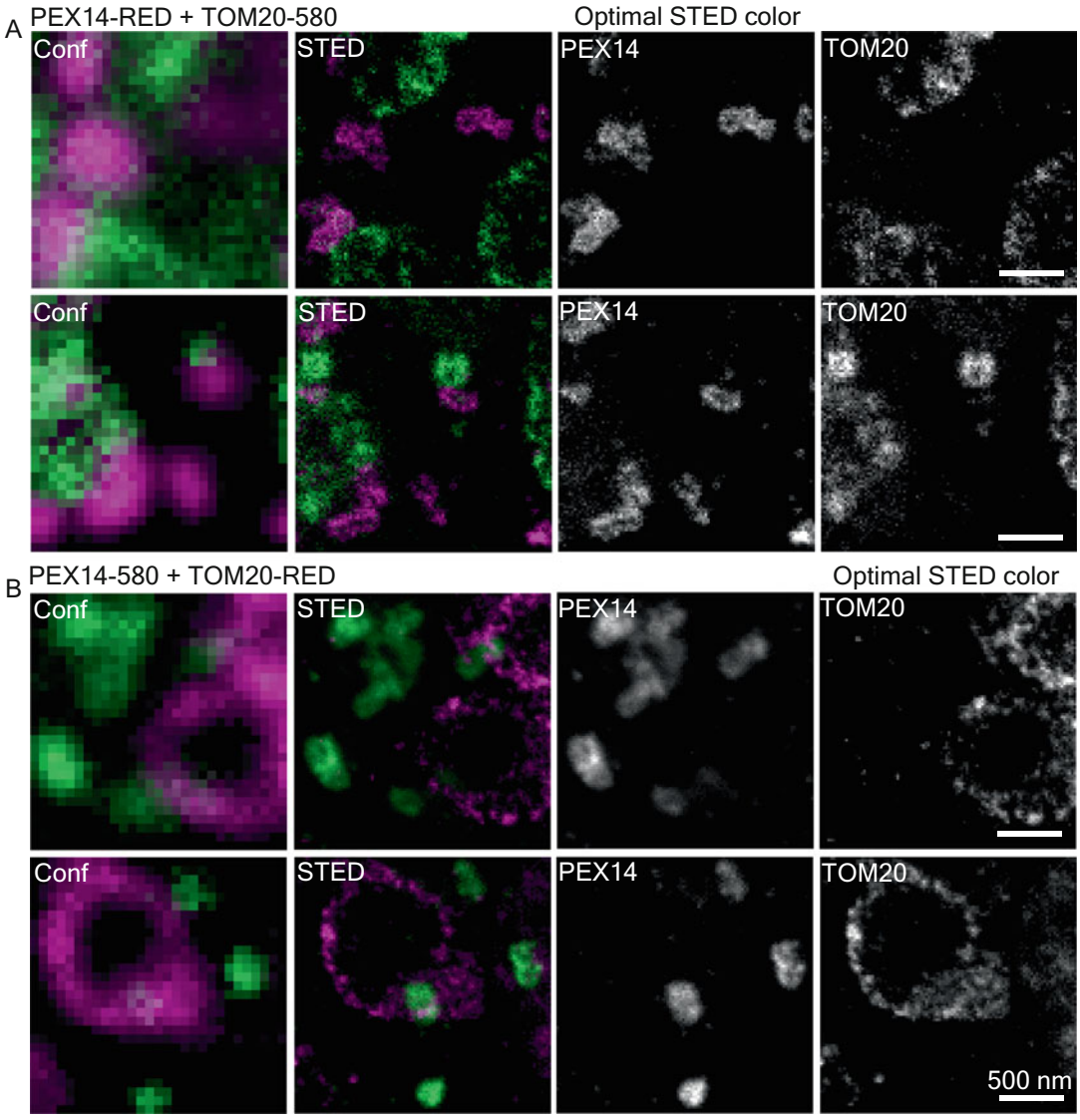

Fig. 5 (Far-)red dyes (~640 nm excitation) are in general more efficiently depleted by the 775 nm STED beam than more orange (~580 nm excitation) dyes. (**a**) Immunofluorescence of peroxisomes (PEX14) and mitochondria (TOM20) in HEK293 cells labeled with STAR RED (638 nm excitation) and STAR 580 (587 nm excitation) secondary antibodies, respectively. The images have optimal STED settings for each specific labeling. The far-red dyes can be depleted more efficiently by the STED beam, leading to a better resolution of the labeled peroxisomes. (**b**) When the labels are reversed (namely, PEX14-STAR580 and TOM20-STAR RED), the mitochondrial labeling (TOM20-STAR RED) shows an improved resolution and the peroxisomal membranes (PEX14-STAR 580) a decrease in quality. All images have the same scale

