Sample Preparation

for

Ground State Depletion (GSD)

Super-resolution imaging

Protocol guide for Leica SR GSD system

Version 1.0
1. Introduction

Ground State Depletion (GSD) is the ultimate super-resolution technique for localization microscopy with a precision of up to 20 nm. The Leica SR GSD system is an integrated solution offering the researcher the possibility to perform:

- Widefield GSD
- TIRF GSD

with
- cells grown on glass coverslips
- embedded tissue sections.

Furthermore, the Leica SR GSD system is a complete Widefield- and TIR-Fluorescence microscope equipped for standard imaging including live cell microscopy (incubator optional). Leica SR GSD is a useful tool for laboratories in a number of scientific research areas, including:

- Cell biology
- Neurobiology
- Virology
- Microbiology
- Biophysics
- Pharmacology
- Physiology

Leica SR GSD is a super-resolution technique based on single molecule localization. To build a high-resolution image the ensemble of overlapping fluorophores in the diffraction-limited image has to be temporally “separated” to allow the high precision detection of single dyes. Ground state depletion (GSD) can be achieved by using high power lasers to transfer fluorophores into long-lived dark states – a non-fluorescent molecule state. Stochastically, as soon as single fluorophores return from the dark state, new bursts of emitted photons are recorded and localized immediately by the system. The positions of these signals are used by the software to reconstruct a super-resolution image with a precision up to 20 nm “online”. This enables the researcher to see the formation of the (super-resolution) image during the acquisition time of typically 2-10 minutes, giving the possibility to optimize the acquisition parameters during the acquisition phase in order to increase the efficiency and maximize the image quality.
This manual is designed to guide researchers through the current specimen preparation methods for Leica SR GSD. All protocols discussed in this guide serve as a starting point to establish and perfect your own protocols.

Important: Read the instruction manual for your Leica SR GSD system and for all other products used in your work before following a protocol. Your lab may have specific protocols that differ from those in the guide. Leica Microsystems’ protocols do not supersede your protocols. This guide does not replace the instruction manual. Leica Microsystems does not guarantee safety and performance of another company’s products that it recommends, nor does it guarantee the safety or successful outcome of experiments using the suggested protocols for your specific work.

You are welcome to contribute to future editions of this guide by sharing your protocols with Leica Microsystems. Please submit the protocol or contact us at:

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We would also appreciate if you could provide us with your peer-reviewed publications.
2. Preparing Biological Specimen for Leica SR GSD

The Leica SR GSD is - in addition to super-resolution microscopy - a multi-purpose widefield system for transmitted light and fluorescence microscopy. In this protocol guide we will focus on preparation of samples used for super-resolution microscopy.

GSD is suitable for many samples. Cells grown on standard glass coverslips can be used after chemical fixation. Furthermore tissue slices can be prepared for GSD and placed on a glass coverslip. The samples are normally fluorescently labeled by immunostaining or other suitable techniques. One advantage of GSD is the broad range of fluorophores suitable for super-resolution imaging, including AlexaFluor® 488, AlexaFluor® 532, Rhodamine 6G, AlexaFluor® 647 and many more. The sample preparation for GSD is based on already established standard protocols but requires in general some optimizations to obtain the highest quality possible.

We will discuss the different protocols in detail in the following sections. Please note that single molecule detection as employed for GSD might have some extra requirements on purity of reagents and handling of samples as well as on the environmental conditions of your microscope. We will point out these requirements in this protocol guide within the protocol section and also in the troubleshooting section.

Important: All protocols serve only as a starting point and may need modifications to obtain the best results from your specimens. Once successfully established, protocols must be strictly followed to assure reproducibility of experiments. Any changes in specimen handling or reagent quality might affect the super-resolution GSD image.
3. Accessories

Coverslips and coverslip holder

Super-resolution imaging requires excellent optical performance throughout the whole optical system – not only within the microscope and objective. The coverslip is a major parameter with high impact to imaging results. To obtain optimal super-resolution image quality a flat coverslip is required. Unfortunately, while being uncritical for standard widefield applications, not all chambered coverslip products can guarantee the degree of flatness required for GSD. Furthermore, chambered coverslip products may not provide the necessary mechanical stability for high-resolution imaging. The respective products should be tested by the researcher for their suitability. A better flatness and high stability can be generally achieved by stress free mounting of a glass coverslip, e.g. in specially designed coverslip holders.

