



NanoBioImaging

Super-Resolution Microscope

SRiS 2.0

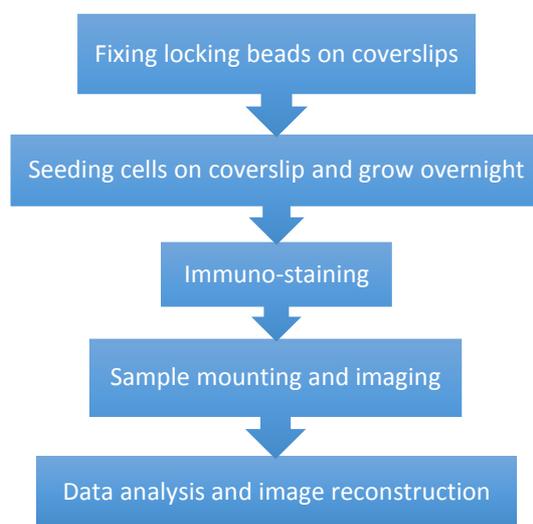
User Manual

Sample Preparation for Super Resolution Microscope

General Guidelines for Super Resolution Imaging System (SRiS)

The SRiS technique is a super-resolution imaging method that uses sequential activation and high-precision localization of individual fluorophore to achieve sub-diffraction-limit resolution. This manual is designed to guide researchers through the validated specimen preparation methods for NBI SRiS. Your lab may have specific protocols that differ from those in the guide. All protocols discussed in this guide serve as a starting point to establish and perfect your own protocols.

Flowchart of Sample Preparation and Imaging



NBI SRiS has a unique active locking system to remove the sample drift in x-y axis. For tracking the motion of sample, polystyrene beads need to be fixed on the coverslips before seeding cells. The biological structure of interest needs to be labeled with photoswitchable probes. In general, your standard immunofluorescence protocol optimized for diffraction limited fluorescence microscopy to stain your structure of interest is your starting point.

Sample Support

Unlike standard widefield or TIRF applications, samples used for super-resolution imaging should be prepared qualified for:

- ✓ Immersed in freshly prepared imaging buffer for realizing photoswitching properties
- ✓ Allowing sample tracking during image acquisition period (several minutes)

We recommend a method for easy coverslip mounting tailored for SRiS imaging. Cell were plated on coverslip and the staining procedure are carried out in 12-well plate (Figure A-2). Suspension cells or tissue section can be stuck to coverslip before IF, too. After IF is done, the coverslip is sealed in an imaging chamber which can hold several hundred microliter of

imaging buffer on top (Figure A-3). The following products proofed to be applicable for this purpose.

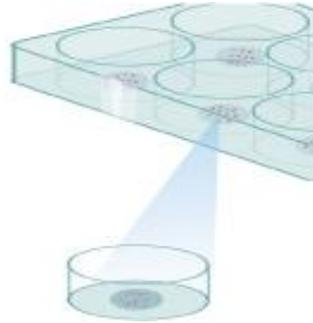


Figure A-2 Cells present on coverslip



Figure A-3 Coverslip sealed within an imaging chamber

- ✓ Coverslips:
Thickness No. 1(0.13 to 0.16 mm)
Circular with 18 mm \varnothing

Recommended:
Marienfeld Superior: www.marienfeld-superior.com
Cat No. 0111580

- ✓ Polystyrene beads for active locking system:
Polystyrene particles, 2.0-2.4 μm

Recommended:
Spherotech: www.spherotech.com
Cat No. PP-20-10

- ✓ Imaging chamber
Steel

Recommended:
NanoBioImaging Ltd.
Cat No.: I-01.

