



Sample Preparation

for

Ground State Depletion (GSD)

Super-resolution imaging

Protocol guide for Leica SR GSD 3D system

Version 2.0

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 3D system is an integrated solution offering the researcher the possibility to perform:

- | | | |
|---|------|--|
| <ul style="list-style-type: none">• Widefield GSD 2D• Widefield GSD 3D• TIRF GSD 2D• TIRF GSD 3D | with | <ul style="list-style-type: none">• Cells grown on glass coverslips• Embedded tissue sections |
|---|------|--|

Furthermore, the Leica SR GSD 3D system is a complete Widefield- and TIR-Fluorescence microscope equipped for standard imaging including live cell microscopy (incubator optional). Leica SR GSD 3D 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.

With the Leica SR GSD 3D, super-resolution, image acquisition is not restricted to two dimensions (x and y) only, but can also be achieved for the third dimension (z). This advantage bases on the usage of an automatically positioned cylinder lens into the emission beam path of the system, leading to an astigmatism. The distortion of the Point Spread Function (PSF) can be used to calculate the original position of the respective fluorescent molecule. The 2D and 3D mode can be switched conveniently with one “click” by the software.

This manual is designed to guide researchers through the current specimen preparation methods for Leica SR GSD 3D. All protocols discussed in this guide serve as a starting point to establish and perfect your own protocols.

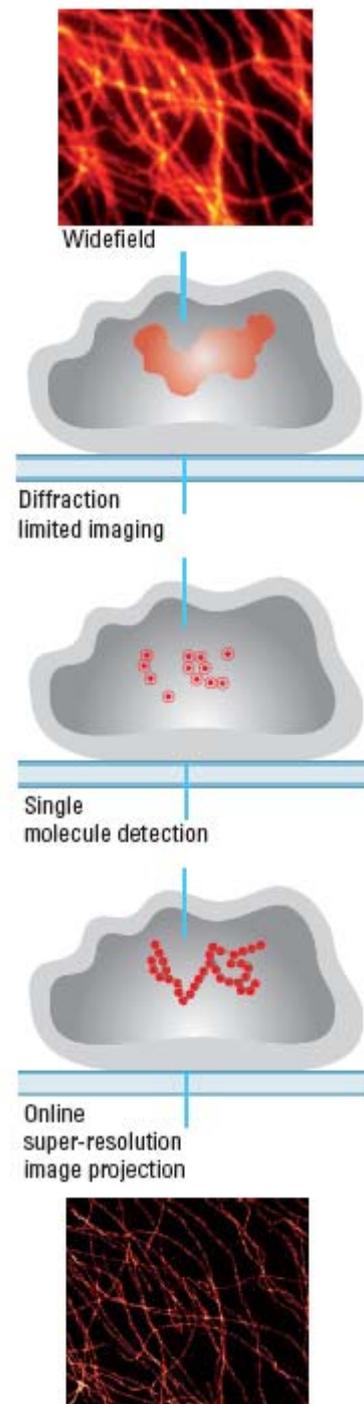
Important: Read the instruction manual for your Leica SR GSD 3D system and for all other products used in your work before following a protocol.

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: Tamara.Straube@leica-microsystems.com

We would also appreciate, if you could provide us with your peer-reviewed publications.



The Leica Science Lab offers an interactive 3D-GSD tutorial:
<http://www.leica-microsystems.com/science-lab/3d-localization-microscopy-with-ground-state-depletion-gsd/>



2. Preparing Biological Specimen for Leica SR GSD 3D

The Leica SR GSD 3D 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. Note: Sample preparation for 2D and 3D imaging are identical.

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® 555, 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

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 (f. e. ibidi® chamber, Lab-Tek™, WilCo-dish®) 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 6** and has additional advantages for sample handling and GSD imaging. The following products proofed to be applicable for this purpose.