We recommend an alternative method for easy and flat coverslip mounting specifically tailored for GSD imaging. The method is described in Section 5 and has additional advantages for sample handling and GSD imaging which will also be discussed in Section 5.

Optional components

Large Volume Incubator

For live cell imaging the Leica SR GSD system can be equipped with an incubator (Large Volume Incubator BLX TIRF, #11532831). For maximum stability and drift reduction the temperature control of the chamber should not be used during GSD experiments. The switching of the heating element within the incubation chamber introduces small but fast temperature changes causing elevated drift of the GSD stage and reduces the super-resolution imaging capability dramatically.

The incubation chamber itself provides an additional thermal buffer around the microscope stage. Leica therefore recommends leaving an existing chamber installed on the microscope even for GSD imaging. In this case the temperature control should be deactivated. After use of the system in a temperature controlled mode it is necessary to equilibrate and stabilize the system. This might take a few hours.
4. Sample Preparation Protocols

4.1. Recommended Fluorophores for GSD

A wide range of organic and genetically encoded fluorophores are suitable for GSD. The inherent properties of each dye in combination with the direct environment of the fluorophor determine the ground state depletion capability as well as the single molecule return rate. Leica Microsystems has tested a number of fluorophores and recommends the following fluorophores (please note: the imaging buffer might differ between fluorophores, for details see section 5):

- AlexaFluor® 488, Atto 488, AlexaFluor® 532, Atto 532, AlexaFluor® 546, Atto 565, AlexaFluor® 647 and Rhodamine 6G
- YFP

For GSD imaging the specimen should be chemically fixed to avoid any movement during image acquisition. Leica Microsystems recommends the use of organic fluorescent dyes. In general, standard and established immunostaining protocols are a good starting point for super-resolution imaging.

We have compiled a list of general recommendations and some example staining protocols successfully used with Leica SR GSD microscopes in the following section. The protocols focus on staining the structure of interest with a sandwich of unlabeled primary antibody and fluorescently labeled secondary antibody. Of course, the use of fluorescently labeled primary antibodies and Fab-fragments increases the localization accuracy of the structure of interest due to the smaller distance between structure of interest and fluorophor. In general, the signal obtained from directly labeled antibodies and Fab fragments are somewhat weaker and a comparison to the signal strength obtained with fluorescently labeled secondary antibodies is advised to find the optimal compromise between number of localized fluorophores (“brightness”) and structure-representation (the distance of fluorophores to the epitope). Both parameters contribute to the maximal achievable resolution within the GSD-image.

If you intend to perform double-staining of your sample please check that your fluorophores can be imaged under the same GSD imaging conditions (Section 5) and crosstalk is negligible. Leica Microsystems recommends the combination of AlexaFluor® 532 and AlexaFluor® 647 for sequential dual-color imaging. Please image the red channel (AlexaFluor® 647) first. Leica does NOT recommend imaging the green/orange channel (AlexaFluor® 532) at the beginning to avoid bleaching of AlexaFluor® 647, which can be - although at very low levels - excited by a 532 nm laser. In addition, other fluorophore pairs (Atto 488 or AlexaFluor® 488 combined with AlexaFluor® 647) have proven excellent performance for dual-color imaging.

Genetically encoded fluorophores like YFP and other labeling technologies like SNAP-tag® are generally suited for GSD.
Note: Leica Microsystems is continuously screening for new fluorophores suitable for GSD and will add these fluorophores to the list as soon as sufficient testing is accomplished.

