



## Sample Preparation for Cell Culture Labeling

This protocol can be used to prepare cell culture samples for super-resolution microscopy applications.

### Buffer

#### PEM stock buffer (10x, pH 7.0)

- 1 M piperazine-N,N'-bis(2-ethanesulfonic acid) sesquisodium salt (PIPES, Fisher, cat. no. BP304-100)
- 10 mM EGTA
- 10 mM MgCl<sub>2</sub>

Adjust to pH 7 with KOH.

Note: Best results obtained with PEM, though PBS can be used instead

### Materials

- PBS (pH ~7.4)
- Paraformaldehyde (PFA-16%): Electron Microscopy Services #15710
- Glutaraldehyde (Glut-8%): Electron Microscopy Services #16019
- Saponin: USB Corporation #21435 100 gram
- Sodium Borohydride (NaBH<sub>4</sub> 99%): Sigma Aldrich #213462-25G
- Triton X-100 (TX-100): Electron Microscopy Services #22140
- Bovine Serum Albumin (BSA): Sigma A3059-10G
- Primary antibody:  $\alpha$ -Tubulin anti-mouse monoclonal antibody (Sigma T5168-0.2 ML)
- Secondary antibody: Alexa Fluor® 647 donkey anti-mouse IgG (H+L)
- Primary antibody: Tom20 (FL-145) mitochondrial protein (Santa Cruz Biotechnology sc-11415)
- Secondary antibody: CF568 donkey anti-rabbit
- Adherent cells grown on No: 1.5 coverslips: BSC-1, Cos-7, Vero, or Ptk2 cells work well

### Reagents

- Fixation buffer: 3% PFA+0.1% glut in 1x PEM buffer
- Post-fixation buffer: 4% PFA in PBS
- Pre-extraction buffer: 0.2% saponin (w/v) in 1x PEM buffer (optional)
- Prepare 0.1% (w/v) NaBH<sub>4</sub> in PBS immediately before use (optional)
- Blocking buffer: 3% BSA+0.2% TX-100 in PBS
- Antibody dilution buffer: 1% BSA+0.2% TX-100 in PBS (used for diluting both primary and secondary antibodies)
- Wash buffer: 0.05% TX-100 in PBS

### Procedure

1. Wash cells one time with PBS warmed to 37°C.
2. Pre-extract with 0.2% saponin in PEM (warmed to 37°C) for 1 minute at room temperature (optional step).
3. Do not rinse just aspirate the saponin mixture.
4. Fix with fixation buffer (3% PFA +0.1% glutaraldehyde) diluted in PEM (warmed to 37°C) for 15 minutes at room temperature.
5. Reduce with 0.1% NaBH<sub>4</sub>
6. for 7 minutes at room temperature (optional for non-Glut fixation).
7. Rinse 3x3 minutes with PBS.
8. Permeabilize and block with blocking buffer for 30 minutes at room temperature gently rocking.
9. Aspirate blocking buffer but do not rinse.
10. Incubate with primary antibody for 1 hour at room temperature gently rocking.
11. Rinse 3x3 minutes at room temperature with wash buffer.
12. Incubate with secondary antibody (with Alexa, CF, Cy3B, Cy3 or ATTO dyes use ~2  $\mu$ g/mL concentration) for 45 minutes at room temperature gently rocking wrapped with aluminum foil.
13. Rinse 3x3 minutes with wash buffer.
14. Post fix for 10 minutes at room temperature.
15. Rinse 3x3 minutes with PBS.
16. Store cells in PBS at 4°C.

## Notes

### Recommended fluorophores:

We recommend Alexa 647 (ThermoFisher), and CF568 (Biotium) as the first choice of probes. Cy3B (GE Healthcare), Alexa 568, ATTO 488 or Alexa 488 are sometimes used but not recommended as a first choice. Cy3B will have to be conjugated to an unlabeled secondary antibody of choice since Cy3B-labeled secondary antibody is not commercially available. For two-color imaging, Alexa 647 and CF568 is a recommended combination and will provide the optimal imaging conditions. Dendra2 or mEos are recommended as fluorescent protein tags. DAPI staining is not typically recommended since it is excited by the 405 activation laser, thereby contributing to the background fluorescence.

### Recommended culturing surface:

Grow cells on #1.5 (0.17 mm thickness) circular coverslips such as 25 mm circular coverglass (Electron Microscopy Services Cat #72225-01), generic 22 mm or 18 mm square coverslips, #1.5 coverslip-bottom 8 well NUNC chambers (Thermo Scientific Cat# 155409), or #1.5H ibidi 8 well chambers (ibidi cat.no 80807). Circular dishes with a No: 1.5 coverslip bottom, such as Mattek 35 mm dishes (Mattek Corporation # P35G-1.5-14-C), will also work. Using the recommended culturing surface will ensure that the sample is in contact with enough fresh switching buffer during imaging. A coverslip mounted on a slide will not work for this protocol.



