1st EUROPEAN (STORM) WORKSHOP AT ICFO

LIGHT FOR HEALTH L4H MADE IN ICFO

I. Sample Preparation: Immunofluorescence

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Sample Preparation

I. Sample Preparation: Immunofluorescence

Reagents List:

- BS-C-1 cells, African Green Monkey Kidney adherent cell line, (ATCC CCL-26)
- Chambered 8-well Coverglasses (Labtek II, Nunc)
- Potassium Hydroxide (1M solution)
- Paraformaldehyde, 16% or 2% Solution, EM Grade (Electron Microscopy Science)
- Glutaraldehyde, 8% Solution, EM Grade (Electron Microscopy Science)
- Dulbeccos Phosphate Buffered Saline (Invitrogen) + Calcium and Magnesium (Sigma Aldrich)
- Sodium Borohydride, 99% (Sigma Aldrich)
- Bovine Serum Albumin, IgG-Free, Protease-Free (Jackson ImmunoResearch)
- Triton-X 100 (Sigma Aldrich)

Antibodies:

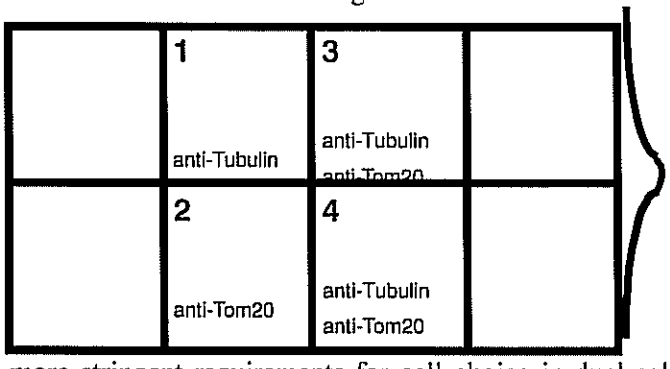
- Anti-β-Tubulin. Rat Monoclonal, [YL1/2]. 1 mg/mL. Abcam (ab6160)
- Anti-Tom20. Rabbit Polyclonal, FL-145. 0.2 mg/mL. Santa Cruz Biotechnologies (sc-11415)
- Labeled secondary antibodies (see Antibody labeling section):
  Donkey-anti-Rat – AlexaFluor405- AlexaFluor647
  Donkey-anti-Rabbit – Cy3-AlexaFluor647

Protocol:

N.B. This protocol has been optimized for BS-C-1 cells with these targets and antibodies. Optimal conditions can be very different for different samples.

(1) Rinse: Remove BS-C-1 cells, plated to ~ 75 % confluency from the cell culture incubator. The center four wells of the eight well chambers are plated with cells. Rinse the each well once with 300 uL PBS + calcium and magnesium (labeled PBS ++).

General Immunofluorescence Plan for Staining:



Since there are more stringent requirements for cell choice in dual-color imaging, it is advantageous to do the staining in duplicate wells.

(2) Fixation:

Add 150 uL of fixation buffer per well. (Fixation buffer: 3% paraformaldehyde (PFA) and 0.1% Glutaraldehyde (GA) diluted in PBS).

Incubate at room temperature (RT) for 10 minutes.

(3) Rinse: Rinse cells twice quickly with PBS (without additional Ca^{2+}/Mg^{2+})

(4) Reduce: Make a fresh solution of 0.1% (1 mg/mL Sodium Borohydride ($NaBH_4$) in PBS immediately before use.

Add ~ 200 uL per well and incubate for 7 minutes at room temperature.

(5) Rinse: Rinse cells 3X quickly with PBS.

Immunostaining:

(6) Block:

After rinsing, add ~ 150 uL per well of blocking buffer.

Blocking buffer: 3% Bovine Serum Albumin (BSA, m/v) and 0.2% TritonX -100 (v/v) in PBS.

Place the cells on the rocking platform.

Normally, this step takes ~ 30- 60 minutes, in the interest of time, we are reducing it to a 20 minute incubation. This will likely increase the non-specific background in the sample, but will still allow for STORM imaging.

(7) Primary Antibody: Dilute primary antibodies for addition.

We will do 2 wells single color staining, beta-tubulin or mitochondria (via **t**ranslocase of the **o**uter **m**embrane 20, or tom20) staining, and 2 wells of two-color staining with both antibodies. We will use ~ 150 uL of staining solution per well.

Final dilutions:

anti-β-tubulin (1:150).

anti-Tom20 (1:50).