Recommended Dyes for SRiS Super-Resolution Imaging

The SRiS technique uses organic dyes that can be photoswitched in imaging buffers. Theoretically, any optically switchable probe compatible with the SRiS laser wavelength will work. However, the photoswitching properties such as on-off duty cycle of a dye in a particular buffer need to be characterized by the researcher for its suitability. Dyes with a high photon yield per switching event, low on-off duty cycles, high survival fraction and a large number of switching cycles are preferential. Please refer to Dempsey et al., 2011 for recommended quantitative characterization procedure.

In the example protocols in this manual, we use Alexa Fluor® 647 and Alexa Fluor® 750 as dye-of-choice for SRiS dual-channel super-resolution imaging. If the experiment involves only one target (single channel), Alexa Fluor® 647 is recommended because of its high photon yield per switching event and low duty cycle (fraction of time in the on state; Dempsey et al., 2011).

If the researchers need to conjugate the dyes to antibodies or nanobodies by themselves, the labeling procedure is provided in Protocol 2. The dyes can be purchased from Life Technologies:

Alexa Fluor® 647 NHS Ester (Succinimidyl Ester), Cat No. A-20006

Alexa Fluor® 750 NHS Ester (Succinimidyl Ester), Cat No. A-20011

Antibodies and Fluorescence Labeling

Specific labeling of biological structures with photoswitchable dyes can be achieved through immunostaining (direct and in-direct immunostaining) or peptide/enzymatic tags (such as the commercially available SNAP-tag, CLIP-tag, etc) genetically fused to the protein of interest.

The secondary antibodies used in immunostaining for SRiS are already commercial available and familiar to researchers. Although not described in this protocol, users may also want to consider direct dye-labeling of primary antibodies, using FAB fragments instead of whole IgGs, or using nanobodies instead of antibodies to reduce the distance between the dye and the target when a better approximation of the target's location is needed. The labeling reaction can be carried out by the researcher following protocol 2 in this manual.

Recommended secondary antibodies

1. Dye-conjugated secondary antibodies (Life Technologies Products):

Alexa Fluor® 647 Goat Anti-Mouse IgG (H+L) Antibody, Cat No. A-21236

Alexa Fluor® 647 Goat Anti-Rabbit IgG (H+L) Antibody, Cat No. A-21245

Alexa Fluor® 750 Goat Anti-Rabbit IgG (H+L) Antibody, Cat No. A-21039

Alexa Fluor® 750 Goat Anti-Mouse IgG (H+L) Antibody, Cat No. A-21037

2. Unconjugated secondary antibodies (Jackson ImmunoResearch Laboratories Products):

Donkey Anti-Rabbit IgG (H+L) Antibody, Unconjugated, Cat No. A16037

Donkey Anti-Mouse IgG (H+L) Antibody, Unconjugated, Cat No. A16019

Goat Anti-Rabbit IgG (H+L) Antibody, Unconjugated, Cat No. A16112

Goat Anti-Mouse IgG (H+L) Antibody, Unconjugated, Cat No. A16080

Post-Fixation Following Antibody Labeling

Post-fixation after staining is also recommended to prevent the detaching and diffusion of label.

Protocol 1: Immobilization of Locking Beads on Coverslips

Materials

- ✓ 70% ethanal
- ✓ 50% ethanol
- ✓ Polystyrene particles, 2.0-2.4 μm (Spherotech, Cat No. PP-20-10)
- ✓ Coverslip, circular with 18 mm \varnothing , Thickness No. 1 (0.13 to 0.16 mm)

Equipment

- ✓ Hot plate with digital temperature display

Procedure

1. Vortex the original bead slurry, and make 1:1000 dilution from it as a stock slurry with sterile distill water. Store at 4°C, as the stock will last for a while.
2. Vortex the stock slurry, and make 1:1000 dilution with 50% ethanol as a working slurry. Prepare 100 μl per coverslip.
3. Turn on the hot plate and set the temperature to be 120°C.
4. Clean coverslips with 70% ethanol, and place the coverslip in the central area of the hot plate.
5. Load 100 μl of the working slurry onto each coverslip. Avoid leaking to the bottom side of the coverslip.
6. After incubation of 10 minutes, carefully transfer the coverslips to dishes or 12-well plates for storage. The side with beads attached should face up and cells should be seeded on this side.

