Protocol

Tubulin Staining Protocol

Fixation

1) Pre-warm extraction and fixation buffers in a 37°C water bath.
2) Incubate cells in extraction buffer for 30-45 sec at 37°C.
3) Replace medium with 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.

Primary antibody

7) Incubate cells with the primary antibody solution for 1 h at RT in the dark.***
8) Wash cells 3x with PBS.

Secondary antibody

9) Dilute Alexa Fluor®647-anti-rabbit, F(ab')2 and Alexa Fluor®647-anti-rat, IgG(H+L) 1:250 in blocking buffer.
10) Incubate cells with the secondary antibody solution for 1 h at RT in the dark.***
11) Wash 3x with PBS.

Post fixation

12) Incubate cells with the post fixation buffer for 10 min at RT in the dark.
13) Wash 3x with PBS.

*Always use freshly made PFA and $NaBH_4$ buffers.
**At this stage, you can store the sample at 4°C or proceed with staining.
***Incubate on a seesaw rocker to achieve even staining across the sample.

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 5 mM EGTA and 2 mM $MgCl_2$ to the PIPES solution.

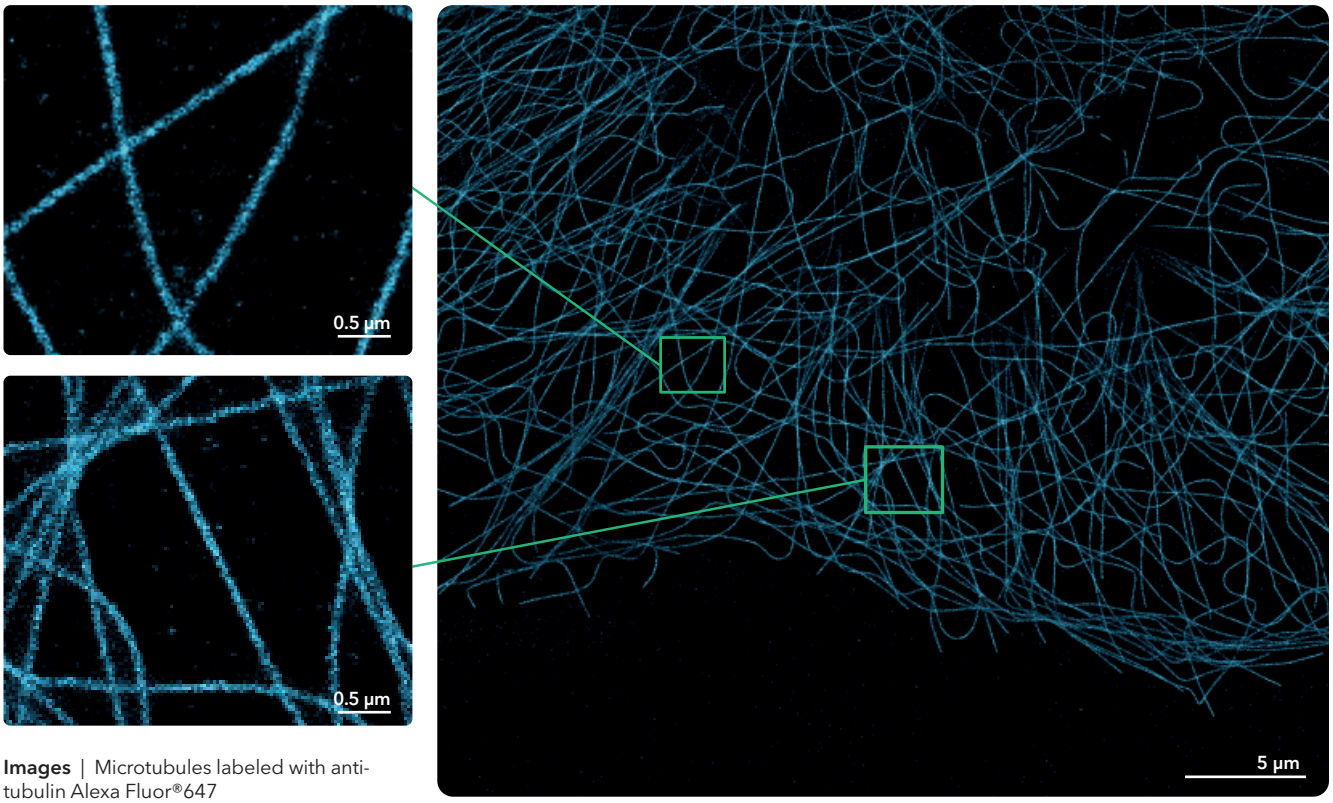
Extraction buffer
0.25% v/v Tx100 and 0.1% GA in PEM.

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. Make fresh.

Post fixation buffer*
4% PFA. Dilute 16% PFA in PBS. Make fresh.

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



Images | Microtubules labeled with anti-tubulin Alexa Fluor®647

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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 (1.5H glass coverslip bottom). Use 400 µl/well for all buffer solutions and 200 µl/well for all antibody 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)

Antibodies
Anti-alpha tubulin antibody (rabbit) (Abcam, ab18251)
Anti-alpha tubulin antibody (rat) (Fisher, 11525382)
Alexa Fluor®647 anti-rabbit, F(ab')2 (Fisher (Invitrogen A21246), 10236552)
Alexa Fluor®647 anti-rat, IgG(H+L) (Fisher (Invitrogen A21247), 10666503)

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**

This protocol was 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

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