



Super Resolution Microscope

N-STORM

STORM Protocol-Sample Preparation



N-STORM

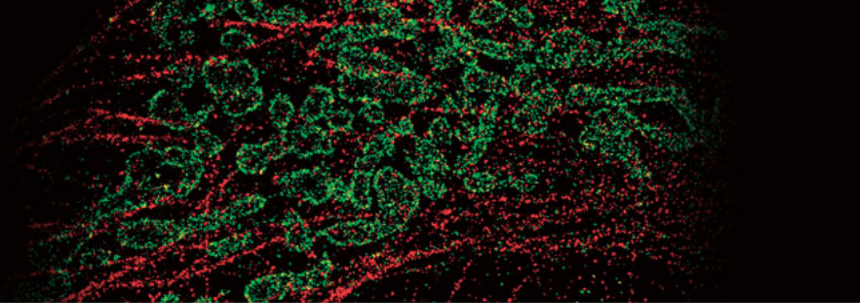
STORM Protocol-Sample Preparation



The STORM technique is a super-resolution imaging method that uses sequential activation and high-precision localization of individual fluorophores to achieve sub-diffraction-limit spatial resolution. To use this method, the biological structure of interest simply needs to be labeled with photoswitchable probes. Any suitable optically switchable probe will work. This generality allows for flexibility during sample preparation. In this protocol, we use photoswitchable dyes as illustrative examples. Specific labeling of biological structures with these dyes can be achieved through immunostaining or peptide/enzymatic tags (such as the commercially available SNAP-tag, CLIP-tag, HaloTag, SorTag, etc) genetically fused to the protein of interest.

In the following, a few example protocols using activator-reporter paired antibodies, conventional (reporter-only) antibodies, and SNAP tags are described. In choosing which method to use, users must carefully consider the specific goal(s) of their STORM experiment. This is particularly important when the experiment involves imaging more than one target. Because the photoswitching properties of dyes vary, the resulting resolution will vary as well. To date, Alexa 647 remains the dye-of-choice for STORM because of its high photon yield per switching event and low duty cycle (fraction of time in the on state; Dempsey et al., 2011). As such, the use of the activator-reporter antibodies with Alexa 647 as the common reporter in dual-channel experiments will result in the highest resolution for both channels. However, the use of conventional antibodies (labeled with the reporter dye only) for STORM is on the rise due to its ease of use, commercial availability and familiarity. As with the activator-reporter method, Alexa-647 is recommended for this reporter-only method. Atto 488, Alexa 568 or Cy3b are recommended for detecting the second channel.

In general any immunostaining protocol may be used for STORM. However, care should be taken to minimize background/non-specific staining while maximizing specific staining density. Post-fixation after staining is also generally recommended to prevent the diffusion of label. Although not described in this protocol, users may also want to consider direct dye-labeling of primary antibodies or using FAB fragments instead of whole IgGs to reduce the distance between the dye and the target when a better approximation of the target's location is needed.



1. IMMUNOSTAINING PROTOCOL USING CONVENTIONAL ANTIBODIES (REPORTER-ONLY METHOD).

The following validated protocol is intended to serve only as a starting guide. For example, blocking conditions will have to be adjusted if secondary antibodies other than goat are to be used. For example, if using donkey secondary antibodies, normal donkey serum should be used instead of normal goat serum. Secondary antibodies conjugated with Alexa 647 (Rockland), Atto 488 (Jackson Immuno), and Alexa 568 (Invitrogen) are recommended. Alexa 568 can also be substituted with Cy3B when a yellow-absorbing dye is needed. Because of its optimal photoswitching properties, Alexa 647 should be used when staining for only one target. Please refer to the antibody manufacturer for appropriate dilutions.

EQUIPMENT

- Cover glass for plating cells. Recommended: Lab-Tek II Chambered Coverglass #155409 [Thermo Scientific Nunc]
- Rocking platform shaker
- Sonicator

REAGENTS

- Phosphate-Buffered Saline (PBS), 1x
- Sodium borohydride (NaBH_4) 99% #213462-25G [Sigma-Aldrich]
- Paraformaldehyde Aqueous Solution-16%*¹, EM grade #15710 [Electron Microscopy Sciences]
- Glutaraldehyde*¹ Aqueous Solution-8%, EM grade #16019 [Electron Microscopy Sciences]
- Bovine Serum Albumin IgG-Free, Protease-Free #001-000-162 [Jackson ImmunoResearch Europe]
- Triton X-100 #T8787-100ML [Sigma-Aldrich]
- Potassium Hydroxide #PX1480 [EMD Chemicals]

PRIMARY ANTIBODIES

- Rat Anti-Tubulin monoclonal antibody (YL1/2)-Loading Control 1.0mg/ml #ab6160 [Abcam].
- Rabbit Anti-Tom20(FL-145) polyclonal antibody 0.2mg/ml #sc11415 [Santa Cruz].
- Mouse Anti-Clathrin monoclonal antibody [X22] -Membrane Vesicle Marker 6.0mg/ml #ab2731 [Abcam].
- Mouse Anti-Clathrin light chain monoclonal antibody [CON.1] 1.0mg/ml #ab24579 [Abcam].

SECONDARY ANTIBODIES

Activator-reporter antibodies as prepared following Protocol 4.