3.1. Coverslips

- Coverslip 1.5 high precision 18 mm round, tolerance +/- 5µm, made by Marienfeld Superior (Art. No: 0117580).
- Coverslip 1.5 high precision 18 x 18 mm rectangular, tolerance +/- 5µm, made by Marienfeld Superior (Art. No: 0107032).

3.2. Depression Slides

Slide size: 76 x 26 x 1.20 – 1.50 mm
Depression depth: 0.6 – 0.8 mm
Depression diameter: 15 – 18 mm

- NeoLab (Art. No: 1 – 6293)
- Marienfeld Superior (Art. No: 1320000)

3.3. Silicone-Glue - Twinsil®

- Picodent (Art. No: 13001000)



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 – **the embedding medium** – of the fluorophore 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 ones (please note: the embedding media might differ between fluorophores, for details see section 5):

- **AlexaFluor® 488, Atto 488, AlexaFluor® 532, Atto 532, AlexaFluor® 546, AlexaFluor® 555, 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. In order to prevent a potential background, provoked by the back pumping laser, please avoid usage of DAPI/Hoechst dyes.

We have compiled a list of general recommendations and some example staining protocols successfully used with Leica SR GSD 3D 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 fluorophore. 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 or AlexaFluor® 555 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 or AlexaFluor® 555) 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 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 3D 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 – Two-Step Process (cell culture)

A two-step process might improve epitope recognition.

Reagents:

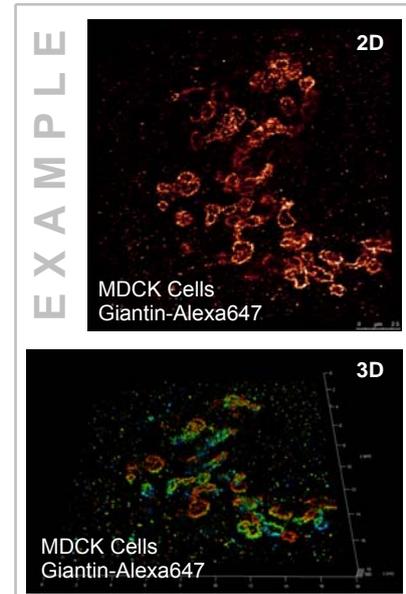
- Phosphate Buffered Saline (PBS)
- 2% Paraformaldehyd (PFA) in PBS
- 0.1% Triton in PBS
- Bovine Serum Albumin (BSA)
- Fetal Bovine Serum (FBS)

Procedure:

All steps are performed at room temperature in a wet chamber.

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

Fixation with Methanol (cell culture)

Reagents:

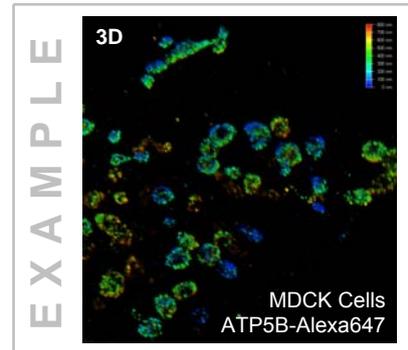
- Methanol (MeOH)
- PBS
- PBS++ (PBS with 100 mM MgCl₂
- and 100 mM CaCl₂)
- Bovine Serum Albumin (BSA)

Procedure:

All steps are performed at room temperature in a wet chamber.

- 1.) Rinse 3x with PBS++
- 2.) Fix the cells with cold (-20°C) MeOH for 4 min
- 3.) Rinse 3x with 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 min with 1% BSA in PBS
- 6.) Incubate with primary antibody for 60-120 min diluted in 1-5% BSA in PBS (BSA concentrations might vary for different antibodies)
- 7.) Rinse coverslips 5x with PBS++
- 8.) Incubate with secondary antibody for 60 min diluted in 1-5% BSA in PBS (BSA concentrations might vary for different antibodies)
- 9.) Rinse coverslips 5x with 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 2 b

Fixation with Methanol (cell culture)

Reagents:

