# Preparation of Neuronal Cultures for Super-Resolution Imaging

Multicolor single-molecule localization microscopy (SMLM) extends the neuroscience and neurobiology toolbox for characterizing protein organization at the nanometer scale.

Here we present a guide on how to prepare neurons to detect synaptic proteins of interest using super-resolution imaging.

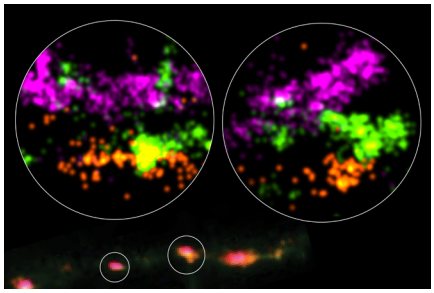


**Figure 1** 3-color dSTORM image of neuronal synapses in rat primary cortical neurons cultured for 14 days. Image shows a neuronal process containing several synapses imaged using fluorescently-labeled antibodies against the presynaptic protein Bassoon (magenta), synaptic vesicle marker VGlut1 (green) and the postsynaptic protein Homer1 (orange).

### 1. NEURONAL CULTURE

Dissociated neurons from brain tissue from rat or mouse embryos (latest days) or from postnatal pups (early days) are the most common sample source for neuronal studies in SMLM. Recently, there is an increased use of human iPSC (induced pluripotent stem cells)-differentiated neurons. The tips provided in this guide, are best suited for dissociated neurons from mouse or rat brain tissue. Any protocol should be optimized depending on the source material and use case.

Seed the neurons in round #1.5H glass coated coverslips (poly-D-lysine- is the most common coating used). Read our dSTORM sample preparation workflow for details on how to prepare coverslips and seed cells.

A density of around 10,000-50,000 cells/cm2 is recommended. For SMLM, a lower confluency is generally recommended such that a significant proportion of single cells can be detected for imaging.

Submerge coverslips in dishes containing Neurobasal medium supplemented with B-27 Supplement. Alternatively, Ibidi 8-well dishes may be used, depending on the growth conditions of the neuronal culture. Coating and culture times can be maintained, but volumes need to be adjusted to 200-300 µl/well.

Culture neurons in an incubator on average for 15 days at 37°C and 5% of CO2.)

### 2. FIXATION

After incubation, fix neurons using pre-warmed (37°C) 4% paraformaldehyde (PFA) and 4% glucose in phosphate buffered saline (PBS) for a 5-10 min at room temperature. Glucose helps maintain the integrity of the neuronal membrane during fixation.

Wash 3 times very gently with PBS after that in order to avoid cell body detachment. In some cases, where synapse morphology is important, 0.1% Glutaraldehyde may be included, but this could be at the expense of epitope binding being affected.

## 3. STAINING AND PERMEABILIZATION

### Staining before permeabilization

Immunostaining of primary antibodies targeting membrane proteins with extracellular binding sites can be performed before cell permeabilization. Dilute antibodies in blocking buffer, e.g. 1 % of bovine serum albumin (BSA) in PBS, and incubated at room temperature for several minutes (~1h) or overnight at 4°C. Wash 3 times very gently with PBS afterwards.

To avoid labeling the intracellular pool, surface-expressed protein staining can be performed before fixation. In this case, incubate neurons with antibodies for 10-15 min at 37°C and fix immediately to avoid protein turnover. Wash very gently as above.

### Permeabilization

Lipid membrane permeabilization is necessary when labeling intracellular structures. Use a detergent, such as 0.1-0.5% Triton X-100 in PBS for 5-10 minutes. Wash 3 times very gently with PBS after that.

### Blocking

The same buffer used for antibody dilution can be used to block the non-specific binding of staining agents and reduce the background fluorescence of the image. Typically ~1h incubation at room temperature is sufficient.

### Staining after permeabilization

Incubate again with the antibodies targeting cytoplasmic binding sites using the same timing as before.

### Secondary staining (if applicable)

The most common method for fluorophore labeling is the use of secondary antibodies conjugated with organic dyes compatible for dSTORM imaging. The same procedure as explained above should be followed. Immunostaining of multiple targets at once is possible, if different spectral fluorophores are used.

