Protocol for immunofluorescence staining of adhesion cells

This is provided as a general protocol. Optimization of concentration or incubation condition of the primary antibody and the secondary antibody for your own specimen is necessary. Please also review the datasheet of the antibody and publications using the same antibody for reference.

Preparation

Coverslip preparation:

- # 1.5 coverslip. In general you should use #1.5 coverslips as most microscope objectives are designed to work optimally with these.
- Size of coverslip. Round 18 mm coverslips in a 12 well plate work well, or 12 mm coverslips in a 24-well plate, or 22 mm square coverslips/25mm round coverslips in a 6-well plate/35mm culture dishes.
- Sterile coverslip. Washing in ethanol or exposing to UV for 60 min.
- **Coating of coverslip.** Many cell lines will grow well on uncoated coverslip but some do better with coated coverslips (e.g. 50 µg/ml poly-lysine for 1 hr, or collagen).

Cell preparation:

- Transfer your cells on the coverslip in plates/culture dishes and culture them (e.g. overnight) so they are well adhered and reach 50-70% confluency.

Reagents preparation:

Note: Always filtration or centrifuge solutions before use to avoid precipitation.

- Phosphate-buffered saline (PBS)
- BSA or Serum from the species that the secondary antibody was raised in
- Fixatives: Formaldehyde/Paraformaldehyde; Methonal; or Acetone
    - Note: Choose the appropriate fixation method according to the application.
    - The goal of fixation is to maintain cellular structure but may damage or mask antigenic sites. You may need to test several fixatives before you find the one that produces the right balance of antibody binding and structural integrity of the sample.
    - Formaldehyde/paraformaldehyde and glutaraldehyde are aldehyde fixatives which cross link proteins and generally preserve cell structure well but cause strong autofluorecence, especially glutaraldehyde. This can be counteracted with reduction of the aldehyde groups with NaBH4 or NH3Cl at 10 mM (See the step of Quenching). 4% formaldehyde/ paraformaldehyde for 10 min is a good starting point for mammalian cells. 4% paraformaldehyde + 0.1% glutaraldehyde is good to preserve cell structure but quenching is required before mounting.
    - Organic solvents like methanol precipitate proteins, which produces a similar outcome to crosslinking with aldehydes but all the small molecules within will be lost during the rest of the protocol. Fixation with methanol denatures proteins, so never use if you have a fluorescent protein that you want to look at in your sample. Using a combination of methanol and acetone can improve immunolabelling lost

in 100% methanol fixation (methanol is best for structure, acetone permeabilizes well and is less damaging). Try 10 min in solvent at -20°C as a starting point.

- Triton/NP-40/Digitonin or Saponin
  - Permeabilization helps the antibodies get into the fixed cells. Cell surface proteins don't require much/any permeabilization. If the target protein is expressed intracellularly, it is very important to permeabilize the cells.
  - Triton X-100 is the most popular detergent for improving the penetration of the antibody. However, it is not appropriate for the use of membrane-associated antigens since it destroys membranes.
- Prepare primary-antibody in 3% BSA/PBS, dilute antibodies according to the recommended manufacturer specification data sheet or reference publications.
- Selection of the secondary antibody is depended on the donor species of the primary antibody and the desired fluorochrome**.**

Procedure

Note: Incubate samples in the dark and cover whenever possible.
It is advisable to run the appropriate negative controls. Negative controls establish background fluorescence and non-specific staining of the primary and secondary antibodies.

- **Rinse** cells with PBS x2
- **Fixation:** fix the cells either in cold methanol, acetone (1-10 min) at -20^{o}C or in 2-4% paraformaldehyde (PFA) (10-20 min) in PBS (freshly prepared) at RT.
- Wash the samples with PBS 10min x3 on shaker
- **Permeabilization:** incubate the samples for 10-15 min with 0.05-0.25% Triton X-100 in PBS (or 100 µM digitonin or 0.5% saponin).
- Wash the samples with PBS 10min x3 on shaker
- **Blocking:** incubate the cells for 30-60min with 1-3% BSA in PBS to block unspecific binding of the antibodies (alternative blocking solutions are 1% gelatin or 10% serum from the species that the secondary antibody was raised in).
- Remove the blocking buffer by holding each coverslip on its edge with forceps and draining it onto a sheet of Kimwipes.
- **Primary Ab:** Incubate cells the primary antibody dilution in 3%BSA/PBS (**keep cells dark in a humidified chamber**) 1-4 hr at room temperature/37 ^{o}C or overnight at 4^{o}C.
  - A simple chamber can be made from a small box with water-soaked filter papers.
  - If it is desirable to examine the co-distribution of two different antigens in the same cell, a double immunofluorescence procedure may be used. Cells may be incubated simultaneously with two primary antibodies, provided they are monospecific and can be distinguished with secondary antibodies conjugated to different fluorochromes (or with primary antibodies directly conjugated to different fluorochromes).

- Wash the samples with PBS 10min x3 on shaker

- **Secondary antibody:** incubate cells with the secondary antibody in 3%BSA/PBS for 30-60min at RT (keep cells dark in a humidified chamber). Most secondary antibodies can be used after 1:200-1:500 dilution
  Note: If the primary antibodies are already conjugated to a fluorochrome, incubation with secondary antibody is not necessary.
- Wash the samples with PBS 10min x3 on shaker.
- **Counter staining**: stain nuclear with DAPI or Hoechst for 5-10min.
- Wash the samples with PBS 10min x3 on shaker.
- **Quenching:** To quench auto-fluorescence, incubate coverslips with 1 mg/ml Sodium borohydride (NaBH4) in PBS for 5min. (This solution is prepared immediately before use.) The solution will foam when PBS is added, so use a large enough tube to avoid overflow.
- Wash the samples with PBS 10min x3 on shaker
- **Mounting**: mount coverslip up site down on slide with a drop of mounting medium
  - Remove the blocking buffer by holding each coverslip on its edge with forceps and draining it onto a sheet of fiber-free paper
  - Remove any excess liquid from the coverlip and apply 1 drop (30-50ul) (depending on the surface area of your sample) of mounting medium on the slide.
  - Carefully lower the coverslip onto the mounting medium to avoid trapping any air bubbles.
  - Allow the mounted sample to cure on a flat surface in the dark. Curing time may vary from a couple of hours to overnight, depending on the thickness of the sample and the relative humidity of the surrounding air.
  - For long-term storage, seal the coverslip to the slide after curing to prevent excessive shrinkage of the mounting medium, which can result in sample distortion. After sealing, store the slide upright in a covered slide box at 4°C.
  - To view the samples immediately, secure the coverslip using nail polish or hot wax to prevent the coverslip from moving.