4.2. Immunofluorescence

Leica Microsystems recommends using your standard immunofluorescence protocol optimized for diffraction limited fluorescence microscopy to stain your structure of interest. A good specimen preparation with your standard protocol should already provide you with stunning GSD images. To obtain optimal GSD images it might be necessary to increase the concentration of primary and secondary antibodies - compared to your standard labeling protocol - to increase the labeling density. Increasing the labeling density ensures that the structure of interest is sufficiently stained with fluorophores. A sparse labeling of structures can lead to a point-like appearance of your image.

In order to reduce unspecific background signals in the GSD image, it might be necessary to increase the time for blocking your sample as well as the concentration of the blocking reagent. It is advised to wash your samples thoroughly (e.g. by increasing the number and time of washing steps).

These parameters have to be carefully determined for each antibody by the researcher.

To use the recommended standard mounting protocol (see section 5) the researcher should seed the cells onto round glass coverslips with a diameter of 18-22 mm (rectangular coverslips are also suitable, but only the center portion can be efficiently used for GSD-imaging).

Protect fluorescently labeled antibodies and samples from light. Perform longer incubation periods in a humid chamber.

Protocols for immunostaining successfully used by researchers performing super-resolution imaging with the Leica SR GSD system are listed in the following.

Please note, that the preservation of a specific cellular structure strongly depends on the fixation method used. The exact fixation protocol should be optimized for your structure of interest. For the majority of structures a Paraformaldehyde / Formaldehyde fixation is sufficient. Small amounts of Glutaraldehyde (0.05% to 0.2% (v/v)) in addition to Paraformaldehyde / Formaldehyde as a fixant can strongly improve structure preservation. Glutaraldehyde can induce a hazy fluorescent background therefore quenching with ammonium, NaBH₄ or other suitable reagents is recommended. Methanol fixation is common for microtubule preservation, but might not be sufficient for other structures.
Protocol 1

Fixation with Paraformaldehyde using a two-step process improving epitope recognition for some proteins (cell culture)

Reagents:
- PBS
- 2% Paraformaldehyd (PFA) in PBS
- 0.1% Triton in PBS
- Bovine Serum Albumin (BSA)
- FETAL Bovine Serum (FBS)

Procedure:

All steps are performed at room temperature.

1. Aspirate the cell culture medium
2. Rinse coverslips with 3x with PBS
3. Pre-fix the cells with 2% PFA in PBS for 20 sec
4. Rinse 1x with PBS
5. Pre-extract cells with 0.1% Triton in PBS for 3 min
6. Rinse 1x with PBS
7. Fix cells with 2% PFA in PBS for 15 min.
8. Rinse 3x times with PBS
9. Wash 3x 5min with PBS
10. Permeabilize cells with 0.1% Triton in PBS for 10 min
11. Rinse 3x with PBS
12. Block 1 hour at room temperature with 2% BSA and 2% FBS in PBS
13. Incubate with primary antibody 1hour at RT or overnight at 4°C
14. Wash 3x 5 min with PBS
15. Secondary 1hr at RT
16. Wash 3x 5min

Store samples at 4°C in PBS. Please note that sample quality might degrade over time and the maximum time of storage before super-resolution imaging should be determined by the researcher. In general, storage overnight is uncritical. Some samples are still usable for GSD after two weeks of storage.
Protocol 2

Fixation with Methanol (cell culture)

Reagents:

- Methanol (MeOH)
- PBS
- Bovine Serum Albumin (BSA)

Procedure:

All steps are performed at room temperature.