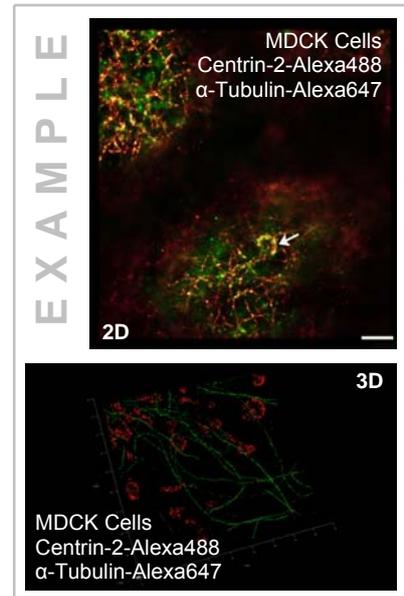
- Methanol (MeOH)
- PBS++ (PBS with 100 mM MgCl₂ and 100 mM CaCl₂)
- Milk powder (enhances cytoskeleton staining)

Procedure:

All steps are performed at room temperature in a wet chamber.

- 1.) Rinse 3x with PBS++
- 2.) Fix the cells with cold (-20°C) MeOH for 4 min
- 3.) Rinse 3x with 1% milk powder in PBS
- 4.) Wash the cells with 1% milk powder in PBS for 3 min and repeat 3 x
- 5.) Incubate for 30-60 min with 1% milk powder in PBS
- 6.) Incubate with primary antibody for 60-120 min diluted in 1% milk powder in PBS
- 7.) Rinse coverslips 5x with PBS++
- 8.) Incubate with secondary antibody for 60 min diluted in PBS
- 9.) Rinse coverslips 5x with 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 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 in a wet chamber.

- 1.) Wash cells 3x 5 min with PBS++
- 2.) Fix the cells in 4% PFA for 20 min at room temperature
- 3.) Wash 3x 5 min with PBS++
- 4.) Incubate 10 min with 0.1% Triton X-100 in PBS
- 5.) Wash 3x 5 min with PBS++
- 6.) Block with 1% BSA and 0.025% Saponin in PBS for 30 min 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 5x 5 min with PBS++
- 9.) Incubate with secondary antibody diluted in 1% BSA for 1 h
- 10.) Wash 5x 5 min with PBS++
- 11.) Fix cells for 10 min with 2% PFA in PBS (**post-fixation** prevents any dissociation of secondary antibody)
- 12.) Wash 3x 5 min 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 a

Fixation with Methanol including post-fixation (cell culture)

Reagents:

- Methanol (MeOH)
- PBS
- PBS++ (PBS with 100 mM MgCl₂ and 100 mM CaCl₂)
- Triton X-100
- Saponin (optional)
- Bovine Serum Albumin (BSA)

Procedure:

All steps are performed at room temperature in a wet chamber.

- 1.) Wash cells 3x 5 min with PBS++
- 2.) Fix the cells with cold (-20°C) MeOH for 6 min
- 3.) Wash 3x 5 min with PBS++
- 4.) Incubate the cells for 10 min with 0.1 % Triton X-100 in PBS
- 5.) Wash 3x 5 min 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 5x 5 min with PBS++
- 9.) Incubate with secondary antibody diluted in 1% BSA for 1 h
- 10.) Wash 5x 5 min with PBS++
- 11.) Fix cells with 10 min with 2% PFA in PBS (**post-fixation** prevents any dissociation of secondary antibody)
- 12.) Wash 5x 5 min 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 b

Fixation with Methanol including post-fixation (cell culture)

Reagents:

- Methanol (MeOH)
- PBS
- PBS++ (PBS with 100 mM MgCl₂ and 100 mM CaCl₂)
- Triton X-100
- Saponin (optional)
- Milk powder (enhances cytoskeleton staining)

Procedure:

All steps are performed at room temperature in a wet chamber.

