*Super Resolution Microscope*

# N-STORM

## STORM Protocol-Sample Preparation

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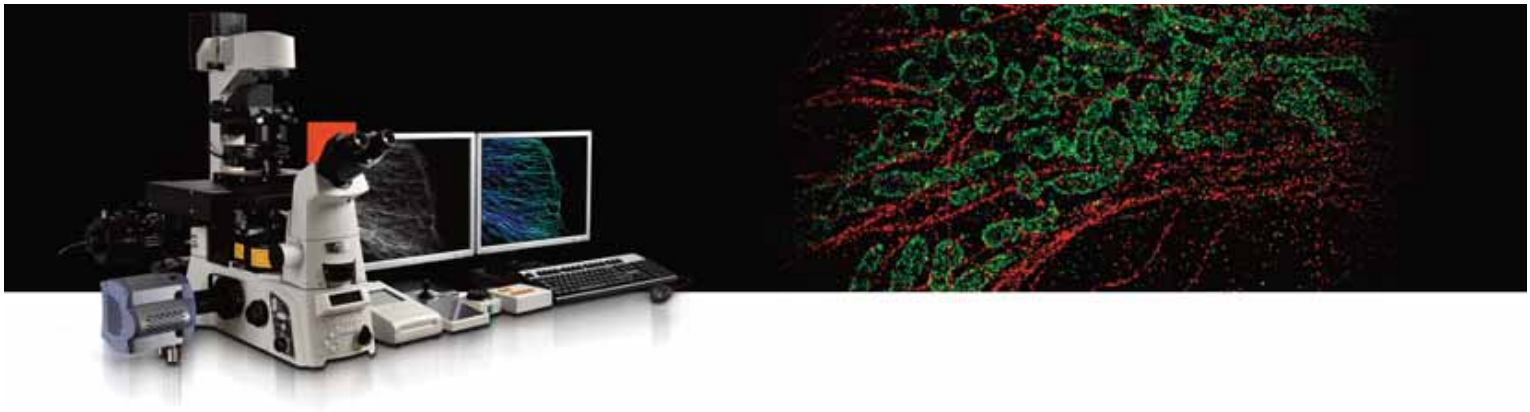
The STORM technique is a super-resolution imaging method that uses sequential activation and high-precision localization of individual fluorophores to achieve sub-diffraction-limit resolution. To use this method, the biological structure of interest needs to be labeled with photoswitchable probes. Any optically switchable probe will work. 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 Fluor®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 Fluor®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 Fluor®647 is recommended for this reporter-only method. ATTO 488, Alexa Fluor®568 or Cy™ 3B are recommended for 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 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 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.**