1.) Remove the cell culture medium
2.) Fix the cells with cold (-20 °C) MeOH for 4 minutes
3.) Rinse 3 x 1% BSA in PBS
4.) Wash the cells with 1% BSA in PBS for 3 min and repeat 3 x
5.) Incubate for 30-60 minutes with 1% BSA in PBS
6.) Incubate with primary antibody for 60-120 minutes diluted in 1-5% BSA in PBS
   (BSA concentrations might vary for different antibodies)
7.) Rinse coverslips 3 x with PBS
8.) Wash antibodies 3 x with 1% BSA in PBS
9.) Incubate with secondary antibody for 60 minutes diluted in 1-5% BSA in PBS (BSA concentrations might vary for different antibodies)
10.) Rinse coverslips 3 x with PBS
11.) Wash antibodies 3 x with 1% BSA in PBS

Store samples at 4 °C in PBS. Please note that sample quality might degrade over time and the maximum time of storage before super-resolution imaging should be determined by the researcher. In general, storage overnight is uncritical. Some samples are still usable for GSD after two weeks of storage.
Protocol 3

Fixation with Paraformaldehyde (PFA) including post-fixation (cell culture)

Reagents:
- 4% Paraformaldehyde (PFA) in PBS
- PBS
- PBS++: PBS with 100 mM MgCl₂ and 100 mM CaCl₂
- Triton X-100
- Saponin
- Bovine Serum Albumin (BSA)

Procedure:

All steps are performed at room temperature.

1.) Wash cells 3 x with PBS ++
2.) Fix the cells in 4% PFA for 20 min. at room temperature
3.) Wash 3 x with PBS ++
4.) Incubate 10 min with 0.1% Triton X-100 in PBS
5.) Wash 3 x with PBS ++
6.) Block with 1% BSA and 0.025% Saponin in PBS for 30 minutes in a humid chamber
7.) Incubate with primary antibody diluted in PBS containing 0.025% Saponin and 1% BSA. The exact dilution of the antibody has to be determined by the researcher.
8.) Wash 5 x with PBS ++
9.) Incubate with secondary antibody diluted in 1% BSA for 1 h
10.) Wash 5 x with PBS ++
11.) Fix cells for 10 min with 2% PFA in PBS (post-fix prevents any dissociation of secondary antibody)
12.) Wash 3 x with PBS ++

Store samples at 4 °C in PBS or PBS++. Please note that sample quality might degrade over time and the maximum time of storage before super-resolution imaging should be determined by the researcher. In general, storage overnight is uncritical. Some samples are still usable for GSD after two weeks of storage.
Protocol 4

Fixation with Methanol (including post-fixation – cell culture)

Reagents:

- Methanol (MeOH)
- PBS
- PBS++: PBS with 100 mM MgCl$_2$ and 100 mM CaCl$_2$
- Triton X-100
- Saponin (optional)
- Bovine Serum Albumin (BSA)

Procedure:

All steps are performed at room temperature.

1.) Wash cells 3 x with PBS ++
2.) Fix the cells with cold (-20 °C) MeOH for 6 minutes
3.) Wash 3 x with PBS ++
4.) Incubate the cells for 10 min with 0.1 % Triton X-100 in PBS
5.) Wash 3 x with PBS ++
6.) Block unspecific binding sites and increase accessibility of epitopes by incubation with 1% BSA and 0.025% Saponin (optional) in PBS++ for 30 min
7.) Incubate with primary antibody diluted in 1% BSA and 0.025% Saponin for 2 hours
8.) Wash 5 x with PBS ++
9.) Incubate with secondary antibody diluted in 1% BSA for 1 h
10.) Wash 5 x with PBS ++
11.) Fix cells with 10 min with 2% PFA in PBS (post-fix prevents any dissociation of secondary antibody)
12.) Wash 5 x with PBS ++

Store samples at 4 °C in PBS or PBS++. Please note that sample quality might degrade over time and the maximum time of storage before super-resolution imaging should be determined by the researcher. In general, storage overnight is uncritical. Some samples are still usable for GSD after two weeks of storage.

Note: BSA might be replaced by milk powder as a blocking reagent, if microtubules are stained with the monoclonal antibody anti-α-tubulin, clone DM1A (Sigma-Aldrich, # T9026).
5. Fluorophores and compatible embedding media

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*Note: Embedding Medium 4 has been reported to be suitable for tissue sections. Testing of LR-White is ongoing and full performance cannot be guaranteed until the tests are completed.