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

5. Fluorophores and compatible embedding media

5.1. Embedding Medium 1 - MEA in PBS

Reagents:

- β -Mercaptoethylamine (MEA) (e.g. Sigma-Aldrich, # 30070, also known as Cysteamine)
- Phosphate Buffered Saline (PBS)
- HCl / NaOH for pH adjustment

Buffer:

PBS containing 10 mM β -Mercaptoethylamine (MEA) adjusted to pH 7.4.

Dissolve the β -Mercaptoethylamine in PBS and adjust 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.

Illumination: EPI and TIRF

Compatible Fluorophores:

Laser [nm]	Dye	Manufacturer
488	AlexaFluor® 488	life technologies
	Atto 488	Sigma
	chromeo 488	Active Motif
	oregon Green 488	life technologies
	chromeo 505	Active Motif
532	Atto 520	Sigma
	AlexaFluor® 532	life technologies
	AlexaFluor® 555	life technologies
	AlexaFluor® 568	life technologies
642	Atto 633	Sigma
	AlexaFluor® 647	life technologies
	Atto 647n	Sigma
	Atto 655	Sigma
	AlexaFluor® 680	life technologies
	AlexaFluor® 700	life technologies

5.2. Embedding Medium 2 – Glucose-Oxidase Mix

Reagents:

- Glucose Oxidase (e.g. Sigma-Aldrich G2133)
- Catalase (e.g. Sigma-Aldrich C1345)
- Glucose
- Phosphate Buffered Saline (PBS)
- HCl / NaOH for pH adjustment

Buffer:

PBS containing 10% (w/v) glucose, 0.5 mg/mL glucose oxidase and 40 µg/mL catalase adjusted to pH 7.4.

Note: It is recommended to prepare a glucose stock solution and mix the reagents freshly before mounting the coverslip.

Illumination: EPI and TIRF

Compatible Fluorophores:

Laser [nm]	Dye	Manufacturer
532	Atto 532	Sigma
	AlexaFluor® 532	life technologies
	AlexaFluor® 555	life technologies
	Rhodamine 6G	Active Motif
	Atto565	Sigma
642	AlexaFluor® 568	life technologies
	Atto 655	Sigma
	AlexaFluor® 680	life technologies

Embedding Medium for YFP

The following aqueous embedding was successfully used to image YFP in fixed and permeabilized mammalian cells:

- Catalase SIGMA, C1345 40 µg/mL
- Glucose Oxidase SIGMA G2133 0.5 mg/mL
- Glucose 10 mg/mL
- β-Mercaptoethylamine (Cysteamine) 1 mM

Diluted in PBS, adjusted to pH 7.4.

5.3. Embedding Medium 3 – PVA

Reagents:

- Polyvinyl alcohol (PVA) with a molecular weight of 25,000; 88 mol% hydrolyzed, e.g. Polysciences, #02975-500
- Phosphate Buffered Saline (PBS)
- HCl / NaOH for pH adjustment

Hardware:

Spincoater

Buffer:

Prepare a solution of 1% PVA in PBS. Adjust the pH to 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.

Illumination: EPI (TIRF not possible)

Compatible Fluorophores:

Laser [nm]	Dye	Manufacturer
488	AlexaFluor® 488	life technologies
	Atto 488	Sigma
	chromeo 488	Active Motif
	oregon Green 488	life technologies
532	Atto 520	Sigma
	AlexaFluor® 532	life technologies
	AlexaFluor® 680	life technologies

5.4. Embedding Medium 4 – Mowiol

Reagents:

- Glycerol (analytical grade)
- Mowiol 4-88 (e.g. Calbiochem #475904)
- Distilled water
- TRIS
- HCl / NaOH for pH adjustment

Embedding Medium:

- Start with 6 g Glycerol (analytical grade)
- Add 2.4 g Mowiol 4-88 (Calbiochem #475904)
- Add 6 ml Aqua dest.
- Add 12 ml 0.2 M TRIS buffer pH 8
- Stir for 4 hours (magnetic stirrer)
- Let suspension rest for 2 hours
- Incubate for 10 min at 50 °C (water bath)
- Centrifuge at 5000 g for 15 min
- Take the supernatant and freeze it in aliquots at -20 °C