CS315105, Promega Corporation). Abberior dyes include Abberior LIVE 460 L, 590, or 610 dyes conjugated to SNAP®, tubulin, actin, or DNA. Alternatively, Spirochrome provides SiR-actin, SiR-tubulin, or SiR-DNA kits.

7. The increased temperature has the effect that samples mounted with Mowiol need longer to dry. However, the 4-88 polyvinylalcohol (Mowiol) dissolves better, which leads to better refractive index matching, and therefore the 50 °C stirring is preferred.
8. For the permeabilization, detergents such as saponin or Triton X-100 are often used. The disadvantage of Triton X-100 is that it is non-selective and may extract proteins along with the lipids from the membrane. Therefore, cells can also be permeabilized using 0.1% saponin (at Subheading 3.2, **item 4**), which only interacts with membrane cholesterol, selectively removing it and leaving holes in the membrane. Saponin is thus a less invasive method that permeabilizes cell membranes without destroying them. However, staining of paraformaldehyde-fixed cells permeabilized with saponin does not allow the detection of proteins in the nucleus.
9. This step can also be performed overnight at 4 °C. Goat serum (use serum from the organism the secondary antibody is raised in) is more specific and thus preferred over regular blocking buffer with milk or BSA, since it might result in less nonspecific labeling. This blocking buffer is thus antibody dependent. If serum from the used organism is not available, BSA can still be used.
10. Antibody or dye dilutions and incubation times depend on the expression of the protein of interest and should be optimized using the recommended concentrations as a guideline.
11. Extensive washing is needed for STED nanoscopy, to reduce the background noise. Nevertheless, the last washing step could be shortened if preferred.
12. Low concentrations of DAPI are needed, especially when the protein of interest is located near the nucleus, as merely the 775 nm STED can excite DAPI to create unwanted background signal in the detector of the proteins of interest.
13. The last wash can be performed with MilliQ® water, to remove the salts from the PBS and prevent crystal formation in the sample.
14. Mowiol completely hardens overnight, but this could be sped up by incubating the slide for 1 h at 37 °C. Immediate storage at 4 °C does not facilitate this solidification. Avoid air bubbles underneath the coverslip, and ensure that the Mowiol is dried completely before examining the sample on the microscope.

15. Samples are best imaged as soon as they are prepared, to obtain the highest resolution. Depending on the used dyes, they can be sufficiently stable to allow imaging (although at lesser quality) several days, or up to several months after preparation, when stored at 4 °C in the dark. Addition of anti-fade reagents may slow down the degradation of the fluorophores.
16. Do not prepare more medium with dye than you will use, and do not prepare this working stock more than 1 h before use, as a water-like environment promotes degradation of the dye in general.
17. Other dyes such as a MitoTracker™ could also be added at this point.
18. Other medium lacking phenol red can be used as well. Special imaging medium may improve the image quality but may also be suboptimal for cell proliferation.
19. Permeabilization/blocking is optional but only necessary for immunofluorescence. It is not needed when using the cell-permeable Halo/SNAP (live) dyes.
20. Staining can be done in PCR tubes, which prevents loss of sample when working with small volumes.
21. Make sure your sample and microscope are at the same temperature (RT or 37 °C), to prevent drift.
22. Bleed-through correction can be performed using a single-labeled control sample. Here, the amount of bleed-through into a second channel is determined while using the actual imaging conditions as used in the multicolor experiment. By comparing the original signal of this single fluorophore (e.g., STAR 460L, with optimal settings, in channel 1), to that of the bleed-through caused by this labeling (e.g., the signal from STAR 460L in channel 2, as produced when optimal setting for Alexa Fluor™ 488 are used), the bleed-through factor can be determined. Next, this factor is used to subtract the background signal in channel 2 and acquire a corrected image (*see* Fig. 6).

Acknowledgments

We would like to thank Ida van der Klei for insightful scientific discussions and for reading the manuscript.