- **NOTE**

After immobilizing the beads, the researcher can't flame to clean the coverslips. Before seeding cells, sterilize the coverslip for at least 15 minutes under UV light in a biosafety cabinet. Cells should grow on the same side as the beads attach.

Protocol 2: Labeling Antibody/Nanobody with Fluorescent Dye

Example: Labeling anti-GFP Nanobody with Alexa Fluor® 647

Materials

- 0.5M freshly made NaHCO₃ solution
- Dimethyl Sulfoxide, anhydrous, ≥99.9%, #276855-100ML [Sigma-Aldrich]
- 10 X Phosphate-Buffered Saline (PBS)
- Alexa Fluor® 647 NHS Ester (Succinimidyl Ester), Life technologies, Cat No. A20006
- GFP nanobody: anti-GFP V_HH purified protein/GFP nanobody (GFP-Trap®, ChromoTek, Cat No. gt-250), 1 mg/ml in PBS and 0.09% sodium azide.
- Zeba™ Spin Desalting Columns, 7K MWCO, 0.5 ml (ThermoFisher, Cat No. 89882)

Equipment

- Bench top centrifuge
- NanoDrop 2000 UV-Vis spectrophotometer

Protocol

1. To aliquot the Alexa Fluor® dyes, dissolve 1.0 mg in 100 µl anhydrous DMSO to get 10 µg/µl. Make aliquot again into tubes for a final 25 µg amount of dye per tube. If an evaporator is available, remove the DMSO and store the aliquots at -20°C. Otherwise, store them at -80°C with parafilm sealing tightly.
2. To remove the original liquid from the Zeba columns, spin down at 1500g for 1 minute.
3. Load the nanobody solution from step 2 into a new dry column and spin down at 1500g for 2 minutes.
4. Load 15 µl GFP nanobody onto the zeba column, and after it gets absorbed, add 15 µl sterilized 1 X PBS buffer on the top to maximize the yield, spin for 2 minutes at 1500g.
5. Incubate GFP nanobody with 25 µg of Alexa 647 (dilute the dye with DMSO if needed) along with NaHCO₃ solution with a final concentration of 0.1M. Put the mixture tube on a rotor for 1 hour at room temperature, light proof. Thus, dye vs. protein ratio is around 5.
6. Pass the reaction through three Zeba columns successively. Spin down for 1 min at 1500g for each time.
7. After the third wash, add 1/10 volume of 10 × PBS, and sodium azide to 0.01% to the GFP nanobody. Store at 4 °C, light-proof.
8. Use NanoDrop 2000 UV-Vis spectrophotometer to measure final protein concentration and Alexa 647 for calculation of labeling efficiency.

Calculation

GFP nanobody molecular weight: 13.5 KDa

50 µg GFP nanobody equals to 3.7×10^{-9} mole molecules

Alexa Fluor® 647 molecular weight: 1300 Da

According to manufacture, when dye vs. protein ratio is 5:1, the labeling efficiency is the highest. Thus, the amount of dye to take is:

$5 \times 3.7 \times 10^{-9} \text{ mole} \times 1300 \text{ Da} = 25 \text{ µg}$

2.5 μ l of the above Alexa Fluor[®] 647 stock in DMSO will be used.

Protocol 3: Indirect Immunostaining Protocol (Cell Culture)

Example targets: microtubules and mitochondria in COS-7 cell line for dual staining.

The following validated protocol is intended to serve only as a starting guide. If other targets or cell types are selected, different fixation, permeabilization and labeling conditions may apply.

