APR 12, 2024

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.kxygxyeeol8j/v1

Protocol Citation: Harper JW, Louis R R Hollingsworth, Chan Lee 2024. Preparing fixed cells for immunofluorescence. **protocols.io** https://dx.doi.org/10.17504/protocols.io.kxygxyeeol8j/v1

Protocol status: Working
We use this protocol and it's working

Created: Apr 09, 2024

Preparing fixed cells for immunofluorescence

Harper JW[1,2], Louis R R Hollingsworth[1], Chan Lee[1]

[1]Department of Cell Biology, Harvard Medical School Boston, MA 02115, USA;
[2]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD 20815, USA

ASAP Collaborative Research Network

Louis R R Hollingsworth
Harvard Medical School

ABSTRACT

This protocol provides a general method to prepare fixed cells for immunofluorescence imaging, e.g., by confocal microscopy.

PROTOCOL REFERENCES

https://www.protocols.io/view/immunocytochemical-analysis-c7g8zjzw
https://www.protocols.io/view/immunofluorescence-of-galectin-3-puncta-after-lyso-x54v9jnmpg3e/v2

Last Modified: Apr 12, 2024

PROTOCOL integer ID: 97980

Keywords: ASAPCRN, immunofluorescence, IF

Funders Acknowledgement:
National Institutes of Health
Grant ID: R01NS083524

MATERIALS

Cell culture -- sterile materials

PBS (Thermo J67802.AP)
24-well glass bottom plate (Cellvis P24-1.5H-N)
0.1% poly-L-lysine solution (Sigma P8920)
Relevant cell culture media

IF

PBS (MedChemExpress HY-K1023)
Triton-X 100 (Sigma X100)
Tween-20 (Sigma 655205)
16% paraformaldehyde (PFA; Electron Microscopy Sciences 15710)
BSA (GoldBio A-420-10)
Glycine (Sigma 410225)
Methanol (Sigma 34860-2L-R)

Prepare-ahead buffers (see protocol for more details)

PBS (from MedChemExpress HY-K1023, filtered)
PBS-T (PBS with 0.02% Tween-20)
PBS-TritonX (PBS with 0.3%-0.4% Triton-X 100)
PBS-PFA (PBS with 4% PFA; prepared fresh)
PBS-Block (PBS with 3% BSA and 22.52 mg/mL)
100% methanol pre-chilled at -20 degrees celsius

Representative secondary antibodies and conjugates:

	A	B	C
	Secondary antibodies and conjugates		
	Antobody	Vendor, Cat#	Dilution
	Anti-mouse 488	Thermo A-11029	1:200
	Anti-mouse 568	Thermo A-11004	1:200
	Anti-mouse 647	Thermo A-21235	1:200
	Anti-rat 488	Thermo A-11006	1:200
	Anti-rat 647	Thermo A-21247	1:200
	Anti-Rabbit 488	Thermo A-11034	1:200
	Anti-Rabbit	Thermo A-11011	1:200

	A	B	C
	568		
	Anti-Rabbit 647	Thermo A-21244	1:200
	Phalloidin-405	Thermo A30104	1:200
	Phalloidin-488	Thermo A12379	1:200

Plate coating and cell seeding

1 Dilute 0.1% poly-L-lysine solution (Sigma P8920) in PBS (0.01%). Make enough solution for 0.5 mL per well, plus some excess.

2 Add 0.5 mL of the diluted poly-L-lysine solution (0.01% in PBS) to each well of a 24-well glass bottom plate (Cellvis P24-1.5H-N). Place the plate back in the cell culture incubator (37 °C, 5% CO_2) for 1 – 24 h to adhere the solution to the plate. 1h

3 Wash the wells 3x with sterile PBS or water.

4 Add 1 mL of cell culture medium to each well, then plate cells at an appropriate density such that they are not more than 70% confluent on the day of the experiment.

Note

Use DMEM for iBMDMs and HEK293(T)s. For these cell types, seed the cells at approximately ~15% confluence for iBMDM or ~30% for HEK293Ts to have an ideal cell density for imaging the next day.

Fixation and permeabilization

5 Prepare fresh solutions for IF including: PBS, PBST (PBS with 0.02% Tween-20), PBS-Triton (PBS with 0.3%-0.4% Triton-X 100) or 100% methanol pre-chilled at -20 degrees celsius, PBST-Block (see step 5.1 below), and PBS-PFA (see step 5.2 below).