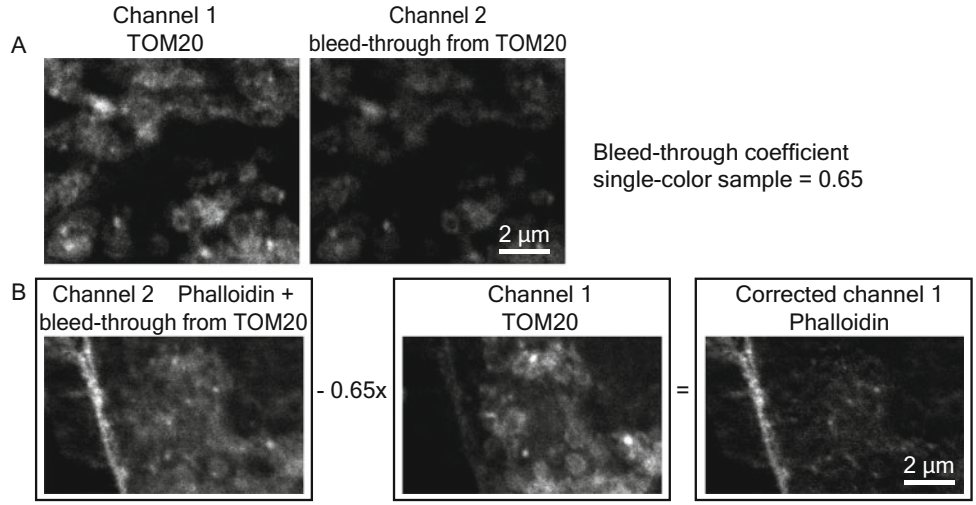

Fig. 6 Bleed-through correction using single-labeled control samples. (**a**) A control sample labeled with only the fluorophore (TOM20 with long Stokes-shift dye Abberior STAR 460L) is used to measure the bleed-through of this fluorophore into the detector used to determine other labels (e.g., Alexa Fluor™ 488 in detector channel 2), which has a partially overlapping emission spectrum. Importantly, instead of using optimal imaging settings for the fluorophore in this single-colored sample (e.g., STAR 460L), now the image acquisition settings of the label intended to be detected by this detector (Alexa Fluor™ 488) are used. The average slope of the correlation regression line of 4 control samples is calculated using the Coloc2 plugin in Fiji [19], whereby a mask of the TOM20 signal was applied. This value (slope) is the bleed-through coefficient to correct the multicolor images [25]. (**b**) The final, corrected image is acquired by subtracting the TOM20 (channel 1) signal multiplied by the bleed-through coefficient (here 0.65), from the phalloidin (channel 2) signal. All images have the same scale

References

1. Smith JJ, Aitchison JD (2013) Peroxisomes take shape. Nat Rev Mol Cell Biol 14(12): 803–817. https://doi.org/10.1038/nrm3700
2. Ferreira AR, Marques M, Ribeiro D (2019) Peroxisomes and innate immunity: antiviral response and beyond. Int J Mol Sci 20(15): 3795. https://doi.org/10.3390/ijms20153795
3. Bjørgo K, Fjær R, Mørk HH et al (2017) Biochemical and genetic characterization of an unusual mild PEX3-related Zellweger spectrum disorder. Mol Genet Metab 121(4): 325–328. https://doi.org/10.1016/j.ymgme.2017.06.004
4. Dursun A, Gucer S, Ebberink MS et al (2009) Zellweger syndrome with unusual findings: non-immune hydrops fetalis, dermal erythropoiesis and hypoplastic toe nails. J Inher Metab Dis 32(Suppl 1):1–4. https://doi.org/10.1007/s10545-009-9010-0
5. Sahl SJ, Hell SW, Jakobs S (2017) Fluorescence nanoscopy in cell biology. Nat Rev Mol Cell Biol 18:685–701. https://doi.org/10.1038/nrm.2017.71
6. Klar TA, Jakobs S, Dyba M, Egner A, Hell SW (2000) Fluorescence microscopy with diffraction resolution barrier broken by stimulated emission. PNAS 97(15):8206–8210. https://doi.org/10.1073/pnas.97.15.8206
7. Hell SW, Wichmann J (1994) Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. Opt Lett 19(11):780–782. https://doi.org/10.1364/OL.19.0780
8. Vlijm R, Li X, Panic M et al (2018) STED nanoscopy of the centrosome linker reveals a CEP68-organized, periodic rootletin network

anchored to a C-Nap1 ring at centrioles. PNAS 115(10):E2246–E2253. https://doi.org/10.1073/pnas.1716840115

