ONI

dSTORM
Discovery
Kit

TECHNICAL GUIDE

Sample preparation & imaging with the ONI Discovery Kit™: dSTORM in cells

dSTORM is a single-molecule localization based super-resolution technology that bypasses the diffraction limit, achieving ≈ 20 nm in resolution. It relies on the detection of spatiotemporally separated single localization events, from targets labeled with photo-switchable fluorophores that switch off-and-on ('blink'), when exposed to high laser and specific buffer conditions. dSTORM can be very sensitive and is well suited to imaging proteins at low abundances.[1]

Sample preparation for dSTORM imaging follows similar protocol steps to standard immunofluorescence staining methods: fixation, optional permeabilization or quenching, blocking, antibody labeling, fixation, and washing in between. Here we share key steps and considerations to prepare samples for super-resolution imaging using ONI's Discovery Kit: dSTORM in cells.

1. Growing your cells

Coverslip and sample holder to use

- Select a microscopy slide that has a glass imaging surface (do not use plastic)
- Choose the correct glass thickness for a 100x objective, with the Nanoimager use #1.5H coverslips (170 µm).
- Coverslips need to be clean and sterile to minimize any debris that could increase background signal.
- Coverslips must not be mounted or sealed to allow the addition of the imaging buffer prior to imaging
- 8-well chambers (iBidi or Lab-Tek), 35 mm glass-bottomed petri dishes, or single coverslips, ideal dimensions 50-75 x 20-25 mm.
- To enhance cell adhesion, cell activation or block non-specific binding of reagents, it can be necessary to coat the coverslips before seeding the cells. Coating can be done with poly-L-Lysine, antibodies or other proteins depending on the experiment requirements.

Cell compatibility

The kit has been tested for the detection of protein targets in immortalized (U2OS, NIH3T3, COS7, Jurkat) or primary (embryonic rat cortical neurons, primary human T-cells) adherent and non-adherent cell lines.

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

2. Controls to Consider

Antibody controls
It is good practice to include an isotype control to evaluate background signals and verify that the antibody specifically recognizes the intended target epitope.

Structure of interest controls
Assess specificity and sensitivity by including a negative control lacking your protein or parts of your structure of interest (if possible) to examine detection efficiency and distinguish intact from disrupted structures.

3. Choosing a fixative in the dSTORM Discovery Kit

The composition of the fixative should be considered knowing that certain structures or epitopes require a stronger fixation to immobilize or preserve ultrastructure. It is important to search the literature to see if optimized fixation conditions have been reported for your protein of interest.

There are two versions of the Discovery Kit:

1. Discovery Kit: dSTORM in cells
   Fixative A (PFA)
2. Discovery Kit: dSTORM in cells with strong fixative
   Fixative A (PFA)
   Fixative B (PFA + glutaraldehyde)

Intracellular Proteins

Almost all non-membrane proteins can be fixed with Fixative A. We, therefore, recommend the standard Discovery Kit (*STORMDISC-M)* for researchers studying intracellular non-membrane proteins. Exceptions are structural proteins, such as tubulin and actin, which are optimally fixed with Fixative B.

Membrane proteins

For many membrane proteins PFA is insufficient to cross-link and many proteins remain mobile in the membrane[2]. Insufficient fixation of membrane proteins can lead to antibody-induced protein clustering and/or impact data quality[3,4]. On the other hand, PFA + Glutaraldehyde has been shown to preserve their native structure (Figure 1). Unfortunately, not all proteins can be fixed with glutaraldehyde as it can disrupt the epitope and impact antibody recognition.

When working with a new antibody or protein target (particularly membrane proteins), we recommend using the Discovery kit with Strong Fixative (*STORMDISC-S*) to test and compare Fixative A and B, and determine optimal biomarker distribution and epitope detection.

If the signal is equal or better when using Fixative B, proceed with this fixative. However, if the signal is weaker with Fixative B than Fixative A, it is recommended to proceed with Fixative A. Extra caution when interpreting results from clustering analysis might be needed in the latter scenario. See the table below for more guidance on how to approach fixation for different targets.