SOLUTIONS

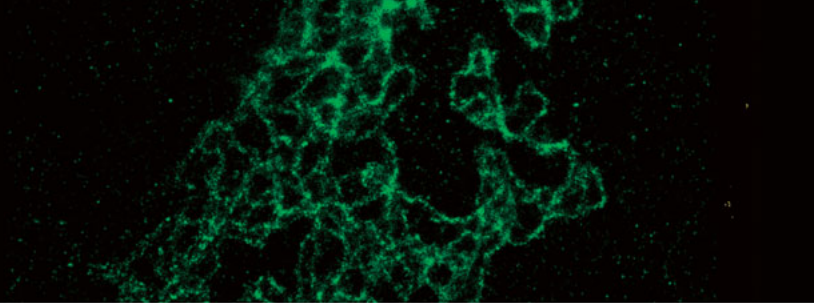
Check box

- Fixation solution: 3% paraformaldehyde (PFA) + 0.1% glutaraldehyde in PBS
- Reducing buffer (prepare immediately before use): 0.1% NaBH₄ in PBS
- Permeabilization buffer: 0.2% Triton X-100 in PBS
- Blocking buffer: 10% normal goat serum (NGS) + 0.05% Triton X-100 in PBS
- Washing buffer: 1% NGS + 0.05% Triton X-100 in PBS
- Antibody dilution buffer: 5% NGS + 0.05% Triton X-100 in PBS

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PROTOCOL

1. Wash with 500 µl PBS once.
2. Fix with 3.0% PFA/0.1% glutaraldehyde for 10 minutes at room temperature.
3. Reduce with 200 µl of 0.1% NaBH₄ (prepared immediately before use) for 7 minutes at room temperature. This step and each following step (except for 12) are performed while shaking.
4. Wash 3 times with PBS, 5 minutes per wash.
5. Permeabilize cells for 15 minutes with 0.2% Triton/PBS.
6. Block cells for at least 90 minutes with 10% NGS/0.05% Triton/PBS at room temperature.
7. Replace blocking buffer with primary antibodies diluted in 5% NGS/0.05% Triton/PBS for 60 minutes at room temperature.
8. Wash 5 times with 1% NGS/0.05% Triton/PBS for 15 minutes per wash at room temperature.
9. Incubate cells with secondary antibodies diluted in 5% NGS/0.05% Triton/PBS for 30 minutes at room temperature.
10. Wash 5 times with 1% NGS/0.05% Triton/PBS for 15 minutes per wash at room temperature.
11. Wash once with PBS for 5 minutes.
12. Post-fix with 3.0% PFA/0.1% glutaraldehyde for 10 minutes without shaking.
13. Wash 3 times with PBS, 5 minutes per wash.
14. Wash 2 times with dH₂O, 3 minutes per wash.



2. IMMUNOSTAINING PROTOCOL USING ACTIVATOR-REPORTER ANTIBODIES.

The following validated protocol is intended to serve only as a starting guide. Please refer to the primary antibody manufacturer for appropriate dilutions if other targets are selected. The fixation, permeabilization and labeling conditions were carefully selected and validated for these specific targets in BS-C-1 cells only. Other target combinations and cell types might require different fixation, permeabilization and labeling conditions.

Example targets: Microtubules and Mitochondria for dual staining and Clathrin for single staining.

SOLUTIONS

Check box

- Fixation solution: 3% paraformaldehyde (PFA) *1 + 0.1% glutaraldehyde*1 in PBS ☐
- Reducing buffer (prepare immediately before use): 0.1% NaBH₄ in PBS ☐
- Blocking buffer: 3% BSA + 0.2% Triton X-100 in PBS ☐
- Washing buffer: 0.2% BSA + 0.05% Triton X-100 in PBS ☐
- Diluted primary antibody solution: To stain for *tubulin and mitochondria*, use a mixture of both antibodies diluted in blocking buffer with a final concentration of 10 µg/ml for the tubulin Ab and 4 µg/ml for the mitochondrial Ab. To stain for *clathrin*, use a mixture of both clathrin antibodies diluted in blocking buffer with a final concentration of 2.0 µg/ml for each antibody. ☐
- Diluted secondary antibody solution: Dilute antibodies 1:100 in blocking buffer for a final concentration of 3.0 µg/ml per antibody. ☐

PROTOCOL

1. Clean Lab-Tek II chambered cover glass by sonicating in 1M potassium hydroxide*¹ for 15 minutes. Rinse thoroughly with Milli-Q water and sterilize for at least 30 minutes under UV light in a biosafety cabinet.
2. Plate cells (about 12,000 cells/well), 50-60% confluency on Lab-Tek II chambered cover glass.
3. Wash with 500 µl PBS once.
4. Fix with 200 µl of 3% PFA*¹ + 0.1% glutaraldehyde*¹ for 10 minutes at room temperature.
5. Reduce with 200 µl of 0.1% NaBH₄ (prepared immediately before use) for 7 minutes at room temperature. This step and each following step (except for 14 and 16) are performed while shaking.
6. Wash 3 times with 500 µl PBS, 5 minutes per wash.
7. Block cells in 200 µl blocking buffer for 20 minutes if staining for microtubules and mitochondria, or 120 mins for clathrin, at room temperature, rocking.
8. Aspirate blocking buffer.
9. Add 150 µl primary antibody dilutions in blocking buffer and incubate 30 for microtubule and mitochondrial staining, or 60 mins for clathrin staining, at room temperature, rocking.
10. Aspirate and wash 5 times with 200 µl washing buffer for 15 minutes per wash at room temperature, rocking.
11. Add 150 µl labeled secondary antibody dilutions in blocking buffer, covered with foil or otherwise protected from light, and incubate for 30 minutes at room temperature, rocking.
12. Aspirate and wash 3 times with 200 µl washing buffer for 10 minutes per wash at room temperature, rocking.
13. Wash once with 500 µl PBS for 5 minutes.
14. Post-fix with 200 µl 3% PFA*¹ + 0.1% glutaraldehyde*¹ for 10 minutes at room temperature.
15. Wash 3 times with 500 µl PBS, 5 minutes per wash.
16. Store in 500 µl PBS. For long term storage, add 20 mM sodium azide*¹.