9. Galiani S, Waithe D, Reglinski K et al (2016) Super-resolution microscopy reveals compartmentalization of peroxisomal membrane proteins. J Biol Chem 291(33):16948–16962. https://doi.org/10.1074/jbc.M116.734038
10. Soliman K, Göttfert F, Rosewich H, Thoms S, Gärtner J (2018) Super-resolution imaging reveals the sub-diffraction phenotype of Zellweger Syndrome ghosts and wild-type peroxisomes. Sci Rep 8:7809. https://doi.org/10.1038/s41598-018-24119-2
11. Stagge F, Mitronova GY, Belov VN, Wurm CA, Jakobs S (2013) Snap-, CLIP- and halotag labelling of budding yeast cells. PLoS One 8(10):e78745. https://doi.org/10.1371/journal.pone.0078745
12. Yofe I, Soliman K, Chuartzman SG et al (2017) Pex35 is a regulator of peroxisome abundance. J Cell Sci 130(4):791–804. https://doi.org/10.1242/jcs.187914
13. Arias JA, Moser AB, Goldfischer SL (1985) Ultrastructural and cytochemical demonstration of peroxisomes in cultured fibroblasts from patients with peroxisomal deficiency disorders. J Cell Biol 100(5):1789–1792. https://doi.org/10.1083/jcb.100.5.1789
14. Nagotu S, Veenhuis M, van der Klei IJ (2010) Divide et impera: the dictum of peroxisomes. Traffic 11:175–184. https://doi.org/10.1111/j.1600-0854.2009.01019.x
15. Jansen R, Santana-Molina C, van den Noort M, Devos DP, van der Klei IJ (2021) Comparative genomics of peroxisome biogenesis proteins: making sense of the PEX proteins. Front Cell Dev Biol 9:654163. https://doi.org/10.3389/fcell.2021.654163
16. Saraya R, Krikken AM, Kiel JAKW, Baerends RJS, Veenhuis M, van der Klei IJ (2012) Novel genetic tools for Hansenula polymorpha. FEMS Yeast Res 12(3):271–278. https://doi.org/10.1111/J.1567-1364.2011.00772.X
17. Faber KN, Haima P, Harder W, Veenhuis M, Ab G (1994) Highly efficient electrotransformation of the yeast Hansenula polymorpha. Curr Genet 25:305–310
18. Van Dijken LP, Otto R, Harder W (1976) Growth of Hansenula polymorpha in a methanol-limited chemostat. Arch Microbiol 111:137–144. https://doi.org/10.1007/BF00446560
19. Schindelin J, Arganda-Carreras I, Frise E et al (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9(7):676–682. https://doi.org/10.1038/nmeth.2019
20. Lau L, Lee YL, Sahl SJ, Stearns T, Moerner WE (2012) STED microscopy with optimized labeling density reveals 9-fold arrangement of a centriole protein. Biophys J 102(12):2926–2935. https://doi.org/10.1016/j.bpj.2012.05.015
21. Cepińska MN, Veenhuis M, van der Klei IJ, Nagotu S (2011) Peroxisome fission is associated with reorganization of specific membrane proteins. Traffic 12(7):925–937. https://doi.org/10.1111/J.1600-0854.2011.01198.X
22. Nyquist H (1928) Certain topics in telegraph transmission theory. Trans AIEE 47:617–644
23. Sakuma T, Nakade S, Sakane Y et al (2016) MMEJ-assisted gene knock-in using TALENs and CRISPR-Cas9 with the PITCh systems. Nat Protoc 11:118–133. https://doi.org/10.1038/nprot.2015.140
24. Erdmann RS, Baguley SW, Richens JH et al (2019) Labeling strategies matter for super-resolution microscopy: a comparison between HaloTags and SNAP-tags. Cell Chem Biol 26(4):584–592.e6. https://doi.org/10.1016/j.chembiol.2019.01.003
25. Jost AP-T, Waters JC (2019) Designing a rigorous microscopy experiment: validating methods and avoiding bias. J Cell Biol 218(5):1452–1466. https://doi.org/10.1083/jcb.201812109