(a) **Well 1:** Anti-β-tubulin (Rat)

☐ 149 uL blocking buffer [3% BSA (m/v) and 0.2% TritonX -100 (v/v) in PBS] + 1 uL of anti-β-tubulin

(a) **Well 2 :** Anti-Tom20 (rabbit)

☐ 147 uL blocking buffer + 3 uL of anti-Tom20

(b) **Wells 3 and 4** : Anti-β-tubulin (mouse) + Anti-Tom20 (rabbit)

☐ 296 uL blocking buffer + 1 uL anti-β-tubulin + 3 uL anti-Tom20

Add ~ 150 uL of the appropriate dilutions to each well.

Incubate at RT on the rocking platform for ~ 30 minutes. Again, in the interest of time we are reducing this incubation. Normally we would allow the staining to proceed for ~ 1 hour.

(8) Wash: Aspirate and quickly rinse 1X with wash buffer [0.2% BSA (m/v) + 0.05% TritonX-100 (v/v) in PBS] and then wash 2X.

Allow each wash to proceed for 5 minutes on the rocking platform.

- ☐ Rinse 1
- ☐ Wash 2
- ☐ Wash 3

(9) Secondary Antibody:

Dilute secondary antibodies for addition. Be cautious of light exposure of the secondary antibodies, protect from light whenever possible. Dilute all secondary antibodies 1:100 to ~ 1-2 ug/mL.

(a) **Well 1:** Donkey-anti-Rat (for anti-β-tubulin)

☐ 148.5 uL PBS+ 1.5 uL of AlexaFluor405-AlexaFluor647 Donkey-anti-Mouse

(a) **Well 2 :** Donkey-anti-Rabbit (for anti-Tom20)

☐ 148.5 uL PBS+ 1.5 uL of Cy3-AlexaFluor647 Donkey-anti-Rabbit

(b) **Wells 3 and 4** : Donkey-anti-mouse (for anti-β-tubulin) and donkey-anti-rabbit (for anti-Tom20)

☐ 294 uL PBS + 3 uL AlexaFluor405-AlexaFluor647 donkey-anti-mouse + 3 uL Cy3-AlexaFluor647 donkey-anti-rabbit

Add ~ 150 uL of the appropriate dilutions to each well.

Incubate at RT on the rocking platform protected from light for ~ 20 minutes.

(10)Wash: Aspirate and rinse 1X with wash buffer. Do 2X subsequent washes in PBS. Allow each wash to proceed for 5 minutes on the rocking platform.

- ☐ Rinse 1 – Wash Buffer
- ☐ Wash 2 – PBS
- ☐ Wash 3 – PBS

(11) Post-fixation: In order to "lock" the antibodies in place and prolong sample lifetime, a post-fixation step is often desirable. Here we'll use 3% PFA and 0.1% GA diluted in PBS again. Incubate at room temperature (RT) for 5 minutes. Note that no reduction step is required here, in fact, $NaBH_4$ reduction will kill the fluorescence of the AlexaFluor647 by disrupting the dye conjugation.

(12) Rinse: Rinse cells 3X quickly with PBS. Store samples at 4°C. If long term storage is desired, it is recommended to store in PBS + 20 mM Sodium Azide (NaN_3) to prevent bacterial contamination.

Coverslip Cleaning:

As an aside, while the chambered coverslips come pre-sterilized, there are remaining fluorescent contaminants. In order to remove them, prior to cell plating, coverslips are sonicated in a tabletop sonicator for ~ 20 minutes in 1 M potassium hydroxide (KOH). They are then rinsed thoroughly in Milli-Q water and sterilized in the biosafety cabinet under ultraviolet light for > 30 minutes

These protocols were developed at Harvard University in the lab of Prof. Xiaowei Zhuang.

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II. Sample Preparation: Antibody Labeling

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II. Sample Preparation: Antibody Labeling

Reagents List:

- Cy3 mono-Reactive Dye Pack (GE Healthcare)
- AlexaFluor647 carboxylic acid, succinimidyl ester (Invitrogen)
- Anhydrous DMSO (Sigma Aldrich)
- 1 M Sodium Bicarbonate Solution
- Donkey-anti-rabbit secondary IgG (Jackson Immunoresearch)
- Dulbeccos Phosphate Buffered Saline (Invitrogen)
- illustra Nap-5 Columns, Sephadex G-25 (GE Healthcare)

Dye Aliquoting: For the AlexaFluor647, dissolve 1mg dye in anhydrous DMSO and aliquot into tubes for a final 0.02 mg amount of dye per tube.

For the Cy dyes, dissolve one dye pack into a sufficient, but preferably small, amount of anhydrous DMSO to allow distribution into 10 new aliquots.

Using an evaporator, remove all DMSO. Store aliquots at -20°C protected from light. (N.B. For AlexaFluor405, the appropriate solvent for making aliquots is H_20)

Protocol:

(1) For labeling, dissolve one aliquot of the activator dye (Cy3) in 10 µL of anhydrous DMSO and one vial of the reporter dye (AlexaFluor647) in 20 µL of anhydrous DMSO.