Note

Different antibodies work with different fixation and permeabilization protocols. Additionally, certain organellar populations are better preserved with alternative fixation/permeabilization protocols. For example, we've found that lysosomes and other vesicles are better resolved with methanol permeabilization instead of PBS-TritonX (see note at step 8 for that particular alternative). Because of the difference in permeabilization temperatures, we recommend using different plates if you intend to conduct experiments in parallel that require different permeabilization protocols.

5.1 To prepare PBST-Block, dissolve 3% BSA and 22.52 mg/mL glycine in PBST (for example, add 1.5 g BSA and 1.126 g glycine to 50 mL PBST). Glycine binds free aldehyde groups (from PFA) to prevent antibody crosslinking and thus high background, whereas BSA reduces nonspecific antibody binding.

5.2  To prepare PBS-PFA, freshly dilute an ampule of 16% paraformaldehyde (PFA; Electron Microscopy Sciences 15710) in PBS to 4% (add the 10 mL ampule to 30 mL of PBS) in a falcon tube. Warm the solution to 37 degrees celsius prior to fixation.

Note

PFA is toxic, so do this step carefully in a chemical hood. Additionally, be careful when breaking the glass ampule.

6 Conduct relevant cellular treatment(s). Stagger any treatments so that all of the wells of the plate are ready to harvest simultaneously.

7 Aspirate the cell culture medium and add 1 mL of pre-warmed PBS-PFA to each well. Incubate for 10 mins 10m with gentle rocking at room temperature.

Note

Certain cell types can be extremely labile, and so use caution when aspirating and adding fixation medium. Alternatively, you can add 8% PBS-PFA directly to the cell culture medium (1:1 volumetric ratio for a final concentration of 4%), which can prevent tricky cells from lifting off of the plate.

8 For Triton-X 100 permeabilization, slowly add 1 mL of PBS-Triton directly to each well then immediately aspirate the solution (this can help prevent the formation of aggregates). Replace with 1 mL PBS-Triton and

incubate at RT for 10 min.

PFA-containing waste should be collected and disposed of properly.

Note

For methanol permeabilization, instead first wash the plate 3x with PBS, prior to adding 1 mL cold methanol (-20 degrees) to each well and placing the plate at -20 degrees celsius for 10 min.

9 Wash cells 3x with 1 mL PBST. For the first wash, aspirate immediately and replace with PBST, and then conduct the subsequent washes over 5-10 mins. 10m

Blocking and antibodies 19h

10 Aspirate PBST, and add 0.5 mL PBST-Block to each well. Incubate at RT for 1 h. 1h

11 Dilute primary antibodies in pre-chilled PBST-Block on ice. Aspirate the wells and add 200 µL of the antibo 18h solution to each well. Rock gently at 4 degrees celsius overnight.

Note

Note that some organelles, like mitochondria, have significant autofluorescence in the 488 channel. Additionally, with multiple primary/secondary combinations, bleed through can occur. Control wells lacking particular primary antibodies (minus-one controls) should always be included at least once to assess bleed-through and autofluorescence with a given set of cells, antibodies, and laser settings.

A useful resource for checking dyes and fluorophores is FPbase: https://www.fpbase.org/

12 The next day, wash wells 3x with 1 mL PBST. For the first wash, aspirate immediately and replace with PBST, and then conduct the subsequent washes over 5-10 mins.

13 Dilute fluorescent secondaries (see materials) 1:200 in PBST-Block. Phalloidin can also be added at thi 1h 30m
step. Aspirate the wells and add 200 µL of secondary antibody solution to each well. Rock gently at RT for 60-90 mins.

Note

Wrap the plate in aluminum foil to minimize fluorophore quenching over subsequent incubation and wash steps.

If possible, spread the secondary antibodies out as far as possible to avoid bleed-through (e.g., 488 and 647).

14 Wash cells 3x with 1 mL PBST. For the first wash, aspirate immediately and replace with PBST, and then 10m
conduct the subsequent washes over 5-10 mins.

15 Aspirate the PBST and add Hoechst 33342 (0.5 mL, 5 µM in PBS) to each well, if desired. Hoechst labels 10m
nuclei in the ~405 channel. Incubate at RT for 10 min.

16 Wash cells 3x with 1 mL PBS. For the first wash, aspirate immediately and replace with PBST, and then 10m
conduct the subsequent washes over 5-10 mins.

17 Keep cells at RT if imaging with the same day (ideal), or transfer the plate to 4 degrees celsius for imaging at a later time. Image the plate within ~3 - 5 d with an appropriate confocal microscope.

Note

Imaging settings highly depend on the cells and antibodies used in the experiment, in addition to the cellular phenomenon. We thus recommend consulting with an imaging core during imaging optimization.