ONI Resources

Protocol

Phalloidin Staining Protocol

Adapted from: A. Jimenez, K. Friedl, C. Leterrier. About samples, giving examples: Optimized Single Molecule Localization Microscopy. Methods, 174 (2020), p. 100-114, 10.1016/j.ymeth.2019.05.008

Fixation

1) Pre-warm extraction and fixation buffers in a 37°C water bath.
2) Incubate cells with the extraction buffer for 30-45 sec at 37°C.
3) Quickly replace it with the fixation buffer for 10 min at 37°C.
4) Incubate cells with the reducing buffer for 7 min at RT.
5) Wash cells 2x with PBS.**

Blocking

6) Incubate cells with the blocking buffer for 30 min at RT.

Actin Staining***

7) Incubate cells with 500 nM of phalloidin-Alexa Fluor® 647 in PBS for either 1 h at room temperature or overnight at 4°C.
   Key factor: Leave samples incubating with phalloidin-Alexa Fluor® 647 staining solution until just before imaging since phalloidin-Alexa Fluor® 647 is more labile than uncoupled phalloidin and can easily detach from actin.
8) Wash cells briefly with PBS.

*Always use freshly made GA and $NaBH_4$ buffers.
**At this stage, you can store the sample at 4°C or proceed with staining.
***Perform any antibody staining prior to phalloidin staining.

Buffers

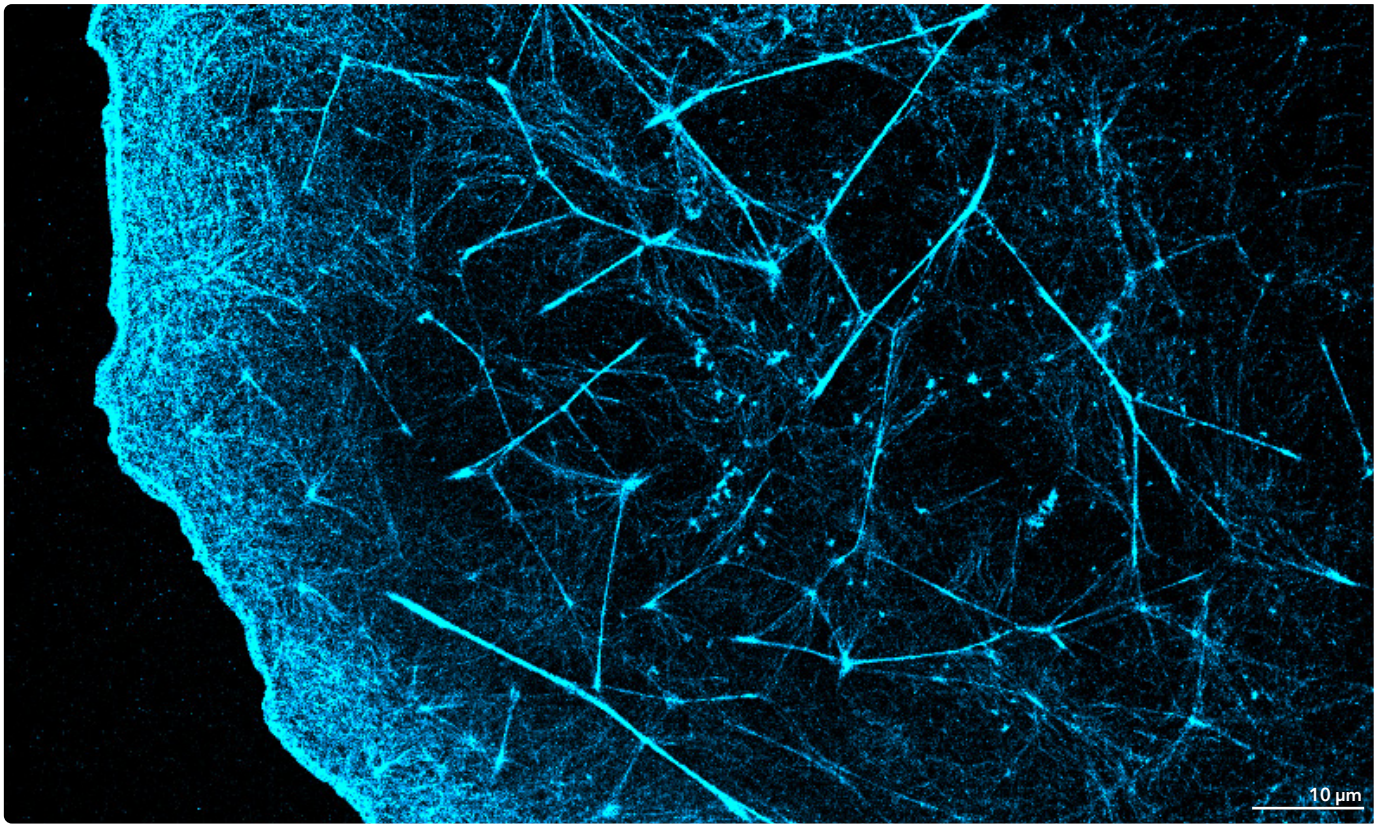
PEM
Use KOH to adjust the pH of PIPES (80 mM) to 6.8. The PIPES solution will appear milky until the pH reaches 6.8. Add EGTA and $MgCl_2$ to yield a final concentration of 5 mM and 2 mM, respectively.