(2) Set up labeling reaction:

Final volume 60 µL:

For Cy3:

50 uL Donkey-anti-Rabbit (1.3 mg/mL)

6 uL 1 M $NaHCO_3$

1.5 uL Cy3 (of 10 uL aliquot)

+ 0.6 uL AlexaFluor647 (of 10 uL aliquot)

Wrap the tube in aluminum foil to protect from light.

Allow the reaction to proceed for up to ~ 40 minutes at RT on a rocking platform.

(If substituting Cy2, use ~ 5 uL, for AlexaFluor405 use ~ 4 uL of a 10 uL aliquot)

(3) While the reaction is progressing, equilibrate a Nap-5 gel filtration column, one per labeling reaction.

Run three column volumes of PBS through the column.

In the interest of time we will end the labeling reaction once the column has been equilibrated. This may result in slightly reduced labeling efficiency.

(4) After the incubation, stop the reaction by the addition of 140 uL of PBS to bring the reaction volume up to 200 uL (the minimum column loading volume) and gently vortex. Add the entire volume to the center of the column.

(5) Allow the sample to enter the column and after the last drip add sufficient PBS to collect the fastest running colored band.

For IgG, 550 uL of PBS should be required.

(6) Add 300 uL PBS and collect the resulting eluent in a 1.5 mL Eppendorf tube. (7) Store the antibody at 4°C protected from light, for up to 6 months.

(8) Calculate the antibody concentration and labeling ratios using the NanoDrop

Calculating Labeling Ratios

Measure the absorbance of the sample at 280 nm (protein, IgG); 550 nm (maximum absorbance of Cy3); 650 nm (maximum absorbance of AlexaFluor647).
Concentrations are calculated according to the Beer-Lambert Law.

Dye	λ (nm)	Abs (1:10 dil)	ε	CF_{280}
IgG	280		210,000	--
Cy3	550		150,000	0.08
AlexaFluor647	650		239,000	0.03

Antibody Concentration (in molarity):

$[IgG] = [(Abs_{280} - (A_{dye1} * CF_{280\text{-}1}) - (A_{dye2} * CF_{280\text{-}2})) * dilution] / \varepsilon_{IgG}$

Dye Concentration (in molarity) [Dye] =

$(Abs_{dye} * dilution) / \varepsilon_{dye}$

Labeling ratio: [Dye]/[IgG]

Sample Calculation:

For a sample of Cy3-Alexa 647 IgG with Abs280 = 0.251; Abs550 = 0.333; Abs650 = 0.156

[IgG] = [0.251- (0.333*0.08) – (0.156*0.03)]/ 210,000 = 1.05×10^{-6} M = 1.05 uM [Cy3] = 0.333/ 150,000 = 2.22×10^{-6} M = 2.22 uM

[AlexaFluor647] = 0.0156/ 239,000 = 6.5×10^{-7} M = 0.65 uM

Labeling ratio: Cy3/IgG = 2.22 uM/1.05 uM = 2.11

AlexaFluor647/IgG = 0.65 uM/1.05 uM = 0.62

Since the molecular weight of IgG ~ 150 kDa, or 150,000 grams: 1.05 uM ~ 0.16 mg/mL

Recommended labeling ratio:

As far as antibodies are concerned, the labeling ratios are fairly forgiving. In general, for secondary antibodies we aim for a labeling ratio of ~ 0.5 – 1 AlexaFluor647 and 2 – 3 Cy3 (or other activator) per antibody.

Samples with higher ratios will certainly work for STORM, and in some cases are preferred. For example, when using a labeled primary antibody, the secondary antibody signal amplification is lost, and therefore a higher labeling ratio maybe helpful.

These higher labeling ratios may result in having to "pre-bleach" the sample to reach the single molecule imaging regime.

Ultimately, while tolerant, the labeling ratio is a parameter that can further be tuned for individual applications.

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Buffers for STORM imaging

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Buffers for STORM imaging:

Organic Fluorophores:

The organic fluorophores used for STORM imaging require a thiol in the buffer to photoswitch effectively.

We commonly use two different thiols for their effects on photoswitching:

β-Mercaptoethanol generates bright, long-lived single molecule photoswitching events. This makes it ideal for single color imaging.

Cysteamine (or MEA) reduces the observed photon number slightly but also reduces the non-specific blinking, making it ideal for multicolor imaging.

Additionally, we use an enzymatic oxygen scavenging system of glucose oxidase and catalase to reduce photobleaching.

