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Super Resolution Microscope

N-STORM

STORM Protocol-Sample Preparation

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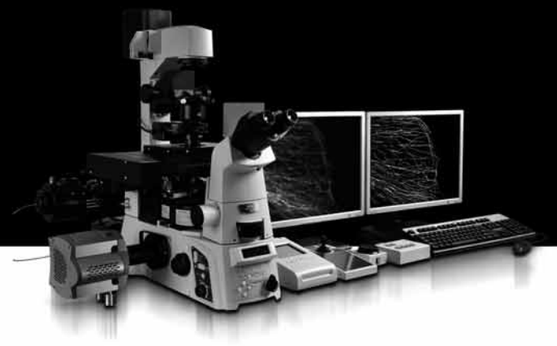


The STORM technique is a super-resolution imaging method that uses sequential activation and high-precision localization of individual fluorophores to achieve sub-diffraction-limit spatial resolution. To use this method, the biological structure of interest simply needs to be labeled with photoswitchable 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, Halo-tag, SorTag, etc) genetically fused to the protein of interest.
In the following, a few example protocols to label microtubules and clathrin-coated pits with photoswitchable dyes using immunolabeling and the SNAP tag are described. The example photoswitchable dyes used here include Alexa Fluor 647 dye alone, and activator-reporter pairs in which Alexa Fluor 647 dye serves as the reporter and Alexa Fluor 405, Cy2 and Cy3 serve as activators.

1. Dye Preparation and Antibody Labeling

MATERIALS AND REAGENTS

Recommended Secondary Antibodies

- AffiniPure Donkey Anti-Rat IgG (H+L) #712-005-153 [Jackson ImmunoResearch Europe]
- AffiniPure Donkey Anti-Rabbit IgG (H+L) #712-005-152 [Jackson ImmunoResearch Europe]
- AffiniPure Donkey Anti-Mouse IgG (H+L) #712-005-151 [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), 1×

EQUIPMENT

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

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 $NaHCO_3$ 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 $NaHCO_3$, 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 - 3 : 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)". A_{280}, A_{405}, A_{647} 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. Furthermore, the Correction Factor at 280 nm of each labeling dye (Cy2, Cy3, Alexa405, Alexa647) is represented by $CF_{280,\ \text{dye name}}$. If you choose Alexa405 as a labeling dye partner for Alexa647, A_{280}(actual) is calculated by the following equation.

- $A_{280}(\text{actual}) = A_{280}(\text{measured}) - CF_{280,\ \text{Alexa405}} \times A_{405}(\text{measured}) - CF_{280,\ \text{Alexa647}} \times A_{647}(\text{measured})$
  $CF_{280,\ Cy2} = 0.15$
  $CF_{280,\ Cy3} = 0.08$
  $CF_{280,\ \text{Alexa405}} = 0.70$
  $CF_{280,\ \text{Alexa647}} = 0.03$

- Concentration of Antibody = $A_{280}(\text{actual}) / \varepsilon_{f280}$
  ε_{f280} = Extinction coefficient at 280 nm

For Dye

The concentration of labeling dye is calculated by absorbance maxima.

- Measured absorbance maxima is equal to absorbance maxima as actual λmax: $A_{\lambda max}$(actual).
- Concentration of labeling dye = $A_{\lambda max}$(actual) / $\varepsilon f_{\lambda max}$

  $\varepsilon f_{\lambda max}$ = Extinction coefficient at the wavelength of absorbance maxima

EXAMPLE EXPERIMENT

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

Cy2 → 0.42 (at 489 nm)
Cy3 → 0.30 (at 550 nm)
Alexa 405 → 0.12 (at 401 nm)
Alexa 647 → 0.16 (at 650 nm)

Extinction coefficients:

Cy2 → 150,000 (at 489 nm)
Cy3 → 150,000 (at 550 nm)
Alexa 405 → 34,000 (at 401 nm)
Alexa 647 → 239,000 (at 650 nm)

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

Cy2 → 0.42/150,000 → 2.8 µM
Cy3 → 0.30/150,000 → 2.0 µM
Alexa 405 → 0.12/34,000 → 3.5 µM
Alexa 647 → 0.16/239,000 → 0.7 µM

If the labeling ratio does not fall within the range given above, 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.

