Immunoflorescence Staining of Cell Lines

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1 Introduction

This protocol aims to describe Immunofluorescence staining of cell lines that are either grown on glass cover-slips or attached to a poly-lysine coated cover-slips.

A note on condition optimization:
Primary antibody concentration optimization is very important. To optimize a robust range to begin with is approximately 1:10,000 to 1:300. Fixation and permeabilization is also a critical step to optimize conditions for. Standard fixation for this protocol is 4% formalin in PBS 15'/RT, and standard permeabilzation is 0.5% Triton X100 in PBS 5'/RT. Considerations such as cell type and protein localization should effect choice of fixation/permeabilization conditions.

Methanol fixation is often better for visualizing big proteins such as microtubules. Saves times because it fixes and permeabilizes the cells in one step. However, it should not be used when staining for membrane (MeOH washes out some lipid components) or when using actin visualization.

2 Reagent List

- 4% Formaldhyde in PBS
- 1X PBS
- IF Blocking Buffer
- IF Permeabilization Buffer
- IF Wash Buffer
- Appropiate 1° and 2° antibodies
- DAPI bath
- Prolong Gold Mounting Gel

3 Protocol

DAY 1

3.1 Attach cells to cover-slips

1. Sterilize round, glass cover-slips* in 70% ethanol under UV light (10' in tissue culture hood). Rinse in sterile PBS.
   ** n.b.* If cell line is non-adherant, be sure to use Poly-D-Lysine coated cover-slips
2. Place clean cover-slips at the bottom of the wells of 24-well tissue culture plate and seed cells at pre-determined density (*e.g.* 20 k cells/well for HeLa).
3. Centrifuge the plate 1000 rcf / 5' (RT).
4. Incubate 37° C, 5% CO_2 for about 24 hr (untill cells are well attached to glass cover-slips).

DAY 2

3.2 Fixation

1. Remove growth media from 24-well plate, and wash with cold, 1X PBS
2. Choose the appropriate fixation buffer, and fix according to steps below.
   (a) **4% Formaldehyde**
      i. Apply 500 µl freshly prepared 4% formaldehyde in PBS to each well of the 24-well plate containing a cover-slip.
      ii. Fix for 15' (RT) under chemical hood.
   (b) **Methanol**
      i. Apply 500 µl of ice-cold methanol (-20° C freezer ideally) to cells.
      ii. Fix for 5' (RT).
3. Wash 3x in ice-cold PBS.

   [Optional] Formaldehyde-fixed cells can be stored at 4° C in PBS overnight as needed.

3.3 Permeabilize

Skip permeabilizeation step if used Methanol Fixation (step 3.2.2.b).

1. Choose permeabilization method based on target protein:
   (a) Standard permeabilization:
      i. Place 500 µl standard permeabilization bufer (0.5% Triton X-100 in PBS) into each well.
      ii. Incubate 5' (RT).
   (b) Membrane protein permeabilization:
      i. Place 500 µl membrane permeabilization bufer (0.1% saponin) into each well.
      ii. Incubate 30-60' (RT).

3.4 Block

Skip blocking step if used Methanol Fixation (step 3.2.2.b) or membrane protein permeabilization (step 3.3.1.b).

1. Remove permeabilization buffer without washing and add 500 µl of blocking buffer (5% BSA in PBS).
2. Incubate 1 hr (RT).

3.5 Immunostain

1. Dilute primary antibodies in appropriate wash buffer with 5% BSA. Typically [1:500] is a usefull starting dilution.
   (a) **Standard** 5% BSA in PBS with 0.1% Triton X100
   (b) **Membrane** 3% BSA in PBS with 0.1% saponin -or- BD Perm/Wash Buffer (BDB554723)
2. Place no more than 50 µl of primary antibody solution on parafilm at the bottom of a humidity chamber. Gently, place cover-slips onto the primary antibody droplet cell-side down.
3. Incubate at 4° C, overnight in a humidity chamber.

DAY 3

3.6 Secondary antibody

1. Apply 50 µl secondary antibody mixture to parafilm on the bottom of a humidity chamber. Secondary antibody should be diluted [1:300] in appropriate wash buffer. Include phalloidin [1:300] in secondary mix if actin visualization required. Take reasonable measures to protect from light from now on.
2. Wash 3X with appropriate wash buffer.
   (a) **Standard** PBS w/ 0.1% Triton X100.
   (b) **Membrane** PBS with 0.1% saponin -or- BD Perm/Wash Buffer.

   Each wash should consist of drop-wise application of 1 ml total wash buffer to the cell side of the cover-slip.
3. Place cover-slip cell side down on secondary antibody droplets in humidity chamber.
4. Incubate 1 hr (RT) in humidity chamber.

3.7 Counterstain and Mount

1. Apply 50 µl 1X DAPI bath solution to a parafilm sheet.
2. Wash 3X with appropriate wash buffer.
3. Place cover-slip cell side down on secondary antibody droplets.
4. Incubate 5’ (RT) in dark.
5. Tapp off excess DAPI bath and mount on glass slides with small drop of Prolong Gold mounting medium.
6. Cure over night (RT) in dark before imaging. Store long-term at 4° C.

4 Recipes

4.1 Permeabilization Buffers

Standard (0.5% Triton X100)

	Stock	Final	Amount
PBS	1X	—	9.75 ml
Triton X100	20%	0.5%	250 μl

Membrane (0.1% Saponin)
A mild detergent for permeabilization, which *preserves membrane structures better*. This buffer is also commercially available: BDB554723

	Stock	Final	Amount
PBS	1X	—	9.75 ml
Saponin	20%	0.1%	50 μl
BSA	100%	3% w/v	1.5 mg

4.2 Blocking Buffer

	Stock	Final	Amount
PBS	1X	—	50 ml
BSA	—	5% w/v	2.5 mg

4.3 Wash Buffer

Standard (0.1% Triton X100)

	Stock	Final	Amount
PBS	1X	—	50 ml
Triton X100	20%	0.1%	50 μl

Membrane (0.1% saponin)
or BD Perm/wash

	Stock	Final	Amount
PBS	1X	—	50 ml
saponin	20%	0.1%	50 μl

5 Notes on Antibodies

5.1 Preferred 1° Antibodies

- Rat α HA-tag [1:500] (4°, primary-2, #27)
- Rabbit α NaK [1:500] (-20°, box 6, #65) *membrane marker
- Rabbit α PDI [1:500] (-20°, box 6, #76) *ER marker
- Rabbit α AIF [1:500] (-20°, box 4, #75) *mitochondrial marker

5.2 Preferred 2° Antibodies

- Goat α Rat AF594 (#5)
- Donkey α Rabbit AF488 (#7)