*¹ Sodium azide, paraformaldehyde (PFA), glutaraldehyde and potassium hydroxide are poisonous materials. Please handle them with particular care.



3. LIVE-CELL LABELING OF PROTEINS WITH SNAP-TAG

SNAP-tag is a two-step tag-based labeling system. First, a fusion protein of the target protein and the short SNAP-tag peptide is expressed in cells. A fluorescently labeled substrate reactive with SNAP is then added to the system and becomes covalently attached to the protein of interest.

The following protocol has been optimized for live-cell labeling of clathrin in BS-C-1 cells using an electroporator (Nucleofector, Lonza) and non-cell permeable Alexa Fluor 647 tag substrates. While the Nucleofector technology is efficient and has a database of optimized protocols for different cells lines, in general, any method of DNA transfection/tag delivery is applicable (lipofection, polymer-mediated delivery, microinjection, bead-loading, etc). Additionally, a variety of cell-permeable photoswitchable SNAP-reactive substrates is available and can be used.

For targets other than clathrin, standard molecular biology techniques can be used to generate a plasmid encoding a fusion protein with the SNAP-tag. A variety of plasmids containing SNAP fusion proteins are commercially available from New England Biolabs. Optimization of conditions for other targets should include transfection conditions, the delay between plasmid and substrate delivery to ensure maximum labeling efficiency, and the amount of SNAP-reactive substrate delivered.

EQUIPMENT

- Nucleofector [Lonza]
- T-25 culture flask #353109 [BD-Falcon]
- Cover glass for plating cells. Recommended: Lab-Tek II Chambered Coverglass #155409 [Thermo Scientific Nunc]
- Sonicator
- Centrifuge

REAGENTS

Transfection Reagents

- SNAP-tag containing plasmid, prepared endotoxin free
- pSNAP-Clathrin, or for added labeling density pSNAP-Clathrin-SNAP, plasmid [Addgene]
- SNAP-Surface Alexa Fluor 647 fluorescent substrate #S9136S [New England Biolabs]
- Cell Line Nucleofector Kit V #VCA-1003 [Lonza]

Other Reagents

- Culture medium for BS-C-1 cells
- Trypsin-EDTA
- Phosphate-buffered saline (PBS), 1x
- Dimethyl sulfoxide, anhydrous, $\geq 99.9\%$ #276855-100ML [Sigma-Aldrich]
- Potassium hydroxide #PX1480 [EMD Chemicals]

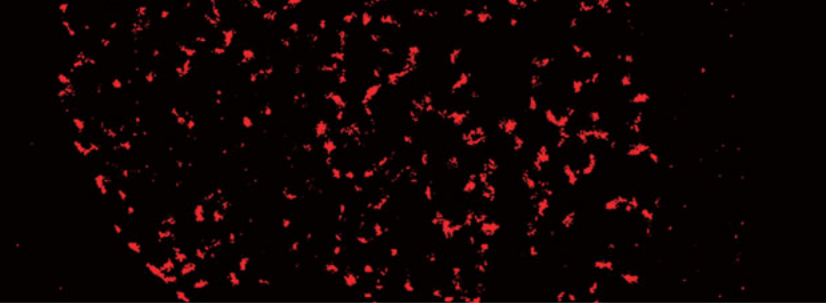
Mixed Reagents

- Labeling solution: 4 mM solution of SNAP-Surface Alexa Fluor 647 dissolved in dimethyl sulfoxide