FIGURE 1.

25 mm circular coverslip that can be imaged in a stage adaptor (Attofluor cell chamber from Invitrogen Cat# A7816) and 8-well NUNC chambers.

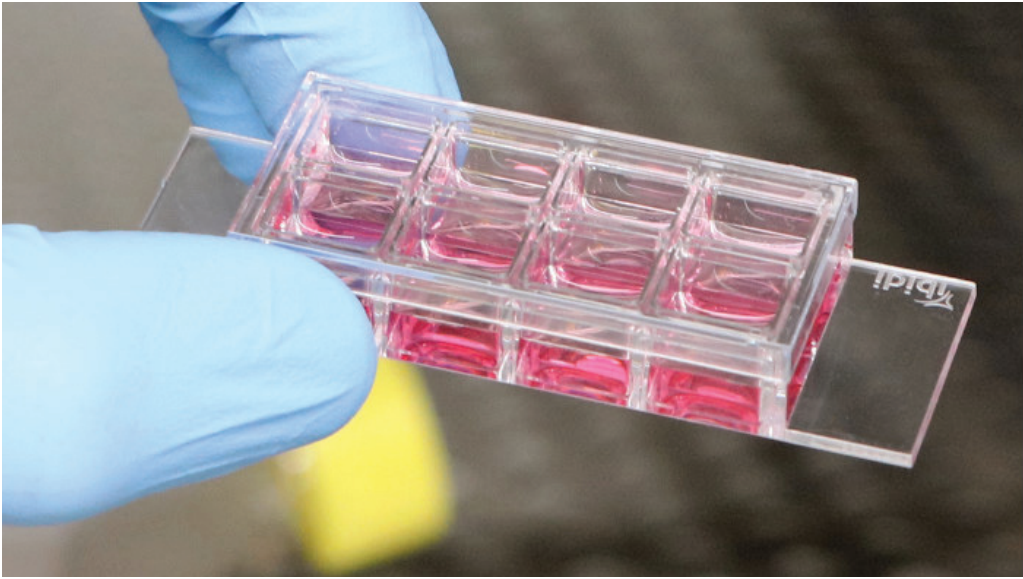


FIGURE 2.

Ibidi  $\mu$ -Slide 8 well high glass bottom chamber (cat.no 80807). Image sourced from ibidi.com.

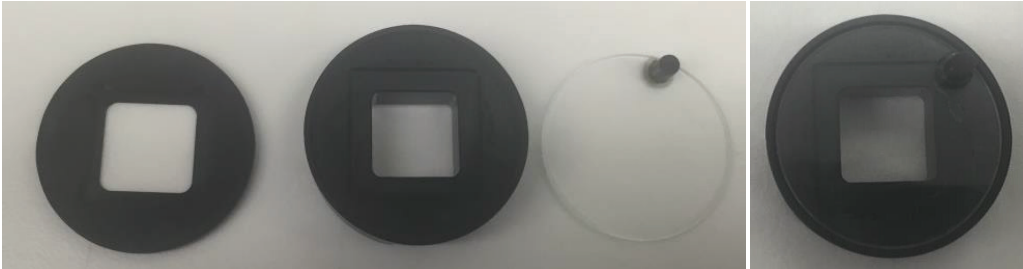


FIGURE 3.

22 mm or 18 mm square coverslips that can be imaged in a stage adaptor.