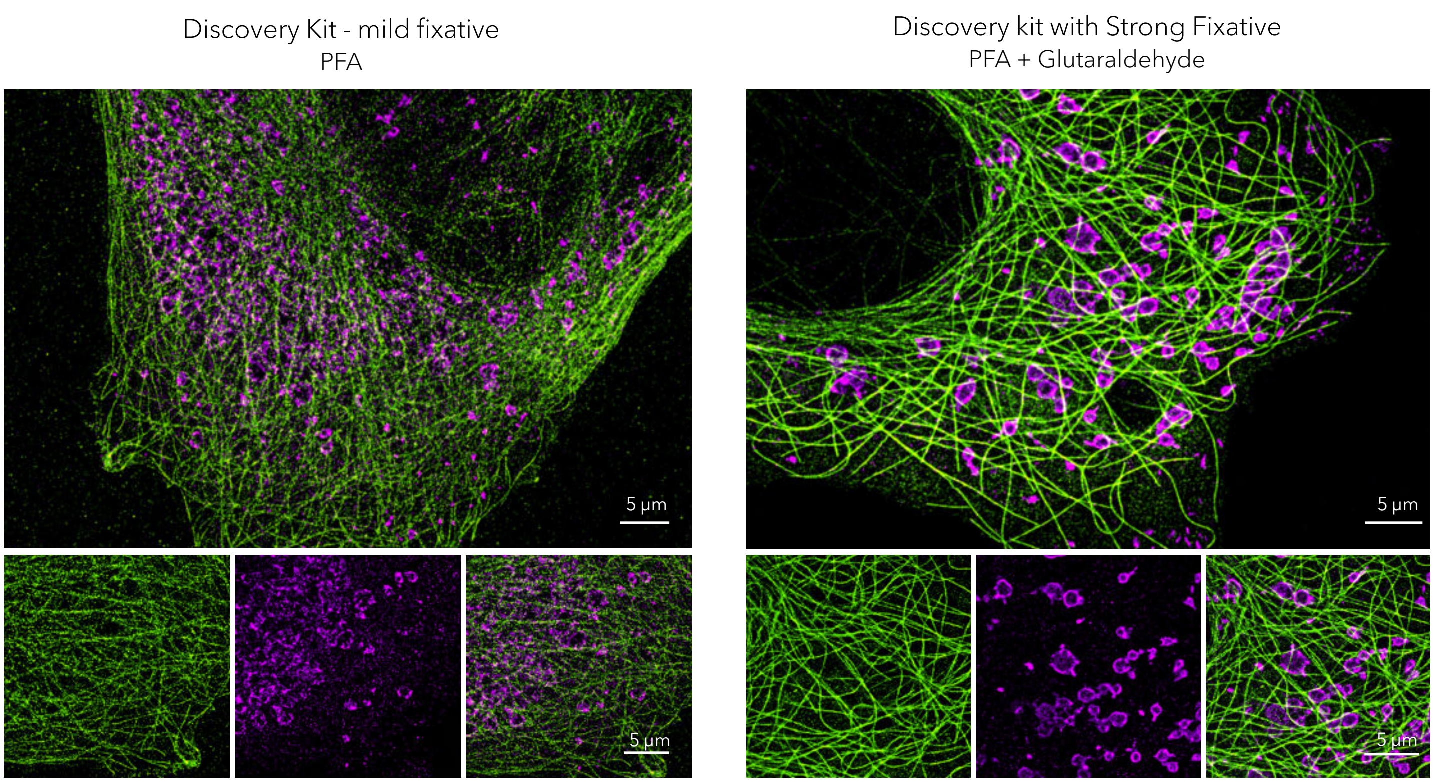

Figure 1. LAMP-1 and Tubulin were labeled with anti-rabbit CF583R (magenta) and anti-rat AZ647 (green) in U2OS cells with both versions of the ONI Discovery Kit: dSTORM in cells, with either Fixative A (mild, left) or Fixative B (strong, right), and imaged using the Nanoimager. This shows that usage of the stronger Fixative B (PFA+Glutaraldehyde) results in better images and maintained integrity of both the vesicular structures labeled with LAMP-1 and microtubule network (right hand side panels).

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4. When to use permeabilization

Permeabilization should be used when the aim is to detect intracellular targets and so, treatment to promote cell permeability is needed to expose antibody epitopes. It is important to consider the experiment setup and the target of interest in order to decide on whether to use a permeabilization buffer during the immunofluorescence staining procedure.

In this case, the Blocking, Staining and Washing Solutions are supplemented with Permeabilization Concentrate. However, when the aim is to exclusively detect cell surface antigens, it is not recommended to permeabilize as the detergent will damage the cell membrane and change the distribution of your protein of interest.

The table below contains some examples of targets and the recommended solution for first use (optimization will be required for each antibody and protein target tested), while Figure 2 shows an example where permeabilization, together with labeling and dSTORM imaging was used to detect two nuclear histone modifications.

