Protocol

Mitochondria Staining Protocol

Fixation
1) Pre-warm buffers in a 37°C water bath.
2) Incubate in fixation buffer for 10 min at 37°C.
3) Incubate cells with reducing buffer for 7 min at RT.
4) Wash cells 2x with PBS.**

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

Primary Antibody
6) Dilute TOMM20 antibody 1:50 in blocking buffer.
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 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.

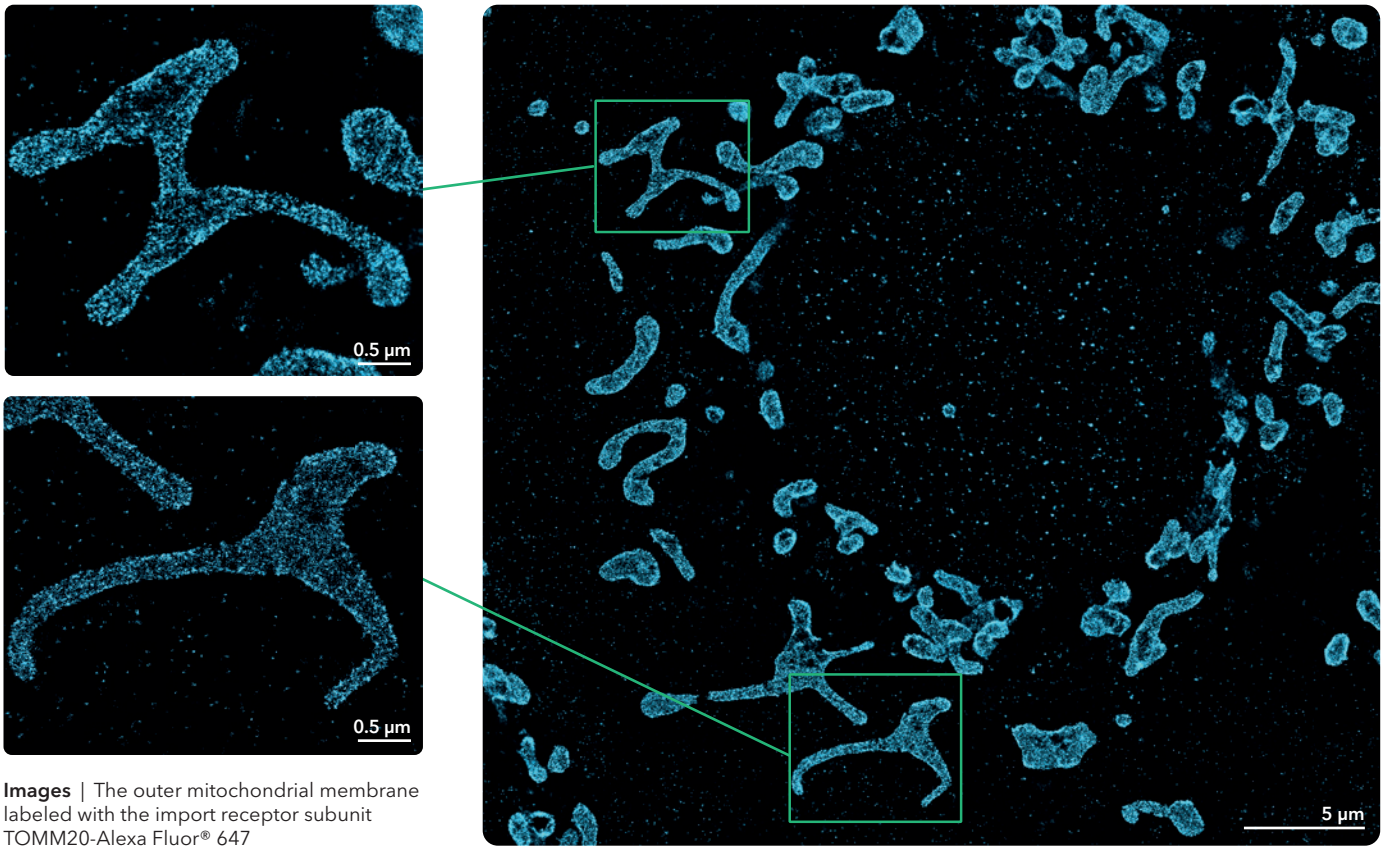
Buffers

Fixation buffer*
4% PFA with 0.2% glutaraldehyde in PBS.

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.

Post fixation buffer*
4% PFA in PBS.



Images | The outer mitochondrial membrane labeled with the import receptor subunit TOMM20-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
Paraformaldehyde (PFA)
TritonX-100 (Tx100) (Sigma, T8787)
Glutaraldehyde (Sigma, G7651- 10ML)
$NaBH_4$ (Sigma, 71320-25G)

Antibodies
TOMM20 antibody (rabbit) (Thermo Scientific, PA552843)
Alexa Fluor® 647 anti-rabbit, F(ab')2 (Fisher (Invitrogen A21246), 10236552)

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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