Materials

- Coverslips with locking beads
- 1 × Phosphate-Buffered Saline (PBS)
- Fixation solution: 3% paraformaldehyde, 0.1% glutaraldehyde in PBS
- 0.1M glycine in PBS solution
- blocking buffer: 3% w/v Normal Goat Serum , 0.2% v/v Triton X-100 in PBS
- rabbit anti-Tom20 antibody (Santa Cruz Biotech, Cat No. FL-145)
- mouse anti- β -tubulin antibody (Sigma-Aldrich, Cat No. T4026)
- Diluted primary antibody solution: dilute both antibodies in blocking buffer at 1:100 ratio
- Diluted secondary antibody solution: dilute secondary antibodies at 1:250 ratios in blocking buffer

Procedure

✓ **Day 1**

1. Immobilize locking beads onto coverslip following Protocol 1.
2. Sterilize the coverslip for at least 15 minutes under UV light in a biosafety cabinet.
3. (Optional) If coating of the coverslips is required, the coating should be added onto the same side as the locking beads attach.
4. Place the coverslips with beads and coating facing up in a 12-well plate. Plate cells to 50-60% confluency.

✓ **Day 2**

5. Wash with 1 ml PBS once at room temperature (RT).
6. Fix with 0.5ml fixation solution for 10 minutes at RT.
7. Wash with 1ml three times, and at the third time, add 0.5 ml of 0.1M glycine and incubate for 7 minutes.
8. Wash 3 times with 1 ml PBS, shaking gently for 5 minutes each wash.
9. Block cells with in 200 μ l blocking buffer for 15 minutes.
10. Incubate with rabbit anti-Tom20 (1:100 dilution) and mouse anti- β -tubulin (1:100 dilution) in 200 μ l blocking buffer at RT for 30 minutes.
11. Wash with 1ml PBS three times, rocking gently for 5 min each wash.
12. Incubate with secondary antibodies at 1:250 dilution in 200 μ l blocking buffer at RT for 40 minutes. Light proof.
13. Wash with 1 ml PBS three times, rocking gently for 5 minutes each wash.
14. Post-fix with the fixation solution for 10 minutes at RT.
15. Wash with 1ml PBS three times, rocking gently for 5 minutes each wash.
16. Store in 1 ml PBS at 4°C. Light proof.

- **NOTE**

Sodium azide, paraformaldehyde (PFA), glutaraldehyde and potassium hydroxide are poisonous materials. Please handle them with particular care.

Protocol 4: Indirect Immunostaining Protocol (Tissue Section)

- **NOTE**

Immunostaining of tissue sections is the same as cell culture. This protocol covers the steps for tissue section preparation, including cardiac perfusion, dissection, embedding and cryostat. These steps will be followed by immunostaining procedure like Protocol 3.

Cardiac Perfusion

Materials

- 1 × Phosphate-Buffered Saline (PBS)
- 4% PFA (paraformaldehyde)

Equipment

- Pump

Procedure

1. Turn on the pump to perfuse the animal with the 1 × PBS buffer to remove the blood. The flow rate should be chosen according to the size of animal. Maintain a steady flow rate throughout the buffer infusion period.
2. When draining fluid is clear, perfuse the animal with 4% PFA (paraformaldehyde) or other fixative in the same manner as with 1 × PBS buffer. When the liver becomes light yellow, the blood is cleared from the body. The colour of the liver is an indicator of a good perfusion.
3. Spontaneous movement (formalin dance) should be observed within seconds after perfusion with 4% PFA.
4. Fixation is almost complete when animal becomes stiff.
5. Stop the perfusion and excise the tissue of interest. The animal organs or tissues are removed from the bodies. Place them in vials containing the same fixation and fix for another 2 more hours on ice or at 4°C before proceeding to dehydration and embedding.
6. For better results, immersion-fix overnight at 4°C.