## 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

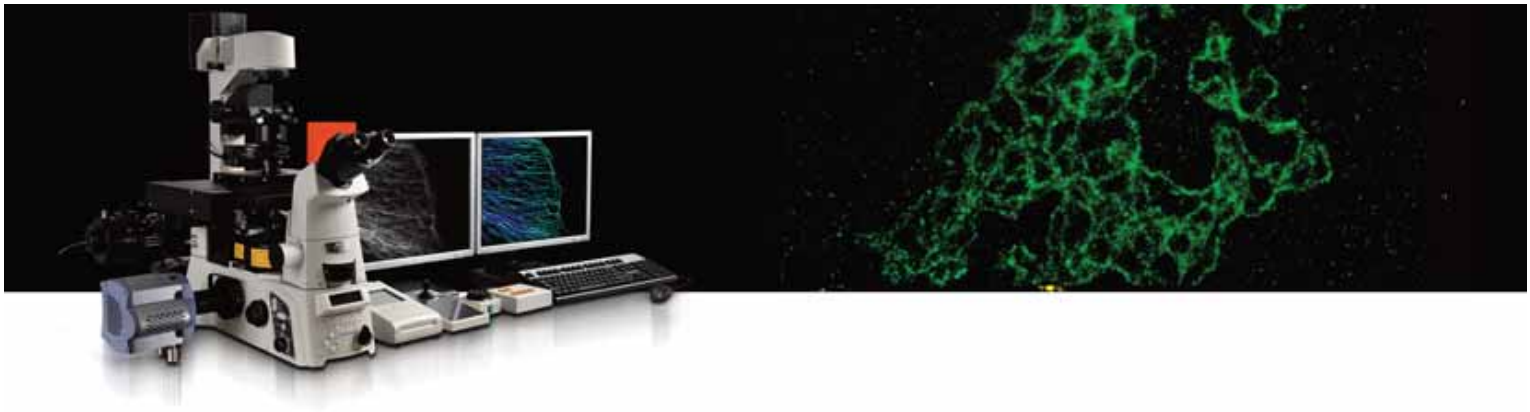
Check box

- Fixation solution: 3% paraformaldehyde (PFA) [*1] + 0.1% glutaraldehyde[*1] in PBS ☐
- Reducing buffer (prepare immediately before use): 0.1% $NaBH_4$ 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 the 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) to ~50-60% confluency on the 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% $NaBH_4$ (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 minutes for microtubule and mitochondrial staining, or 60 minutes 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.



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

The following validated protocol (optimized for goat secondary antibodies) 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. If using donkey secondary antibodies, normal donkey serum should be used instead of normal goat serum. Secondary antibodies conjugated with Alexa Fluor®647 (Invitrogen), ATTO 488 (Jackson Immuno), and Alexa Fluor®568 (Invitrogen) are recommended. Alexa Fluor®568 can also be substituted with Cy™ 3B when a yellow-absorbing dye is needed. Because of its optimal photoswitching properties, Alexa Fluor®647 should be used when staining for only one target. Please refer to the antibody manufacturer for appropriate dilutions.

## SOLUTIONS

Check box

- Fixation solution: 3% paraformaldehyde (PFA) + 0.1% glutaraldehyde in PBS ☐
- Reducing buffer (prepare immediately before use): 0.1% $NaBH_4$ 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 ☐

## PROTOCOL

1. Wash with PBS once.
2. Fix with 3.0% PFA/0.1% glutaraldehyde for 10 minutes at room temperature.
3. Reduce with 0.1% $NaBH_4$ (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 $dH_2O$, 3 minutes per wash.

# 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 ubstrate delivered.

## EQUIPMENT

- Nucleofector [Lonza]
- T-25 culture flask #353109 [BD-Falcon]
- Cover glass for plating cells. Recommended: 8-well 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, ≥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 ~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. 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.
7. Incubate the cells for 18 hours, or until maximal SNAP-tag protein expression.
8. In the interim, clean the 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.
9. 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.
10. Add 3.5 ml culture medium and pipette up and down gently to mix the cell suspension.
11. Take 3.5 ml of the resulting cell suspension and spin at 90× g for 10 minutes at room temperature.
12. 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.
13. 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.
14. 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.
15. Transfer mixture to supplied nucleofection cuvette. Tap the cuvette gently to ensure there are no bubbles in the solution.
16. Using the Nucleofector device, electroporate the cuvette using program T-030.
17. 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.
18. Plate 25-30 µl of the cell suspension per well of the pre-equilibrated Lab-Tek® dish, depending on desired cell density.
19. Incubate for ~ 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]
- Cy™ 2 bis-Reactive Dye Pack 5 vials #PA22000 [GE Healthcare]
- Cy™ 3 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

## PROTOCOL

1. To aliquot the Alexa Fluor® 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 Fluor®647 alone, dissolve one Alexa Fluor®647 aliquot in 10 µl anhydrous DMSO. For labeling with an activator-Alexa Fluor®647 pair, dissolve one activator (Alexa Fluor®405, Cy™ 2 or Cy™ 3) aliquot in 10 µl anhydrous DMSO and one Alexa Fluor®647 aliquot in 10 µl anhydrous DMSO.
5. Recommended labeling mixtures:
   Labeling with Alexa Fluor®647 alone: Mix 50 µl secondary antibody (1.25 mg/ml in PBS) with 6 µl of 1M $NaHCO_3$ and 1.5 µl Alexa Fluor®647.
   Labeling with an activator-Alexa Fluor®647 pair: Mix 50 µl secondary antibody (1.25 mg/ml in PBS) with 6 µl of 1M $NaHCO_3$, 1.5 µl Cy™ 3 (or 4 µl Alexa Fluor®405 or 5 µl Cy™ 2) and 0.6 µl Alexa Fluor®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. Measure absorbance of labeled secondary antibody on UV/Visible spectrophotometer.