PROTOCOL

1. After splitting BS-C-1 cells for routine maintenance and while they are in solution, spin $\sim 8 \times 10^5$ BS-C-1 cells at $90 \times g$ for 10 minutes at room temperature.
2. During the spin, allow Nucleofector Solution V to come to room temperature and pre-equilibrate a T-25 culture flask with 5 ml culture medium in the incubator.
3. Once the spin is complete, carefully aspirate the culture medium from the cell pellet and resuspend the cells in 100 μ l of Nucleofector Solution V.
4. Add 2 μ g of SNAP-tag containing plasmid DNA. Pipette up and down several times to mix.
5. Transfer the mixture to the supplied nucleofection cuvette. Tap the cuvette gently to ensure there are no bubbles in the solution.
6. Using the Nucleofector device, electroporate the cuvette using program X-001.
7. Immediately add 500 μ l of pre-warmed culture medium and use the supplied transfer pipette to place the mixture into the pre-equilibrated T-25 culture flask.
8. Incubate the cells for 18 hours, or until maximal SNAP-tag protein expression.
9. In the interim, clean Lab-Tek II chambered cover glass by sonicating in 1M potassium hydroxide*² for 15 minutes. Rinse thoroughly with Milli-Q water and sterilize for at least 30 minutes under UV light on a clean bench.
10. Aspirate the medium from the T-25 flask, rinse once with PBS, and add 1.5 ml trypsin. Incubate at 37°C for 1-3 minutes or until the cells detach.
11. Add 3.5 ml culture medium and pipette up and down gently to mix the cell suspension.
12. Take 3.5 ml of the resulting cell suspension and spin at $90 \times g$ for 10 minutes at room temperature.
13. During the spin, allow Nucleofector Solution V to come to room temperature and, adding 800 μ l of culture medium per well, place the Lab-Tek dish in the incubator.
14. Once the spin is complete, carefully aspirate the culture medium from the cell pellet and resuspend the cells in 100 μ l of Nucleofector Solution V.
15. Protecting the SNAP-Surface Alexa Fluor 647 substrate from light, add 2.5 μ l to the cell suspension and pipette up and down to gently mix.
16. Transfer mixture to supplied nucleofection cuvette. Tap the cuvette gently to ensure there are no bubbles in the solution.
17. Using the Nucleofector device, electroporate the cuvette using program T-030.
18. Immediately add 500 μ l of pre-warmed culture medium and use the supplied transfer pipette to place the mixture into a 1.5 ml Eppendorf tube.
19. Plate 25-30 μ l of the cell suspension per well of the pre-equilibrated Lab-Tek dish, depending on desired cell density.
20. Incubate for $\sim$ 18 hours before imaging.

*² Potassium hydroxide is poisonous material. Please handle with particular care.



4. LABELING ANTIBODIES WITH SINGLE OR TANDEM DYE PAIRS

EQUIPMENT

- NAP-5 Columns #17-0853-02 [GE Healthcare]
- Evaporator (e.g. Vacufuge® EF27220B [Eppendorf])
- Shaking platform
- UV/Visible absorption spectrophotometer

REAGENTS

Recommended Secondary Antibodies

- AffiniPure Donkey Anti-Rat IgG (H+L) #712-005-153 [Jackson ImmunoResearch Europe]
 - AffiniPure Donkey Anti-Rabbit IgG (H+L) #711-005-152 [Jackson ImmunoResearch Europe]
 - AffiniPure Donkey Anti-Mouse IgG (H+L) #715-005-151 [Jackson ImmunoResearch Europe]
- Or other secondary antibody of your preference

Reporter Dye

Alexa Fluor 647 carboxylic acid, succinimidyl ester, 1 mg #A20006 [Invitrogen]

Activator Dyes

- Alexa Fluor 405 carboxylic acid, succinimidyl ester, 1 mg #A30000 [Invitrogen]
- Cy2 bis-Reactive Dye Pack 5 vials #PA22000 [GE Healthcare]
- Cy3 Mono-reactive Dye Pack 5 vials #PA23001 [GE Healthcare]

Other Reagents

- Dimethyl Sulfoxide, anhydrous, ≥99.9% #276855-100ML [Sigma-Aldrich]
 - Phosphate-Buffered Saline (PBS), 1x
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-
-

PROTOCOL

1. To aliquot the Alexa dyes, dissolve 1.0 mg in anhydrous DMSO and aliquot again into tubes for a final 0.02 mg amount of dye per tube. For the Cy dyes, dissolve one dye pack into a sufficient amount of anhydrous DMSO to allow distribution into 10 new aliquots.
2. Using an evaporator, remove all DMSO.
3. Store aliquots at -20°C.
4. For labeling with the photoswitchable dye Alexa 647 alone, dissolve one Alexa 647 aliquot in 10 µl anhydrous DMSO. For labeling with an activator-Alexa 647 pair, dissolve one activator (Alexa 405, Cy2 or Cy3) aliquot in 10 µl anhydrous DMSO and one Alexa 647 aliquot in 10 µl anhydrous DMSO.
5. Recommended labeling mixtures:
Labeling with Alexa 647 alone: Mix 50 µl secondary antibody (1.25 mg/ml in PBS) with 6 µl of 1M NaHCO₃ and 1.5 µl Alexa647.
Labeling with an activator-Alexa647 pair: Mix 50 µl secondary antibody (1.25 mg/ml in PBS) with 6 µl of 1M NaHCO₃, 1.5 µl Cy3 (or 4 µl Alexa 405 or 5 µl Cy2) and 0.6 µl Alexa 647.
6. Allow the reaction to proceed for 30 minutes at room temperature, wrapped in foil or otherwise protected from light, on a shaking platform.
7. During the reaction equilibrate NAP-5 gel filtration columns, one per labeling reaction, by running three column volumes of PBS.
8. Bring the reaction volume up to 200 µl with PBS (add ~ 140 µl) and gently vortex.
9. Add the entire volume to the center of the column.
10. Allow the sample to enter the column and after the last drip, add 550 µl PBS to wash.
11. Add 300 µl PBS and collect the eluent into a 1.5 ml Eppendorf tube.
12. Repeat for all reactions.
13. Measure absorbance of labeled secondary antibody on UV/Visible spectrophotometer.