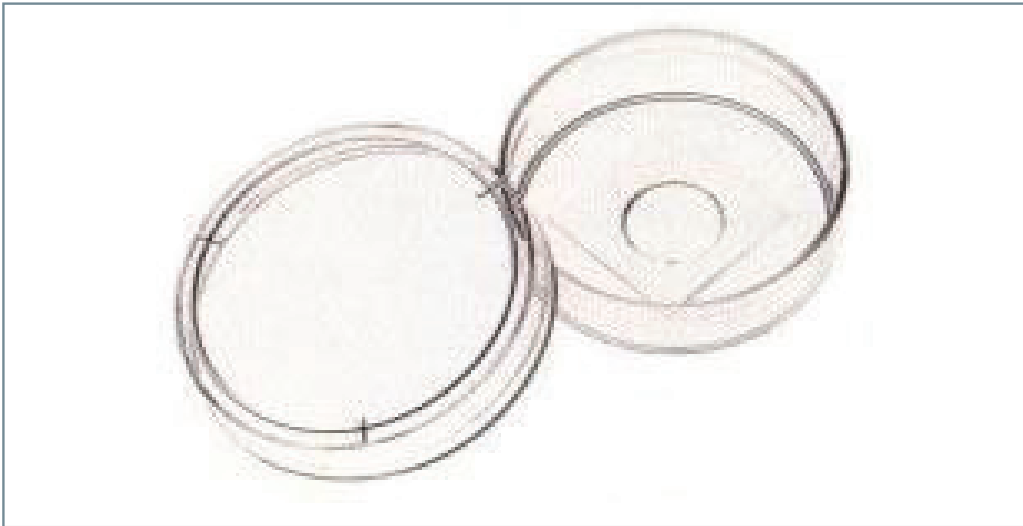


FIGURE 4.

35 mm Mattek dishes.

**Fixation reagent:**

Fixation agents other than PFA, such as glutaraldehyde, methanol, or acetone, can also be used. Glutaraldehyde has been shown to induce autofluorescence, therefore use sodium borohydride to quench autofluorescence. Fixation protocols that have worked in the past for confocal imaging can be used initially and then further optimized for super-resolution imaging.

**Primary antibody concentration:**

It is strongly recommended that users optimize the primary antibody concentration for their biological system of interest. The manufacturer's recommendation is a good starting point. We have observed that a concentration  $\sim 1 \mu\text{g/mL}$  typically works for most primary antibodies when labeling cells.

**Secondary antibody concentration:**

Once again, optimization by the user is recommended while considering the manufacturer's recommendation. A secondary antibody concentration of 1 to 2  $\mu\text{g/mL}$  has worked well for us when labeling cells. Typically, less dye is needed for super-resolution imaging when compared to confocal imaging. For example, if in confocal imaging a 1:400 dilution of Alexa 647 works, then for super-resolution imaging, 1:1000 to 1:2000 dilutions of the same dye seem to work well.

It is critical to wash the samples thoroughly after primary and secondary antibody incubation to minimize background that can be caused due to non-specific labeling. We have also found that quenching with sodium borohydride reduces autofluorescence-induced background. Post-fixing samples after secondary labeling keeps them stable longer.

**Preferred photoswitchable fluorescent proteins (genetically-encoded).** The following table contains information regarding recommended fluorescent labels.

TABLE 1.

Preferred photoswitchable fluorescent proteins (genetically-encoded).

Probe	Type (photoconversion)	$\lambda_{\text{PA}}$ photoactivation (nm)	$\lambda_{\text{x}}$ excitation (nm)	$\lambda_{\text{em}}$ emission (nm)
mMaple*	A (505) $\rightarrow$ B (583) (Irrev)	405	566	583
Dendra2	A (507) $\rightarrow$ B (573) (Irrev)	405	553	573
mEos3.2	A (516) $\rightarrow$ B (580) (Irrev)	405	569	580

\*And its derivatives, mMaple2 and mMaple3.

Note: The fluorescent proteins above switch irreversibly from green emission to red emission upon photoactivation. They must be used separately.

## FAQ

### What dye is recommended for one-color imaging?

Alexa 647

### What dyes are recommended for two-color imaging?

The best combination of dyes to use for two-color imaging is Alexa 647 and CF568.

### What is recommended when using photoswitchable proteins?

Dendra2, mEos3.2, or mMaple3.

### What kind of coverslips/chambers should I use for growing cells or attaching tissue?

Using No: 1.5 coverslips or No: 1.5 coverslips bottom dishes is necessary. No: 0 or No: 1 coverslips will NOT work. 25 mm circular, 22 mm or 18 mm square, NUNC chambers and Mattek dishes are the commonly used configurations.

### Can I mount my sample in Vectashield, Prolong Gold, or similar anti-fade reagents?

No, because samples need to be accessible for adding fresh imaging buffer just prior to imaging.

### How can I store or ship samples?

Postfixation with 4% PFA at room temperature for 10 minutes is recommended to enable long-term storage of samples in PBS for cells. Samples are shipped in buffer; and coverslips are sent in 6 well plates. Samples are either shipped at 4°C or room temperature.

Long-term storage in PBS with azide can help prevent microbial growth.

Plastic bottles, such as the one shown here, can be used to ship chambered coverslips, such as ibidi, NUNC, or labtek chambers by filling with PBS and inserting the chamber into the bottle without its lid on. It is recommended to secure the lid of the bottle with parafilm before shipping.



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