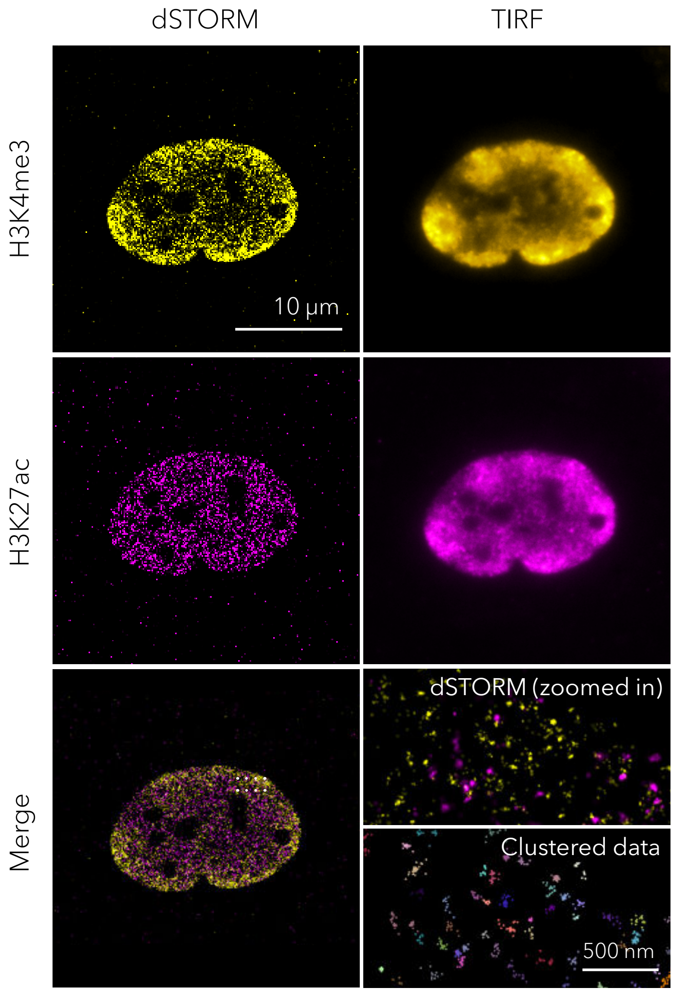

Figure 2. Permeabilization used to detect intracellular proteins with the dSTORM Discovery Kit. Two different histone modifications H3K43me3 and H3K27ac were labeled with anti-rabbitCF583R (yellow, left) and anti-mouse AZ647 (magenta, center) using the ONI Discovery Kit™: dSTORM in cells (PFA). The comparison between diffraction-limited TIRF imaging (top right images) and dSTORM (left) is shown. Zoomed in insets (bottom right) set show the lack of colocalization, as expected, between both histone modifications at the nanoscale level (dSTORM at the top, clustered data with CODI at the bottom).

Target type/location	Fixative recommended	Permeabilization needed?
Structural protein (e.g. tubulin)	Fixative B	Yes
Membrane protein (e.g. CD63)	Fixative B	No
Nuclear protein (e.g. histone)	Fixative A	Yes
Organelle (e.g. mitochondria)	Fixative A/B*	Yes
Structural protein (tubulin) + intracellular target (mitochondria)	Fixative B	Yes
Structural protein (tubulin) + membrane protein (LAMP-1)	Fixative B	Yes

*Target dependent, optimization with both fixatives recommended

5. Screening primary antibodies

Candidate validation

High-quality primary antibodies are essential for obtaining reliable results when using single-molecule imaging based detection of nanoscale protein distribution. When labeling a protein for the first time, it is recommended to carry out a literature review to identify antibodies used in dSTORM imaging for your target of interest. If none are available, screening of antibodies should be done prioritizing high-affinity binders that have been validated using knockout cell lines.

For any new antibody it is recommended to perform a titration experiment to determine optimal concentration with the 1-15 µg/ml range. Including a negative control without a primary antibody or an isotype control should be included to determine the background levels from non-specific binding of the antibodies or anything autofluorescent in the sample. This is particularly important when working with low expression proteins when the number of localizations are closer to the background.

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. 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.

6. Secondary antibodies and probes

Target-fluorophore distance

The fluorescently-labeled secondary Fab’2 antibodies included in the Discovery Kit: dSTORM in cells kit are compatible with the detection of primary antibodies raised in mouse, rabbit and rat hosts. The concentration of the antibodies has been validated for a broad range of targets and provides optimal labeling density and signal-to-noise ratio.