Recommended Labeling Ratio

Labeling with Alexa 647 alone – Antibody : Dye = 1:1-3

Labeling with an activator-reporter pair

Activator Dye : Antibody : Reporter Dye = 2.0 - 3.0 : 1 : 0.6 - 1

The above ratios are suggested for the specific dyes described here, but optimal dye ratios can vary for different photoswitchable probe molecules. In general, the conditions provided here serve as a starting point and can be tuned according to the user's results.

CALCULATIONS FOR CONCENTRATION OF AN ANTIBODY AND DYES

The labeling ratio is calculated by concentrations of the antibody, activator dye and reporter dye. These concentrations are calculated using Beer-Lambert's law.

For Antibody

The concentration of antibody is calculated by "A_{280(actual)}". A₂₈₀, A₄₀₅, A₆₄₇ show absorbance at 280nm, 405nm and 647nm, respectively. A_{280(actual)} shows actual absorbance of labeling antibody and A_{280(measured)} shows a measured value by UV/Visible absorption spectrophotometer. Also, Correction Factor at 280 nm of each labeling dye (Cy2, Cy3, Alexa405, Alexa647) is represented by CF_{280, dye name}. If you choose Alexa405 as a labeling dye pair of Alexa647, A_{280(actual)} is calculated by following equation.

$$A_{280}(\text{actual}) = A_{280}(\text{measured}) - CF_{280, \text{Alexa405}} \times A_{405}(\text{measured}) - CF_{280, \text{Alexa647}} \times A_{647}(\text{measured})$$

$$CF_{280, \text{Cy2}} = 0.15$$

$$CF_{280, \text{Cy3}} = 0.08$$

$$CF_{280, \text{Alexa405}} = 0.70$$

$$CF_{280, \text{Alexa647}} = 0.03$$

$$\text{Concentration of Antibody} = A_{280}(\text{actual}) / \epsilon_{280}$$

ϵ_{280} = Extinction coefficient at 280 nm

E ϵ

For Dye

The concentration of labeling dye is calculated by absorbance maxima.

Measured absorbance maxima is equal to absorbance maxima as actual λ_{max} : A _{λ_{max} (actual)}.

$$\text{Concentration of labeling dye} = A_{\lambda_{\text{max}}}(\text{actual}) / \epsilon_{\lambda_{\text{max}}}$$

$\epsilon_{\lambda_{\text{max}}}$ = Extinction coefficient at the wavelength of absorbance maxima

EXAMPLE EXPERIMENT

Absorbances: $A_{\lambda_{\max}}(\text{actual}) = \text{Measured absorbance maxima}$

Cy2 $\rightarrow$ 0.42 (at 489 nm)

Cy3 $\rightarrow$ 0.30 (at 550 nm)

Alexa 405 $\rightarrow$ 0.12 (at 401 nm)

Alexa 647 $\rightarrow$ 0.16 (at 650 nm)

Extinction coefficients:

Cy2 $\rightarrow$ 150,000 (at 489 nm)

Cy3 $\rightarrow$ 150,000 (at 550 nm)

Alexa 405 $\rightarrow$ 34,000 (at 401 nm)

Alexa 647 $\rightarrow$ 239,000 (at 650 nm)

Concentrations: $A_{\lambda_{\max}}(\text{actual}) / \text{Extinction coefficient of dye}$

Cy2 $\rightarrow$ $0.42/150,000 \rightarrow 2.8 \mu\text{M}$

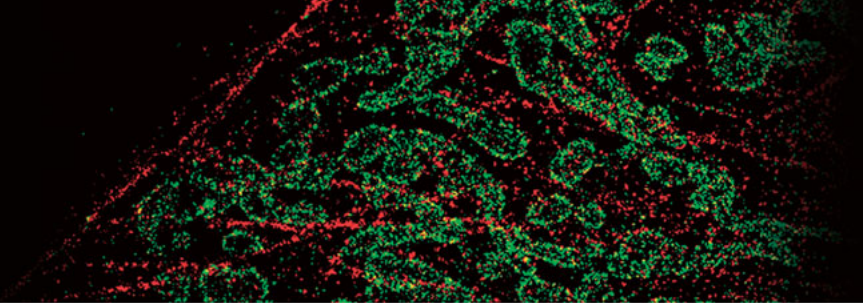
Cy3 $\rightarrow$ $0.30/150,000 \rightarrow 2.0 \mu\text{M}$

Alexa 405 $\rightarrow$ $0.12/34,000 \rightarrow 3.5 \mu\text{M}$

Alexa 647 $\rightarrow$ $0.16/239,000 \rightarrow 0.7 \mu\text{M}$

If the labeling ratio does not fall within the range in previous page, repeat the labeling reaction and adjust the amount of dye added accordingly. For example, if you obtained a reporter dye ratio of 0.35, then double the reporter dye volume for the subsequent labeling.

We recommend the use of labeled secondary antibody as soon as possible that is stored at 4°C protected from light.



5. STORM IMAGING BUFFER PROTOCOL

Several types of imaging buffers are outlined in this section. In general, imaging buffer containing 2-mercaptoethanol is recommended for dyes Alexa 647 and Cy3B. However, if Atto 488 or Alexa 568 dyes are being used, MEA containing imaging buffer is preferable. When the experiment involves dual staining with Alexa 647 and Atto 488 or Aelxa 568, MEA-containing imaging buffer is recommended. Please refer to Dempsey et al. (2011) for more details.