2a. Immunofluorescence Protocol A

The following validated protocol is intended to serve as a guide to get started.
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.
In general, any immunofluorescence protocol may be used. When developing a protocol for super-resolution microscopy, in comparison to conventional fluorescence microscopy, users should take particular care that the cellular ultrastructure is preserved and that the final labeling density is sufficient to accurately represent the structure of interest.

Example: Microtubules and Mitochondria

MATERIALS AND REAGENTS

Recommended Primary Antibody

- 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]

Labeled Secondary Antibody

Other Reagents

- Phosphate-Buffered Saline (PBS), 1×
- Sodium borohydride $NaBH_4$ 99% #213462-25G [Sigma-Aldrich]
- Paraformaldehyde Aqueous Solution-16%, 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]

Mixed Reagents

- Fixation solution: 3% paraformaldehyde (PFA)[*1] + 0.1% glutaraldehyde[*1]
- Blocking buffer: 3% BSA + 0.2% Triton X-100 in PBS
- Washing buffer: 0.2% BSA + 0.05% Triton X-100 in PBS

EQUIPMENT

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

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. Immediately before use prepare 0.1% $NaBH_4$ in PBS.
6. Reduce with 200 µl of 0.1% $NaBH_4$, 7 minutes at room temperature.
7. Wash 3 times with 500 µl PBS.
8. Block cells in 200 µl blocking buffer for 20 minutes at room temperature, rocking.
9. Aspirate blocking buffer.
10. Add 150 µl primary antibody dilutions in blocking buffer and incubate 30 minutes at room temperature, rocking.
    Example experiment: 6.0 µl rat anti beta-tubulin (final conc. 10 µg/ml) and 12.0 µl rabbit anti Tom20 (final conc. 4 µg/ml) were diluted in 600 µl blocking buffer.
11. Aspirate and wash 3 times with 200 µl washing buffer for 10 minutes per wash at room temperature, rocking.
12. 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.
    Example experiment: All labeled secondary antibody were diluted 1:100 (final conc. 3.0 µg/ml) in 600 µl blocking buffer.
13. Aspirate and wash 3 times with 200 µl washing buffer for 10 minutes per wash at room temperature, rocking.
14. Wash with 500 µl PBS once.
15. Post-fix with 200 µl 3% PFA[*1]+0.1% glutaraldehyde[*1] for 10 minutes at room temperature.
16. Wash 3 times with 500 µl PBS.
17. Store in 500µl 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.

2b. Immunofluorescence Protocol B

The following validated protocol is intended to serve as a guide to get started.
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: Clathrin

MATERIALS AND REAGENTS

Recommended Primary Antibody

- 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]

Labeled Secondary Antibody

Other Reagents

- Phosphate-Buffered Saline (PBS), 1×
- Sodium borohydride $NaBH_4$ 99% #213462-25G [Sigma-Aldrich]
- Paraformaldehyde[*2] Aqueous Solution-16%, EM grade #15710 [Electron Microscopy Sciences]
- Glutaraldehyde[*2] 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]

Mixed Reagents

- Fixation solution: 3% paraformaldehyde (PFA)[*2] + 0.1% glutaraldehyde[*2]
- Blocking buffer: 3% BSA + 0.2% Triton X-100 in PBS
- Washing buffer: 0.2% BSA + 0.05% Triton X-100 in PBS

EQUIPMENT

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

PROTOCOL

1. 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 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[*2] + 0.1% glutaraldehyde[*2] for 10 minutes at room temperature.
5. Immediately before use prepare 0.1% $NaBH_4$ in PBS.
6. Reduce with 200 µl of 0.1% $NaBH_4$, 7 minutes at room temperature.
7. Wash 3 times with 500 µl PBS.
8. Block cells in 200 µl blocking buffer for 120 minutes at room temperature, rocking.
9. Aspirate blocking buffer.
10. Add 150 µl primary antibody dilutions in blocking buffer and incubate 60 minutes at room temperature, rocking.
    Example experiment: 20 µl mouse anti-clathrin X22 (final conc. 2.0 µg/ml) and 50 µl mouse anti-clathrin CON.1 (final conc. 2.0 µg/ml) were diluted in 600 µl blocking buffer.
11. Aspirate and wash 5 times with 200 µl washing buffer for 15 minutes per wash at room temperature, rocking.
12. 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.
    Example experiment: Labeled secondary antibody were diluted 1:100 (final conc. 3.0 µg/ml) in 600 µl blocking buffer.
13. Aspirate and wash 3 times with 200 µl washing buffer for 10 minutes per wash at room temperature, rocking.
14. Wash with 500 µl PBS once.
15. Post-fix with 200 µl 3% PFA[*2] +0.1% glutaraldehyde[*2] for 10 minutes at room temperature.
16. Wash 3 times with 500 µl PBS.
17. Store in 500 µl PBS. For long term storage, add 20 mM sodium azide[*2].