- **NOTE**

1. Take care not to introduce air bubbles while transferring from one solution to the other.
2. In general, an adult rat would require around 30-60 minutes of perfusion time but this may vary depending on the size of the animal and technique.
3. An initial indicator of the success of the perfusion is the clearing of extremities such as the nose, ears, and paws and internal organs such as the thymus gland and liver (IHC World). Gross inspection of the brain reveals the blood vessel void of blood (white to pale yellow appearance). This will be also true within tissue sections destined for staining and immunohistochemistry. The final indicator of outcome of the perfusion is the condition of the ultrastructure in the tissue.

Dissection Procedure

1. Gently and carefully separate interested tissues to avoid injury and remain the intact

tissue.

2. Operators have to be aware of their tissue interactions, their advantages and limitations.
3. We will give the sample of dissecting brain of rodents.

Brain Dissection

Materials

- Fine straight scissors
- Forceps
- Rongeurs
- Spatula

Procedure

1. Remove the head using a pair of scissors.
2. Make a midline incision along the integument from the neck to the nose and expose the skull.
3. Trim off the remaining neck muscle so that the base of the skull is exposed; remove any residual muscle using scissor or rongeurs.
4. Place the sharp end of a pair of iris scissors into the foramen magnum on one side, carefully sliding the scissors along the inner surface of the skull.
5. Make a cut extending to the distal edge of the posterior skull surface. Make an identical cut on the contralateral side. Use the rongeurs to clear away the skull around the cerebellum.
6. Carefully slide the scissors along the inner surface of the skull as the tip travels from the dorsal distal posterior corner to the distal frontal edge of the skull, lifting up on the blade as you are cutting to prevent damage to the brain. Repeat for opposite side.
7. Use rongeurs to peel the dorsal surface of the skull away from the brain. Trim away the sides of the skull using rongeurs as well.
8. Use a spatula to sever the olfactory bulbs and nervous connections along the ventral surface of the brain.
9. Gently tease the brain away from the head, trimming any dura that still connects the brain to the skull using iris scissors.
10. Remove the brain and place it in a vial of fixative containing fluid at least 10× the volume of the brain itself.
11. Keep the brain in fixative for 24 hours at 4 °C, swirling occasionally.

Embedding (for Cryostat)

Materials

- 10%, 20%, 30% sucrose
- OCT
- Isopentane
- Dry ice
- Petri dish
- Cryomold
- Metal beaker

- Foil paper

Equipment

- Cryostat

Procedure

1. After fixation in 4% PFA, the tissue is transferred to 10% sucrose in 1 × PBS buffer for 2 hours at 4°C. The containing fluid should be at least 10x the volume of the tissue itself.
2. Then transfer tissue into 20% sucrose until the tissue sinks to the bottom of containers, finally 30% sucrose (also sink to the bottom).
3. After 30% sucrose, submerge the brain into OCT twice in order to remove the residue of sucrose.
4. Acclimate tissue to OCT – cover tissue for a few minutes in OCT in a labeled small petri dish or small weigh boat.
5. Transfer and orientate in fresh OCT in a labeled Cryomold – with just enough OCT to cover the tissue.
6. A metal beaker is ½ filled with isopentane, placed in a foam cooler or laboratory ice bucket and surround with crushed dry ice. Add a few pieces of dry ice to the isopentane and wait until boiling stops. Add more isopentane if necessary.
7. With forceps freeze the cryo mold prepared sample in the isopentane, do not fully submerge.
8. Alternatively: Freeze cryomold prepared sample by surrounding it in finely powder crushed dry ice alone, or in a dry ice methanol or 100% Ethanol slurry.
9. Transfer frozen sample to a covered foam cooler of dry ice while continuing on to other samples.
10. Wrap all samples in labeled foil, place in a bag sealed bag.
11. Store the sample at -80 °C after OCT is completely frozen.

- **NOTE**

1. The following procedure may be modified according to different tissues.
2. The procedure Not for enzyme study method.
3. Avoid bubbles in the OCT – especially near the tissue. Sectioning surface is the bottom of the Cryomold.