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 are recommended to ensure that the fluorophore is close to the target. The fluorescent secondary Fab’2 antibodies provided in the kit are smaller than full length IgG, which reduces the distance between the fluorophore and the antigen, decreasing linkage error.

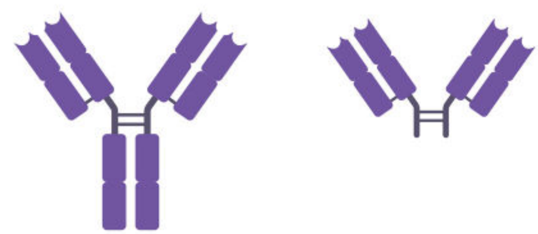

Figure 3. Schematic representation of the size difference between a full IgG antibody (left), the Fab’2 antibodies (center) used in the ONI Discovery Kit: dSTORM in cells, and F(ab) (right).

Fluorophores

To enable 2-color dSTORM detection the antibodies are conjugated to either CF583R or AZ647, the best fluorophores for dSTORM in their respective channels. The dyes exhibit optimal brightness, photostability and blinking kinetics with minimal spectral overlap that allows high-quality dSTORM imaging.

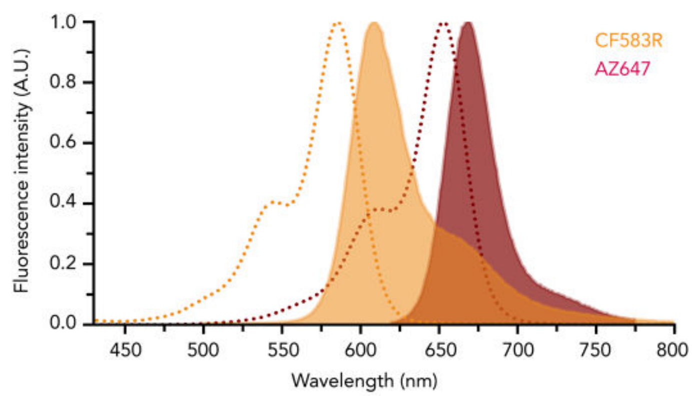

Figure 4. Excitation and emission spectra of the CF583R (orange, left) and AZ647 (red, right) dyes included in the ONI Discovery Kit: dSTORM in cells.

7. Image acquisition

STORM buffer

The Discovery Kit: dSTORM in cells includes ONI's BCubed imaging buffer. This in-house made dSTORM imaging buffer provides excellent blinking and anti-bleaching activity. Low-oxygen and reducing conditions allow for fluorophore blinking and contribute to high-quality super-resolution images. Do not use hard mounting media.

Illumination angle

To minimize background fluorescence, employ total internal reflection fluorescence (TIRF) microscopy. TIRF selectively excites fluorophores within a thin layer in the Z direction, enhancing axial resolution and decreasing interference from out-of-focus fluorophores. Adjust the laser illumination angle to achieve TIRF conditions.

Laser power

Laser power directly impacts the blinking behavior of fluorophores, which is essential for high-resolution dSTORM imaging. While higher laser power can enhance blinking, it also increases the risk of premature photobleaching, leading to signal loss. Therefore, it's crucial to optimize laser power for each fluorophore and imaging experiment, balancing high resolution with appropriate rates of photobleaching.

References

Nerreter T, Letschert S, Götz R, Doose S, Danhof S, Einsele H, Sauer M, Hudecek M. Super-resolution microscopy reveals ultra-low CD19 expression on myeloma cells that triggers elimination by CD19 CAR-T. Nat Commun. 2019; 10(1): 3137. doi: 10.1038/s41467-019-10948-w.

Tanaka, K., Suzuki, K., Shirai, Y. et al. Membrane molecules mobile even after chemical fixation. Nat Methods. 2010; 7, 865–866. https://doi.org/10.1038/nmeth.f.314

Stanly TA, Fritzsche M, Banerji S, García E, Bernardino de la Serna J, Jackson DG, Eggeling C. Critical importance of appropriate fixation conditions for faithful imaging of receptor microclusters. Biol Open. 2016; 5(9): 1343-50. doi: 10.1242/bio.019943.

Werner C, Sauer M and Geis C. Super-resolving Microscopy in Neuroscience. Chem. Rev. 2021; 121 (19): 11971–12015.

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Interested in learning more?

ONI, Linacre House, Jordan Hill Road, Oxford, OX2 8TA, UK
ONI Inc. 11045 Roselle St. Suite 120, San Diego, CA 92121, USA

@ONI @oniHQ @oni_bio @oni-bio