Aqueous embedding media:

- **Embedding Medium 1 - MEA in PBS**
  PBS containing 10 mM β-Mercaptoethylamine (MEA) (e.g. Sigma-Aldrich, # 30070, also known as Cysteamine) adjusted to pH 7.4. The MEA concentration can be varied over a wide range to optimize the GSD image – the usable range is in general between 10 and 100 mM (recommend concentrations 10 / 30 /100 mM).
  **MEA solutions must be freshly prepared before use.** Alternatively, stock solutions can be prepared and stored at -20°C and freshly thawed before use. The frozen aliquots can generally be stored for several weeks.

- **Embedding Medium 2 – glucose-oxidase mix**
  PBS containing 10% (w/v) glucose, 0.5 mg/mL glucose oxidase (e.g. Sigma-Aldrich G2133) and 40 µg/mL catalase (e.g. Sigma-Aldrich C1345) adjusted to pH 7.4.
  Note: It is favourable to prepare a glucose stock solution and mix the reagents freshly before mounting the coverslip.
Hardening Embedding Media:

- **Embedding Medium 3 - PVA**
  Prepare a solution of 1% PVA (Polyvinyl alcohol / MW 25,000, 88 mol% hydrolyzed, e.g. Polysciences, #02975-500) in PBS (adjusted to pH 7.4). Place the coverslip on the spincoater and dry the coverslip with brief 5-10 sec spin at approx. 3000 rpm. Drop 50 µl of the PVA-solution onto the sample and spin the sample at approx. 3000 rpm for approximately 30s. Let the PVA-film dry for a few minutes and mount the sample (see Section 6). The sample can be stored at room temperature and used for GSD imaging for up to three months. No additional mounting medium is required. The PVA-film protects the sample.

- **Embedding Medium 4 – LR-White**
  The embedding medium LR-White should be suitable for imaging tissue sections (typically 50 - 1000 nm) with GSD. Please follow the respective standard protocol for embedding and sectioning of tissue.

### 5.1. Mounting stained samples for GSD

Leica Microsystems recommends to mount the coverslip with the sample directly on depression slides (e.g. depression slide 76x26 mm, neoLab® # 1-6293). The coverslip should be fixed to the depression slide and sealed with two-component silicone-glue Twinsil® (Picodent, Wipperfürth, Germany, #13001000). Twinsil® is non-toxic, fast hardening and can be later on easily removed, e.g. to re-mount the sample. Nail polish is less suitable because it contains organic solvents.

The suggested mounting method has the following advantages:

1) The sample is fixed on the supportive glass coverslip and bending of the coverslip is avoided. Bending of the coverslip impairs the optical system and reduces the quality of the GSD image.

2) The depression slide can be easily fixed on the stage practically abolishing drift of the mounted sample.

3) **For aqueous imaging buffers:** The imaging buffer is protected from oxygen avoiding the oxidation of the reducing agent MEA. Therefore the sample can be used for GSD imaging for an extended period (up to 6 hours). Note: Re-mounting of the coverslip is possible to further extend the lifetime of the sample by adding fresh, un-oxidized buffer.
5.2. Mounting procedure:

1.) Only for imaging with aqueous embedding media (Media 1 (MEA in PBS) & 2 (Glucose-Oxidase-Mix)): Add 75 µl of embedding medium into the cavity of a clean depression slide. For embedding media 3 (PVA) & 4 (LR-White): leave the cavity of the depression slide empty.

2.) Place the glass coverslip with your sample onto the cavity of the depression slide. The sample should face the cavity. The coverslip should cover the depression completely. If aqueous embedding medium is filled in the cavity, ensure that no air bubbles are present. Otherwise carefully remove the coverslip and repeat the procedure.

3.) No liquid should be present at regions, which should be covered by the glue (Twinsil®). Carefully remove remaining liquid with filter paper. For Embedding Media 1 & 2: Ensure that no buffer is soaked out from the reservoir.