Illumination: EPI (TIRF not possible)

Compatible Fluorophores:

Lasers [nm]	Dye	Manufacturer
488	Atto 488	Sigma
	chromeo 505	Active Motif
532	AlexaFluor® 532	life technologies
	Rhodamine 6G	Active Motif
	Atto 647n	Sigma
642	Atto 655	Sigma
	AlexaFluor® 700	life technologies

5.5. Embedding Medium 5 - Vectashield®

Reagents:

- Vectashield® (e.g. Vector Laboratories, # H-1000)
- 50mM Tris in Glycerol
- HCl/NaOH for pH adjustment

Buffer:

50mM Tris/Glycerol buffer containing 10 % Vectashield® adjusted to pH 7.4.

Dissolve Tris in Glycerol to prepare a 50 mM stock solution. Mix 10% Vectashield® with 90% Tris/Glycerol buffer. Adjust to pH 7.4. Samples can be stored over **several weeks** at 4°C.

Illumination: EPI (TIRF not tested yet)

Compatible Fluorophores:

Lasers (nm)	Dye	Manufacturer
532	AlexaFluor®555	life technologies
642	Alexa Fluor®647	life technologies

Leica FluoScout™

For more details concerning the combination of fluorescence filter cubes, fluorophores and light sources please visit our online tool:



<http://www.leica-microsystems.com/applications/fluorescence/learn-more-about-the-leica-fluoscout/>



5.6. Embedding Medium 6 – 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.

Note: Embedding Medium 6 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.

6. 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 the 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:

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

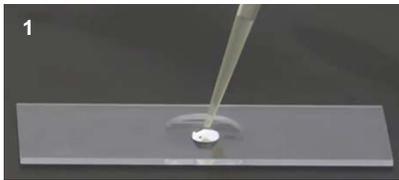
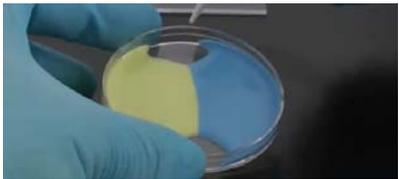
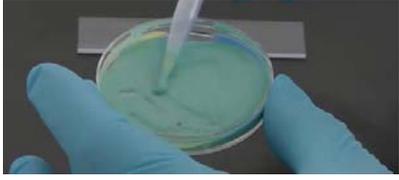
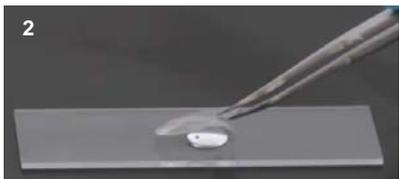
6.1. Mounting Procedure

- 1.) Add 90 µl of aqueous embedding medium (MEA, Glucose-Oxidase-Mix or Vectashield®) into the cavity of a clean depression slide.
(For embedding media PVA and 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.
- 3.) 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.
- 4.) 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 and 5: Ensure that no buffer is soaked out from the reservoir.
- 5.) Mix the yellow and blue component of Twinsil® in a quantity of 1+1 thoroughly and apply it to the edges of the glass coverslip without directly touching the coverslip.
For aqueous embedding media: Seal the coverslip completely with Twinsil®.

For embedding media PVA and LR-White: Seal the coverslip to approx. 75% and leave the remaining quarter open to allow ventilation and avoid condensation on the sample.

- 6.) 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).