REAGENTS

Recommended Reagents

- 2-Mercaptoethanol #63689-100ML-F [Sigma-Aldrich]
- Cysteamine (MEA) #30070-50G [Sigma-Aldrich]
- Glucose Oxidase from *Aspergillus niger*-Type VII, lyophilized powder, $\geq 100,000$ units/g solid #G2133-250KU [Sigma-Aldrich]
- Catalase from bovine liver -lyophilized powder, $\geq 10,000$ units/mg protein #C40-100MG [Sigma-Aldrich]
- 1M Tris pH 8.0
- 1N HCl*³
- Phosphate-Buffered Saline (PBS), 1x
- NaCl
- Dulbecco's Modified Eagle Medium (DMEM) , High Glucose, HEPES, no Phenol Red #21063-029 [Invitrogen]
- HEPES, 1M, pH 7.4 #15630-106 [Invitrogen]

SOLUTIONS

Check box

- | | |
|---|--------------------------|
| • Buffer A : 10 mM Tris (pH 8.0) + 50 mM NaCl | ☐ |
| • Buffer B : 50 mM Tris-HCl (pH 8.0) + 10 mM NaCl + 10% Glucose | ☐ |
| • Buffer C for live cell : DMEM + 75 mM HEPES + 2% Glucose | ☐ |
| • GLOX solution (250 µl) | ☐ |
| 1. Prepare 100 µl of 70mg/ml Glucose oxidase solution with Buffer A. Gently mixed by pipetting. | |
| 2. Prepare 100 µl of 17mg/ml Catalase solution with Buffer A. Gently mixed by pipetting. | |
| 3. Mix 100 µl of Glucose oxidase solution and 25 µl of Catalase solution. | |
| 4. Spin down and use supernatant. | |
| • 1M MEA (1 ml) | ☐ |
| 77 mg MEA / 1.0 ml of 0.25N HCl*3 | |
| Store at 4°C for up to 1 month | |
| • MitoTracker® Red CMXRos | ☐ |
| Dissolve the MitoTracker Red powder with anhydrous dimethylsulfoxide (DMSO) to stock concentration (1mM). Dilute 1mM stock solution of MitoTracker Red solution to the final concentration (500nM – 1µM) in Buffer C. | |

Method A

STORM Imaging Buffer with MEA

1. On ice, add 7.0 µl GLOX, 70 µl 1M MEA and 620 µl Buffer B to a 1.5 ml Eppendorf tube and vortex gently to mix. Keep the GLOX and MEA stored on ice or at 4°C.
2. Add sufficient imaging buffer in the well: for example 700 µl per well of Lab-Tek II chambered cover glass.
3. The samples can be used in imaging buffer for up to several hours.

Method B

STORM Imaging Buffer with 2-mercaptoethanol

1. On ice, add 7.0 µl GLOX, 7.0 µl 2-mercaptoethanol and 690 µl Buffer B.
2. Add sufficient imaging buffer in the well: for example 700 µl per well of Lab-Tek II chambered cover glass.
3. The samples can be used in imaging buffer for up to several hours.

Method C

Live Cell STORM Imaging Buffer with 2-Mercaptoethanol

1. Combine 7 µl GLOX, 3.5 µl 2-mercaptoethanol and 690 µl Live Cell Imaging Buffer. Keep the GLOX and 2-mercaptoethanol stored on ice or at 4°C.
2. Add in excess to the sample, for example 700 µl per well of Lab-Tek II chambered coverglass.
3. Use once for up to 60 minutes, depending on cell viability under the given imaging conditions, in a well-sealed sample.

Method D

Live Cell STORM Imaging Buffer with MEA

1. Add 7 µl GLOX, 4.2 µl 1M MEA and 690 µl Live Cell Imaging Buffer to a 1.5 ml Eppendorf tube and vortex gently to mix. Keep the GLOX and MEA stored on ice or at 4°C.
2. Add in excess to the sample: for example 700 µl per well of Lab-Tek II chambered coverglass.
3. Use once for up to 60 minutes, depending on cell viability under the given imaging conditions, in a well-sealed sample.

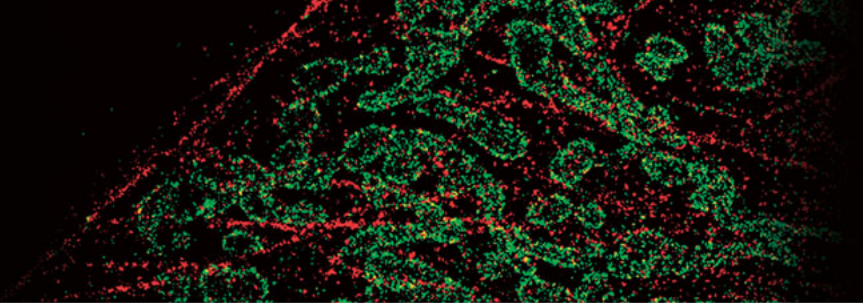
Method E

Live Cell STORM Imaging with MitoTracker

Imaging chamber is filled with MitoTracker Red working solution in Buffer C. Incubate at 37°C for 30 sec – 1 min, then wash with DMEM, 3 times. After washing, chamber is filled with Buffer C with GLOX. (Buffer C with GLOX; 100:1). The chamber should be sealed with vacuum grease.