4.) Mix the yellow and blue component of Twinsil® in a ratio of 1+1 thoroughly and apply it to the edges of the glass coverslip without directly touching the glass coverslip. For Embedding Media 1 & 2: Seal the coverslip completely with Twinsil®. For Embedding Media 3 & 4: Seal the coverslip to approx. 75% and leave the remaining quarter open to allow ventilation and avoid condensation on the sample.

5.) After 5-10 minutes the two-component glue is hardened and the sample is ready for GSD imaging.

The glue can be easily removed without leaving traces on the glass after hardening (e.g. to exchange the imaging buffer).
6. Preparing tissue sections for GSD

Leica Microsystems SR GSD enables the researcher to use (thin) sections of resin-embedded cells and tissue (e.g. in LR-White, Section 5) for super-resolution fluorescence microscopy. Please follow your standard protocol to embed the samples into the resin, cut the sample into the respective slices (usually between 50-1000 nm) and carefully place them on glass coverslip. Perform the immunostaining on the glass coverslip using your proprietary protocol and mount the coverslip onto a depression slide (as described in section 5).

The same protocol can be generally used for electron microscopy and super-resolution fluorescence microscopy samples. Please keep in mind that glutaraldehyde fixation might induce high fluorescent background in samples. As an alternative a mixture of paraformaldehyde / formaldehyde and glutaraldehyde can be used.
7. Troubleshooting

7.1. During GSD image acquisition the number of single molecules detected is decreasing quickly and cannot be increased by changing the power of the GSD laser or the 405 nm laser – the image appears “pointillist”

- Check the recommended combination of embedding medium and fluorophor (Section 5).

- The staining in the widefield image should be easily visible without high laser power and “noise” free. If not, try to improve the staining density by optimizing the antibody concentration during staining. To exclude other causes (e.g. the ones listed below), image a well-known reference sample in parallel (e.g. a microtubule stain).

- For embedding media 1 & 2: Check the quality of components (especially MEA and enzymes). In doubt use fresh batches. MEA is hygroscopic and can degrade already as a powder if not protect from humid air. Store MEA in a desiccator at 2 to 8°C if necessary.

- Check pH of buffers and working solutions

- For embedding medium 1: Compare different MEA concentrations

- For embedding medium 1: Check pH of imaging buffer after adding MEA
7.2. The GSD image does not reflect the structure in the widefield image; the GSD image appears “noisy”

Contamination of imaging buffer with freely diffusing fluorescent contamination or dye molecules, released from the sample:

- If you are not using a recommended dye, please consider the possibility that the dye might not be stably bound for prolonged time periods and might be released from the sample. Compare your dye to a proven and recommended dye before taking any further measures.

- Check imaging buffers for contamination. It might help to filter the buffers. Otherwise test all reagents systematically and replace the source of contamination. If the water is the source of contamination, consider to use commercially available water (e.g. for HPLC or Spectroscopy).

- If the imaging buffer is not the source of contamination, wash your samples (more) thoroughly before GSD imaging to remove unbound fluorescently labeled antibodies. If post-fixation after immunolabelling was not included in the sample preparation consider to do so.
7.3. Clearly defined structures in the diffraction limited widefield image are “smeared” in GSD image

Drift of the sample is the main cause of (directional) “smearing” and broadening of otherwise distinct structures. The resolution of the GSD image is not improved or even worse compared to the widefield image. Drift might be caused by temperature fluctuations in the room, vibrations and not tightly mounted samples.

Vibrations:
- Check the anti-vibration equipment, e.g. if the active vibration table is activated and properly working.
- Identify and remove or deactivate the vibration source – please consider that vibrations might be linked to temporary construction work in the building. If the source cannot be identified and other causes (see below) can be excluded, consider to repositioning the microscopy system e.g. to a new room (e.g. basement).