		MOUNTING PROCEDURE	
<p>Pipet approx. 90 µl of liquid embedding medium (e.g. MEA solution) into the cavity of the depression slide.</p>	 <p>1</p>	<p>Mix the two components – hardening of <i>twinsi</i>® starts with mixing.</p>  	
<p>Place the round coverslip - holding the immunostained sample - onto the medium and avoid the formation of air bubbles.</p>	 <p>2</p>		
<p>Gently press onto the coverslip to remove air bubbles (if necessary) and excess of buffer.</p>	 <p>3</p>		
<p>Carefully remove liquid without soaking buffer from the reservoir in the depression slide.</p>	 <p>4</p>		
<p>Seal the coverslip by carefully applying <i>twinsi</i>®. Do not touch or move the coverslip!</p>	 <p>5</p>		
<p>When the hardened (approx. 5 min) <i>twinsi</i>® is touched gently with a tip, it will not stick nor can it be pushed aside.</p>	 <p>6</p>		



See the movie at <http://www.leica-microsystems.com/science-lab/video-tutorial-how-to-optimize-sample-preparation-for-gsd-microscopy/> or scan QR code.

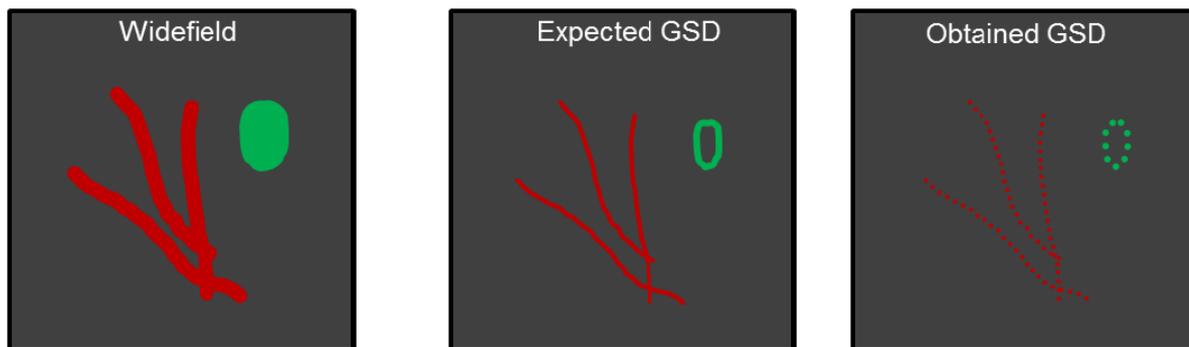
7. Preparing tissue sections for GSD

Leica Microsystems SR GSD 3D 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 a 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.

8. Troubleshooting

- 8.1. 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 fluorophore (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

8.2. 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.

8.3. 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.

8.4. 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.

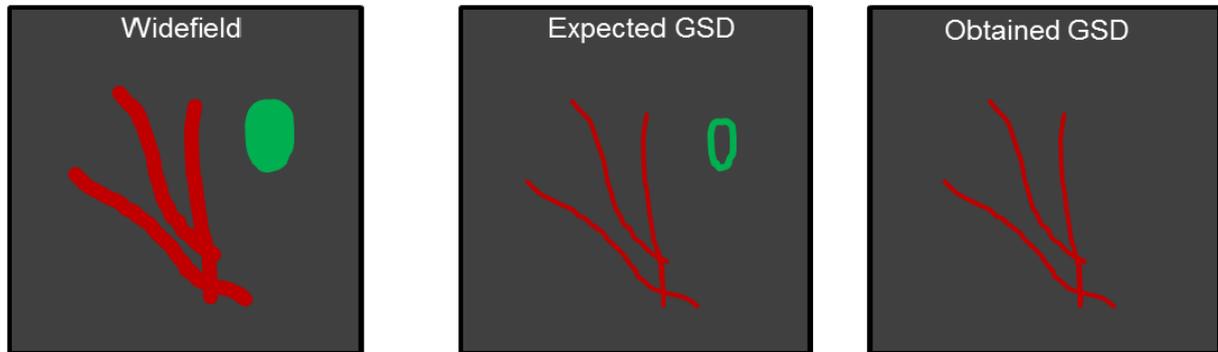
If you are using primary cells:

- 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 of glutaraldehyde by using a mixture of paraformaldehyde / formaldehyde and glutaraldehyde (e.g. 3.8% formaldehyde and 0.2% glutaraldehyde).

8.5. Bright structures in the widefield image appear dark in the GSD image



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.