Extraction buffer
0.25% v/v Tx100 and 0.1% GA* in PEM.

Fixation buffer
0.25% v/v Tx100 and 0.5% GA* in PEM.

Reducing buffer*
0.1% w/v. Mix 10 mg of $NaBH_4$ into 10 mL of PBS.

Blocking buffer
5% BSA and 0.1% Tx100 in PBS.



Images | Actin labeled with phalloidin-Alexa Fluor® 647.

@oniHQ @ONI

ONI, Linacre House, Jordan Hill Road, Oxford, OX2 8TA, UK
ONI, ONI Inc. SPACES, 101 Jefferson Drive, Menlo Park, CA, 94025 USA

Top tips

See this ONI **blogpost** for additional information on how to optimize your immunostaining protocol.

Sample holder

- We recommend using the ibidi µ-Slide 8 Well Glass Bottom (#1.5 glass coverslip bottom). Use 400 µl/well for all buffer solutions and 200 µl/well for all phalloidin solutions.
- If you don't use the ibidi slides, ensure you do not mount the coverslip with mounting media as this will interfere with the autofocus function of the microscope.

Sample Storage (µ-Slide 8 Well Glass Bottom)

1) Wash sample 3x with PBS.
2) Fill each well to the top with PBS.
3) Place parafilm over the wells to seal in the sample. Ensure there are no air bubbles.
4) Place the lid onto the sample firmly.
5) Store at sample at 4°C.

Chemicals
PIPES (Sigma, P1851-25G)
EGTA (Sigma, E4378)
$MgCl_2$ (Fisher (Invitrogen AM9530G), 10418464)
TritonX-100 (Tx100) (Sigma, T8787)
Glutaraldehyde (GA) (Sigma, G7651-10ML)
$NaBH_4$ (Sigma, 71320-25G)

Imaging probe
Phalloidin-Alexa Flour® 647 (ThermoFisher, A22287)

Materials	
#1.5H coverslips	170 µm thickness, 35-75 mm (X dimension) by 15-25 mm (Y dimension)
Multi-well chambers	• ibidi (µ-Slide 8 Well Glass Bottom, No. 1.5) available from **here** • Nunc™ Lab-Tek™ II Chambered Coverglass (Thermofisher, 154534PK) available from **here**

ONI, Linacre House, Jordan Hill Road, Oxford, OX2 8TA, UK
ONI, ONI Inc. SPACES, 101 Jefferson Drive, Menlo Park, CA, 94025 USA