Thermal fluctuations: Fast temperature changes are the main cause of drift.
- Position the microscope away from any fast-changing source of temperature fluctuations or airflow (e.g. in the proximity of doors). It might be necessary to fine-tune air-conditioning and / or guide the air flow away from the GSD system. Alternatively or in combination, use the Large Volume Incubator BLX TIRF (#11532831) to create an additional thermal buffer around the GSD-stage.
- The microscope stand should not be switched off overnight if possible. After switching on the microscope stand it takes up to three hours to equilibrate the system and during that time severe sample drift can occur.
- Bead fixed to a glass coverslip or other reference probes can be used to control for drift by taking time-lapse series in widefield mode for extended periods. It is advised to check temperature changes at the microscope in parallel to identify a possible correlation.
Sample drift: It might be that the sample is not tightly coupled to the microscope stage.
- Check that the sample on the glass coverslip is tightly connected to the stage and no movement of any component is possible. If you are not using the standard mounting protocol for GSD, we would like to suggest testing it. If you are already using it, ensure that the Twinsil® glue is correctly hardened.

Bubbles in the immersion Oil: Sometimes moving air bubbles in the immersion oil can lead to an effective sample drift - especially in axial, but also in lateral direction. These bubbles may not be seen by eye. Simply remove the immersion oil completely from sample and objective with a suitable tissue and apply a new drop of oil.
7.4. **Strong fluorescence structures are still visible after extended pumping period (tips valid for mammalian cells)**

Cellular autofluorescence is defined here as fluorescence not brought into the sample by actively incorporating fluorophores and therefore present in the sample before adding any fluorescent labeling reagents. Cells can exhibit a strong autofluorescence (e.g. lipofuscin-like) that prevents detection of single molecule fluorescence signals. This kind of fluorescence might be hard to bleach and is often multi-spectral. Cellular autofluorescence should not display strong if any single molecule return behavior (“blinking”).

If you are using cell lines:
- Thaw a new aliquot of cells and try to reproduce the results. Anecdotal reports suggest that changing the batch of serum and / or using phenol red free medium might improve the situation. The cells should be grown from the time point of thawing on in the new cell culture medium.
- Screen different cell lines to find a cell line with similar properties, not displaying any cellular autofluorescence.
- Use a fluorophore with different spectral properties compared to the cellular autofluorescence, e.g. if the autofluorescence is strong in the green, try to use a red fluorophore.

If you are using primary cells:
- Use a fluorophore with different spectral properties than the cellular autofluorescence, e.g. if the autofluorescence is strong in the green, try to use a red fluorophore.
- Screening for different culture conditions might be less effective, because the autofluorescence might be already induced in the host organism.

Autofluorescence can be induced by different fixation and sample preparation procedures.
- Staining with glutaraldehyde can induce severe background fluorescence. Try to quench e.g. with Ammonium, and / or reduce the amount glutaraldehyde by using a mixture of paraformaldehyde / formaldehyde and glutaraldehyde (e.g. 3.8% formaldehyde and 0.2% glutaraldehyde).
7.5. Bright structures in the widefield image appear dark in the GSD image

![Widefield](image1)
![Expected GSD](image2)
![Obtained GSD](image3)

The GSD image is an accumulation of individually and precisely localized single fluorescent molecules. To ensure that only *bona fide* single molecules are localized, the software algorithm employs a number of (partially user adjustable) parameters to reject spurious events. For example, events that overlap in one frame are ignored to avoid imprecise localization of the fluorophores. Rejected, overlapping events can therefore “mask” a real, strongly stained structure leading to the phenomenon of a “Black hole” in GSD image - in clear contrast to the well visible structure in the Widefield image.

Not all molecules have been pumped into the dark state and therefore a high background of existing fluorophores leads to overlapping events.

- Increase laser power and / or time of the pumping step.
- Reduce exposure time.

The structure is labeled with a too high density of fluorophores, therefore more than one molecule within a resolvable spot returns from the dark state in one frame. Overlapping fluorescence signals are rejected by the software.

- Reduce labeling density of the structure by decreasing the primary and secondary antibody during immunostaining.