The buffer can be used for ~1hr with large buffer volume (~800µL, i.e. no air supplying O₂) in the chamber sealed with vacuum grease and topped with a glass cover-slide.

*3 HCl is a poisonous material. Please handle with particular care.



6. FLUORESCENT BEADS AS FIDUCIAL MARKERS: ATTACHMENT TO THE GLASS SURFACE

The dilutions listed are for 20nm beads (2% solid). Other beads can be used, but the dilution rate will vary. Melting is not recommended for larger beads because the shape of the bead can deform significantly with heating, resulting in inaccurate fitting of the position.

Care should be taken to maintain sterility of the culture dishes and all reagents throughout the labeling process. Culture dishes should be UV irradiated prior to bead attachment, and any subsequent irradiation would result in photo-bleaching of the fluorescent of the fluorescent beads.

Method 1 : Beads Melting

Reagents:

- FluoSpheres® Carboxylate-Modified Microsphere, 0.02 μm , Red Fluorescent (580/605), 2% solids (Invitrogen, F-8786)
- 1M Magnesium Chloride solution

Equipment:

- Heating block, preheated to 96°C
- Cells cultures dishes (previously KOH cleaned and UV sterilized)

Protocol:

1. Make a 1:500 stock solution of beads with dH₂O. Sonicate for several minutes to break up any aggregates.
2. Make a working dilution of 1:400 from the stock solution with dH₂O (final dilution factor 20,000x) and sonicate the working solution.
3. Add a 1:20 dilution of 1M MgCl₂ to the desired amount of bead solution, for final concentration of 50mM MgCl₂.
4. Under sterile conditions, add the bead solution to the culture dishes (~200 μL per well for a LabtekII 8-well chambered cover-glass). Cover the dishes with foil to protect from the light and incubate for 15 minutes at room temperature.
5. Place the dishes on the heating block with a layer of foil between the heating surface and the dishes and a second layer of foil covering the dishes. Incubate for 30 minutes at 96°C
6. Remove the dishes from the heating block and cooling at room temperature for 30 minutes with lids.
7. Under sterile conditions, rinse the dishes 3 times with dH₂O by pipetting. (Do not use a vacuum line to aspirate the water. This can disrupt the cooling beads).
8. Dry up the dishes after rinse. Wrapped with a foil for protecting from the light. Once dry up, cells may be plated directly on the top of the beads.

Method 2: Chemical Conjugation with EDAC

Reagents:

- FluoSpheres® Carboxylate-Modified Microsphere, 0.02µm, Red Fluorescent (580/605), 2% solids (Invitrogen, F-8786)
- Poly-L-lysine solution (0.01%, cell culture tested, Sigma Aldrich, P4832)
- EDAC (1-Ethy-3-(3-Dimethylaminopropyl) carbodiimide, Hydrochloride, Invitrogen, E-2247)
- 50mM MES (2-[N-morpholino] ethane sulfonic acid) solution (cell culture grade, Sigma Aldrich)
 - pH solution to 5.5
 - Sterilize with 0.2 µm membrane filter
- 0.5% Glycine solution (cell culture grade, Sigma Aldrich)

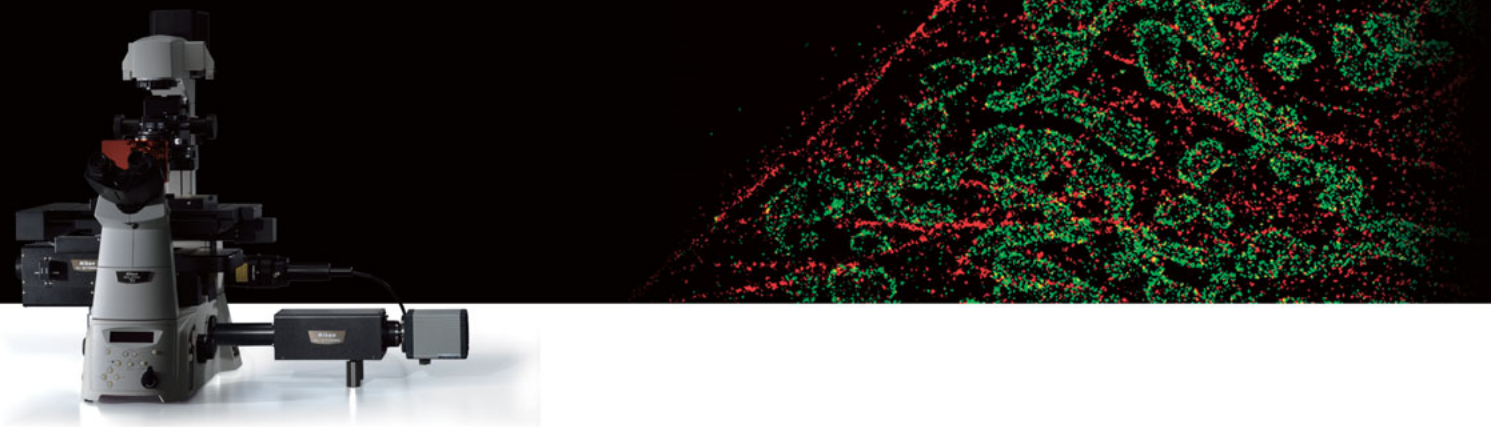
Equipment:

Cells cultures dishes (previously KOH cleaned and UV sterilized)

Rocking platform

Protocol:

1. Make a 1:500 stock solution of beads in water. Sonicate for several minutes to break up any aggregates.
2. Under sterile conditions, add the poly-L-lysine solution to the culture dishes (~200 µL per well for a LabtekII 8-well chambered cover-glass). Replace the lids on the cultures dishes and incubate for 5 minutes at room temperature on the rocking platform.
3. Immediately before use prepare EDAC. Allow EDAC warm up to room temperature before opening. Dissolve the desired amount of EDCA in 50mM MES to a concentration of 0.2 mg/mL.
4. Dilute beads stock 1:300 into EDAC solution (final bead dilution factor 15,000x). Vortex to mix.
5. Under sterile conditions, add EDAC/beads solution to the culture dishes. Replace lids, cover with foil to protect from light. Place on the rocking platform and incubate for 60 minutes at room temperature.
6. Under sterile conditions, rinse the dishes 3 times with MilliQ water. Do not use a vacuum line to aspirate the water as this can disrupt the beads. Instead use a pipette to both add and aspirate the water while rinsing.
7. Under sterile conditions, add 0.5% glycine solution to culture dishes. Replace lids, cover with foil and place on the rocking platform for 60 minutes.
8. Under sterile conditions, rinse the dishes 3 times with MilliQ water, using a pipette to both add and aspirate the water.
9. After the final rinse store the dishes under MilliQ water, protected from light. Aspirate water immediately before adding cell suspension to plates.



7. REFERENCES AND RECOMMENDED READING

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Shim, S-H., Xia, C., Zhong, G., Babcock, H.P., Vaughan, J.C., Huang, B., Wang, X., Xu, C., Bi, G-Q., Zhuang, X. (2012) Super-resolution fluorescence imaging of organelles in live cells with photoswitchable membrane probes. *PNAS*, 109 (35): 13978-83.

Note: ® is a registered mark, and ™ is a trade mark.

Specifications and equipment are subject to change without any notice or obligation on the part of the manufacturer. June 2015 ©2010-15 NIKON CORPORATION



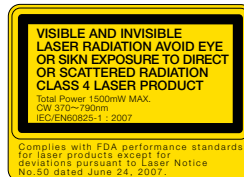
WARNING

TO ENSURE CORRECT USAGE, READ THE CORRESPONDING MANUALS CAREFULLY BEFORE USING YOUR EQUIPMENT.

Monitor images are simulated.

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NIKON CORPORATION

Shinagawa Intercity Tower C, 2-15-3, Konan, Minato-ku, Tokyo 108-6290, Japan
phone: +81-3-6433-3705 fax: +81-3-6433-3785
<http://www.nikon.com/instruments/>

NIKON INSTRUMENTS INC.

1300 Walt Whitman Road, Melville, N.Y. 11747-3064, U.S.A.
phone: +1-631-547-8500; +1-800-52-NIKON (within the U.S.A. only)
fax: +1-631-547-0306

<http://www.nikoninstruments.com/>

NIKON INSTRUMENTS EUROPE B.V.

Tripolis 100, Burgenweeshuispad 101, 1076 ER Amsterdam, The Netherlands
phone: +31-20-7099-000 fax: +31-20-7099-298

<http://www.nikoninstruments.eu/>

NIKON INSTRUMENTS (SHANGHAI) CO., LTD.

CHINA phone: +86-21-6841-2050 fax: +86-21-6841-2060
(Beijing branch) phone: +86-10-5831-2028 fax: +86-10-5831-2026
(Guangzhou branch) phone: +86-20-3882-0550 fax: +86-20-3882-0580

NIKON SINGAPORE PTE LTD

SINGAPORE phone: +65-6559-3651 fax: +65-6559-3668

NIKON INSTRUMENTS KOREA CO., LTD.

KOREA phone: +82-2-2186-8400 fax: +82-2-555-4415

NIKON CANADA INC.

CANADA phone: +1-905-602-9676 fax: +1-905-602-9953

NIKON FRANCE S.A.S.

FRANCE phone: +33-1-4516-45-16 fax: +33-1-4516-45-55

NIKON GMBH

GERMANY phone: +49-211-941-42-20 fax: +49-211-941-43-22

NIKON INSTRUMENTS S.p.A.

ITALY phone: +39-55-300-96-01 fax: +39-55-30-09-93

NIKON AG

SWITZERLAND phone: +41-43-277-28-67 fax: +41-43-277-28-61

NIKON UK LTD.

UNITED KINGDOM phone: +44-208-247-1717 fax: +44-208-541-4584

NIKON GMBH AUSTRIA

AUSTRIA phone: +43-1-972-6111-00 fax: +43-1-972-6111-40

NIKON BELUX

BELGIUM phone: +32-2-705-56-65 fax: +32-2-726-66-45

NIKON INSTECH CO., LTD.

Shinagawa Intercity Tower C, 2-15-3, Konan, Minato-ku, Tokyo 108-6290, Japan
phone: +81-3-6433-3705 fax: +81-3-6433-3785