[*2] 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.

MATERIALS AND 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

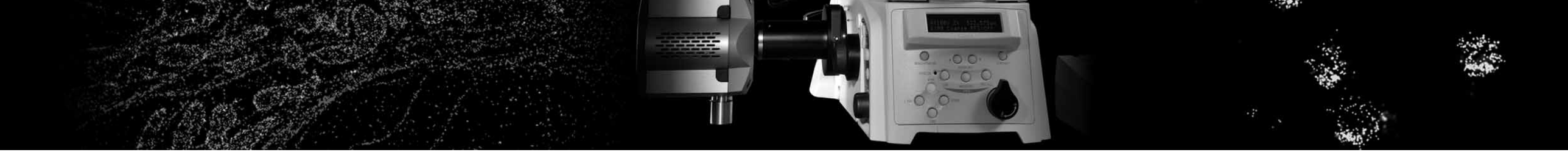
- Culture medium for BS-C-1 cells
- Trypsin-EDTA
- Phosphate-buffered saline (PBS), 1x
- Dimethyl sulfoxide, anhydrous, ≥99.9% #276855-100ML [Sigma-Aldrich]
- Potassium hydroxide #PX1480 [EMD Chemicals]

Mixed Reagents

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

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



PROTOCOL

1. After splitting BS-C-1 cells for routine maintenance and while they are in solution, spin ~8× 10^5 BS-C-1 cells at 90× 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 µl of Nucleofector Solution V.
4. Add 2 µg 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 µl 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[*3] 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 37ºC 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× g for 10 minutes at room temperature.
13. During the spin, allow Nucleofector Solution V to come to room temperature and, adding 800 µl 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 µl of Nucleofector Solution V.
15. Protecting the SNAP-Surface Alexa Fluor 647 substrate from light, add 2.5 µl 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 µl 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 µl of the cell suspension per well of the pre-equilibrated Lab-Tek dish, depending on desired cell density.
20. Incubate for ~ 18 hours before imaging.

[*3] Potassium hydroxide is poisonous material. Please handle with particular care.

4. STORM Imaging Buffer Protocol

MATERIALS AND 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, ≥ 100,000 units/g solid #G2133-250KU [Sigma-Aldrich]
- Catalase from bovine liver -lyophilized powder, ≥ 10,000 units/mg protein #C40-100MG [Sigma-Aldrich]
- 1M Tris pH 8.0
- 1N HCl[*4]
- Phosphate-Buffered Saline (PBS), 1×
- NaCl
- Dubecco's Modified Eagle Medium (DMEM) #21063 [Invitrogen]
- HEPES buffer solution, 1M, pH 8.0

Mixed Reagents

- 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
- GLOX solution (250 µl)
  14 mg Glucose Oxidase + 50 µl Catalase (17 mg/ml) + 200 µl Buffer A
  Vortex to dissolve Glucose Oxidase
  Spin down 14,000 rpm
  Only use supernatant
  Store at 4°C for up to 2 weeks
  In case of reusing spin down at 14,000 rpm again
- 1M MEA (1 ml)
  77 mg MEA + 1.0 ml 0.25N HCl[*4]
  Store at 4°C for up to 1 month
- Live Cell Imaging Buffer
  DMEM + 75 mM HEPES + 2% Glucose

Method A
STORM Imaging Buffer with MEA

1. On ice, add 7.0 µl GLOX, 70 µl 1M MEA GLOX 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.

[*4] HCl is a poisonous material. Please handle with particular care.

Memo

Memo