Protocol

dSTORM Sample Preparation Workflow

1

Coverslip and Sample Holder

Coverslip thickness

#1.5H coverslips (170 µm) should always be used. This thickness of glass is compatible with the working distance of the 100x objective.

- 8-well chambers (iBidi or Lab-Tek), 35 mm glass-bottomed petri dishes, or single coverslips, ideal dimensions 50-75 mm by 20-25 mm.
- Coverslips must not be mounted to allow the addition of the imaging buffer prior to imaging.

2

Preparing the Coverslip

Cleaning

The coverslips used in any experiment, particularly for TIRF illumination, need to be thoroughly cleaned to remove any debris which can increase background signal.

Sterilization

When working with live cells, the coverslips should be sterilized before use and all steps should be performed in a biosafety cabinet with sterile technique.

Coating

To enhance cell adhesion, cell activation or to block non-specific binding of reagents, it can be neccessary to coat the coverslips before seeding the cells. Coating the surface can be done with PLL, antibodies or other proteins depending on the requirements of the experiments.

3

Cell Seeding

Cell density

The optimal level of cell confluency will depend on the specific experiment. For dSTORM, a lower confluency is generally recommended such that a significant proportion of single cells can be detected for imaging.

Cell spreading

Allow cells an appropriate time to spread to help to resolve structures in a single plane.

Media

Use phenol red free media to reduce background florescence.

4

Fixation

An optimized fixation protocol is crucial. If the sample is not fixed properly, the fluorophore labels and/or biomolecules of interest may remain mobile during imaging, resulting in low-resolution images.

Fixation buffer

Paraformaldehyde (PFA) and Glutaraldehyde (GA) are the most common fixation agents. GA contains two aldehyde groups, as opposed to PFA's one, which aids in linking distant proteins. Therefore it is beneficial to use GA when fixing structures with distant protein neighbors (e.g. membrane receptors). Always use freshly made fixation buffers.

Timing

Samples should be fixed for the shortest amount of time possible that still results in full fixation. Longer fixation times can lead to artificial protein clustering, masking of epitopes, and excessive autofluorescence.

Quenching autofluorescence

PFA and GA both have autofluorescence and therefore it is necessary to incubate fixed samples with an autofluorescence quenching buffer, such as sodium borohydride.

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ONI, Linacre House, Jordan Hill Road, Oxford, OX2 8TA, UK
ONI, ONI Inc. SPACES, 101 Jefferson Drive, Menlo Park, CA, 94025 USA

5

Permeabilization

Permeabilization of the lipid membranes is necessary when labeling intracellular structures.

Triton X-100
Commonly used permeabilization buffer. Triton X permeabilizes all lipid bilayers, however, high concentrations or long incubation times can lyse cells.

Saponin/digitonin
Provides reversible permeabilization and is not able to permeabilize the nuclear membrane.

6

Blocking

This step is used to block non-specific binding of the staining agent and to reduce the background fluorescence of the image. Typically, bovine serum albumin (BSA) is used for blocking.

7

Staining

Choose a staining method carefully based on the structure you are trying to visualize.

Labeling density
A high labeling density ensures that all epitopes are labeled. If full coverage is not achieved, a continuous structure will appear as punctate, or not all subunits in a structure may be visualized.

Labeling method
When labeling a structure with many epitopes, antibodies may not be able to reach the target due to overcrowding and steric hindrance. This will result in non-uniform sample labeling, and an inability to visualize the full structure of interest. The size of the label can artificially increase the size of a target structure due to the placement of the fluorophore further from the target. Direct labeling approaches ensure that the fluorophore is close to the target. In contrast, indirect labeling approaches can place the fluorophore 10-30 nm away from the target. Therefore to measure the true size of a structure, it is recommended to use direct labeling methods such as nanobodies, primary conjugated antibodies or self-labeling tags such as Halo-, CLIP-, or SNAP-tag®.

Fluorophores
The photophysical properties of fluorophores influence the quality and resolution of dSTORM images. Two important considerations are the number of photons emitted per blinking event (a higher photons count results in a higher localization precision) and the duty cycle of the blinking (a lower duty cycle results in a higher quality image).

Dye recommendations
Multi-color imaging requires selecting two or more dyes that can be spectrally resolved. We recommend the following combinations.

One-color: Alexa Fluor® 647

Two-color: Alexa Fluor® 647 and Alexa Fluor® 555

Three-color: Alexa Fluor® 647, Alexa Fluor® 555, and Atto 488

For help designing multi-color dSTORM experiments, our table on Popular Fluorophores for dSTORM Imaging will guide you towards choosing the best dye for each laser line.

8

Post fixation

Post fixation of samples ensures that the label and the labeled proteins are stationary during image acquisition and that the sample can be preserved for longer periods of time. This step is necessary for dSTORM imaging since any movement will reduce localization precision and image resolution.

9

Sample quality check

Before performing dSTORM imaging, it is useful to check the quality of the sample. Check that:

- There is minimal background and non-specific binding of the labeling agent
- The correct structures are stained
- There is appropriate cell confluency and spreading of cells

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ONI, Linacre House, Jordan Hill Road, Oxford, OX2 8TA, UK
ONI, ONI Inc. SPACES, 101 Jefferson Drive, Menlo Park, CA, 94025 USA