Reagents:

50% Glucose stock (m/v)

Dilution Buffer

10 mM Tris, pH 8.0

50 mM NaCl

Oxygen Scavenger (GLOX)

14 mg Glucose Oxidase (Sigma Aldrich)

200 µL of Dilution Buffer

50 µL of catalase (20 mg/mL; Sigma Aldrich)

Dissolve glucose oxidase in PBS, vortex to mix.

After mixing the catalase suspension well, add catalase to glucose oxidase solution.

Centrifuge at maximum speed for 1 min.

Catalase may visibly precipitate out and remain at the bottom of the tube.

Use the yellow supernatant for imaging buffers.

Store at 4°C for up to 2 weeks

MEA

77 mg of MEA (a.k.a. Cysteamine, Sigma Aldrich/BioChemika)

Dissolve in 1 mL of 360 mM hydrochloric acid.

Store at 4°C for up to 1 month

Imaging Buffer Base

10 % glucose (m/v)

50 mM Tris, pH 8.0 (100 mM Tris or pH 8.5 can be used for stronger buffer strength)

10 mM NaCl

Store at room temperature for up to 6 months.

Live-Cell Imaging Buffer Base

10 mL DMEM, high glucose, no phenol red (Gibco)

750 µL 1M HEPES, pH adjusted to 8.0

400 µL 50% Glucose

Store at 4°C for up to 2 weeks.

Buffer Compositions:

STORM buffer using βME: Fixed Sample

100x Imaging Buffer Base

\+ 1x (v/v) βME (Sigma Aldrich, 14.3 M pure stock solution)

\+ 1x (v/v) Oxygen Scavenger

This buffer typically lasts for about 0.5 hour in an open environment due to the acidification of the buffer by the oxygen scavenging system via the production of gluconic acid by glucose oxidase. In a well sealed sample, the buffer can facilitate imaging for up to several hours.

STORM buffer using MEA: Fixed Sample

80x (v/v) almost any buffer (Imaging Buffer Base, water, PBS, etc.)

+ 10x (v/v) 1M MEA

+ 10x (v/v) 50% glucose

+ 1x (v/v) Oxygen Scavenger

(N.B. if using Imaging Buffer Base, omit glucose)

This buffer typically lasts for about one hour in an open environment, and up to several hours in a well sealed sample.

While we will not have time to cover it in extensively in this workshop, STORM imaging is entirely compatible with live cell imaging. Below are some examples of STORM imaging buffers optimized for AlexaFluor647 imaging in mammalian cells.

STORM Buffer using βME: Live Sample

100.0x Live-cell imaging buffer base

+ 0.5x (v/v) βME

+ 1.0x (v/v) Oxygen Scavenger

This buffer typically lasts for ~ 30 minutes in an open environment.

STORM Buffer using MEA: Live Sample

100.0x Live-cell imaging buffer base

+ 0.6x (v/v) 1 M MEA

+ 1.0x (v/v) Oxygen Scavenger

This buffer typically lasts for ~ 30 minutes in an open environment.

Photoactivatable/Photoswitchable Fluorescent Proteins

Since fluorescent proteins do not depend on thiols to switch, nor seem to primarily bleach through an oxygen dependent pathway, the optimal buffer is simply situated at the appropriate pH to best facilitate photoconversion.

For EosFP and its derivatives, such as mEos2, the protonated form of the unactivated (green) chromophore is crucial for photoconversion and therefore a low pH buffer is optimal (McKinney et al., Nat. Methods, 2009. 6(2): p.131-3; Wiedenmann, PNAS, 2004 101(45): p15905-10).

We commonly use PBS (pH 7.4) as we do not find the photoactivation rate limiting for imaging, but one could use a lower pH buffer if slow photoconversion were a concern.

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June 29-30, 2012
Barcelona, Spain

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1st EUROPEAN STORM WORKSHOP AT ICFO: USEFUL REFERENCES

TECHNIQUES

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PALM: Betzig et al, Science, 2006

FPALM: Hess et al, Biophysical Journal, 2006

GSDIM: Folling et al, Nature Methods, 2008

PROBES

Bates et al., *Phys Rev Lett*, 2005

Dempsey et al., *JACS*, 2009

Fernandez-Suarez, M., *Nat Rev Mol Cell Bio*, 2008

Dempsey et al., *Nature Methods*, 2011

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MULTI-COLOR Super-Resolution

Bates et al., *Science*, 2007

MULTI-COLOR Super-Resolution cont'd...

Shroff et al., *PNAS*, 2007

Dempsey et al., *Nature Methods*, 2011

Gunewardene et al, *Biophysical Journal*, 2011

3D Super-resolution

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Live Cell Super-resolution

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Super-resolution Data Analysis

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Smith et al, *Nature Methods*, 2010

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Quantitative Super-resolution

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