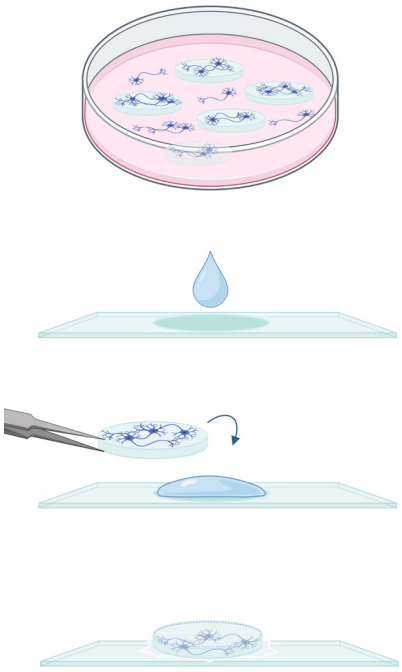
### Alternative fluorophore labeling

Primary antibodies directly conjugated to a fluorophore are also available in the market, in which case, a secondary staining is not necessary. Other approaches for labeling include HaloTag® or SNAP-tag® 1, nanobodies, or Fab fragments.

In neuronal SMLM experiments, it is also possible to use regular tagging methods like in other fluorescence microscopy modalities, such as transfection, viral transduction or electroporation. These methods are recommended for tagging neuronal targets with fluorescent proteins. Photoactivatable Localization Microscopy (PALM) or single-particle tracking (SPT) imaging benefit from this method. Importantly, for PALM imaging, you will need to use photoconvertible fluorescent proteins in order to achieve super-resolution imaging. Read more on fluorophores for PALM imaging.

More recently, DNA-PAINT or uPAINT have proven to be very suitable for neurological studies 2, 3. In this case, oligomers tagged with fluorophores transiently bind to the proteins of interest during imaging.

#### 4. MOUNTING



There are different imaging approaches for single-molecule localization microscopy to study synaptic protein organization at the nanoscale level.

**dSTORM and DNA-PAINT**

It is necessary to have the cells in a cavity that allows the corresponding buffers to be added. Since neurons are typically grown on coverslips, this can be challenging. There are, however, different solutions in the market. The easiest one is the use of cavity slides: coverslips containing the neuronal cultures can be flipped and mounted in cavity slides containing the STORM buffer and then sealed with a sealing reagent, such as nail polish or dental cement.

Alternatives to that can be magnetic, silicon or stainless steel (Ludin) chambers, but it is important to previously check any leakage before putting the sample on the microscope, and verify that they are compatible with the stage to be placed onto.

**For live-cell imaging, like live PALM or SPT**

It is highly recommended to use a buffering agent that maintains the physiological pH despite changes in carbon dioxide concentration, such as HEPES. In this case, the chosen mounting method should be the one that does not require sealing, but allows buffer interchange.

#### 5. IMAGING

The Nanoimager is capable of performing up to 4-color imaging, two of them simultaneously, allowing imaging of multiple targets in one acquisition. This feature is very convenient for the study of protein complexes at the neuronal synapse.

- dSTORM is performed using a high-power laser (typically powers at 1-10 kW/cm2 at the sample) and low exposure time (10 - 30 ms/frame depending on the density of the labeling).
- DNA-PAINT is performed similarly to dSTORM, with the advantage that fluorophores are not affected by photobleaching.
- For PALM or experiments in live cells, the same power is necessary, but exposure time can be reduced to 5-10ms for fast processes.
- For SPT, laser power needs to be much lower, as high power will cause the fast photobleaching of the fluorophore. The density of labeled molecules has to be such that allows molecules to be individually tracked.

#### 6. ANALYSIS

Single-molecule localizations acquired during imaging are used to reconstruct a super-resolved image of neuronal protein and synaptic receptor organization 4,5. Clustering algorithms are required to characterize these maps of localizations and extract both structural and quantitative information. CODI, ONI's cloud-based data analysis platform, can be used to characterize the organization of synaptic proteins at the nanometer scale. Information, such as cluster size, area, or number of localizations per cluster, is extracted. Interestingly, it is also possible to extract information regarding co-localization of synaptic proteins and stoichiometry.

## References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