Recommended Labeling Ratio
Labeling with Alexa Fluor®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 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. Also, Correction Factor at 280 nm of each labeling dye (Cy™ 2, Cy™ 3, Alexa Fluor®405, Alexa Fluor®647) is represented by $CF_{280,\ \text{dye name}}$. If you choice Alexa Fluor®405 as a labeling dye pair of Alexa Fluor®647, $A_{280}$(actual) is calculated by following equation.

- $A_{280}(\text{actual}) = A_{280}(\text{measured}) - CF_{280,\ Alexa405} \times A_{405}(\text{measured}) - CF_{280,\ Alexa647} \times A_{647}(\text{measured})$
  $CF_{280,\ Cy2} = 0.15$
  $CF_{280,\ Cy3} = 0.08$
  $CF_{280,\ Alexa405} = 0.70$
  $CF_{280,\ Alexa647} = 0.03$
- Concentration of Antibody = $A_{280}$(actual) / $\varepsilon_{f280}$
  $\varepsilon_{f280}$ = 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 $\lambda$ max: $A_{\lambda max}$(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

Cy™ 2 → 0.42 (at 489 nm)
Cy™ 3 → 0.30 (at 550 nm)
Alexa Fluor®405 → 0.12 (at 401 nm)
Alexa Fluor®647 → 0.16 (at 650 nm)

Extinction coefficients:

Cy™ 2 → 150,000 (at 489 nm)
Cy™ 3 → 150,000 (at 550 nm)
Alexa Fluor®405 → 34,000 (at 401 nm)
Alexa Fluor®647 → 239,000 (at 650 nm)

Concentrations: A $\lambda$ max(actual) / Extinction coefficient of dye

Cy™ 2 → 0.42/150,000 → 2.8 µM
Cy™ 3 → 0.30/150,000 → 2.0 µM
Alexa Fluor®405 →0.12/34,000 → 3.5 µM
Alexa Fluor®647 → 0.16/239,000 → 0.7 µM

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

Several types of imaging buffers are outlined in this section. In general, imaging buffer containing 2-mercaptoethanol is recommended for dyes Alexa Fluor®647 and Cy™ 3B. However, if ATTO 488 or Alexa Fluor®568 dyes are being used, MEA containing imaging buffer is preferable. When the experiment involves dual staining with Alexa Fluor®647 and ATTO 488 or Alexa Fluor®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, ≥ 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[*3]
- Phosphate-Buffered Saline (PBS), 1x
- NaCl
- Dulbecco's Modified Eagle Medium (DMEM) #21063 [Invitrogen]
- HEPES buffer solution, 1M, pH 8.0

## SOLUTIONS

Check box

- Buffer A : 10 mM Tris (pH 8.0) + 50 mM NaCl ☐
- Buffer B : 50 mM Tris (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 at 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[*3] ☐
  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 µl GLOX and 70 µl 1M MEA to 620 µl Buffer B in 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 a 8-well 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, combine 7 µl GLOX, 7 µl 2-mercaptoethanol and 690 µl Buffer B.
2. Add sufficient imaging buffer in the well: for example 700 µl per well of 8-well 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 an 8-well Lab-Tek® II chambered coverglass.
3. Cells can be imaged in the buffer for up to 60 minutes in a well-sealed sample. Imaging buffer should not be reused.

Method D

**Live Cell STORM Imaging Buffer with MEA**

1. Add 7 µl GLOX and 4.2 µl 1M MEA to 690 µl of Live Cell Imaging Buffer in 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 8-well Lab-Tek® II chambered coverglass.
3. Cells can be imaged in the buffer for up to 60 minutes in a well-sealed sample. Imaging buffer should not be reused.

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

## 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.


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