Indirect Immunofluorescence

Equipment and reagents

- Glass coverslips, 22x22mm
- 6-well plates
- Paraformaldehyde (granular; Electron Microscopy Sciences)
- Parafilm
- Forceps, Dumont, GG (Electron Microscopy Sciences)
- Fluorescently labeled secondary antibody (Vector Laboratories)
- Mounting medium (Molecular Probes)
- Microscopy coverslide

Method

1. Prepare sterile 22x22mm No. 1.5 glass coverslips by soaking them in 95% ethanol[a] and gently flaming them. For multiple samples use 6-well plates[b]
2. Grow cells on coverslips to 50-70% confluence[c]
3. Fix cells in 3-4 ml 2% formaldehyde in PBS, pH 7.4 for 15 min. at room temperature[d,e]
4. Quickly rinse cells in 4 ml PBS, pH 7.4 and wash the coverslips in 2 ml PBS, pH 7.4, 2 X 5 min. at room temperature[f]
5. Permeabilize cells by removing the wash solution and adding 2 ml of 0.5% Triton X-100 in PBS, 7.4 for 5 minutes on ice.
6. Quickly rinse cells in 4 ml PBS, pH 7.4 and wash the coverslips in 2 ml PBS, pH 7.4, 3 X 8 min. at room temperature.
7. Place a piece of Parafilm large enough to easily accommodate all coverslips on a flat surface.
8. For each coverslip, place 50 ul of the appropriate concentration of primary antibody on the Parafilm. Make sure to separate the drops generously.
9. Remove the coverslip from the 6-well plate using fine forceps. Remove excessive PBS by blotting the edges of the coverslip gently against a piece of filter paper.
10. Invert the coverslip with the cells facing down onto the antibody drop.

11. Incubate the coverslips for 1h at room temperature inverted onto a piece of Parafilm. Cover the Parafilm with a light-tight lid.
12. Remove the coverslip from the Parafilm by squirting 1 ml of PBS, pH. 7.4 under the coverslip to float it up. Place the coverslip with the cells facing up in the 6-well plate.
13. Wash the coverslip in 2 ml PBS, pH 7.4, 3 X 8 min. at room temperature.
14. Repeat steps 6-9 with a drop of appropriately diluted secondary antibody (2-4 ug/ml in PBS, pH 7.4).
15. Incubate the coverslip for 1 h at room temperature inverted onto a piece of
16. Wash the coverslip in 2 ml PBS, pH 7.4, 3 X 8 min. at room temperature[h]
17. Place 10 ul of mounting medium on a glass microscopy slide. Remove the coverslip from the 6-well plate. Remove excessive PBS by blotting the edges of the coverslip gently against a piece of filter paper.
18. Invert the coverslip cells facing down onto the mounting medium[i].
19. Remove excessive mounting medium by holding two pieces of filter paper against two opposing edges of the coverslip[j].

[a]It is most convenient to store a large number of coverslips in 95% ethanol.

[b]All volumes given are optimized for 6-well plates and 22x22 mm coverslips.

[c]Confluent lawns of cells result in increased background signal.

[d]2% formaldehyde is made up fresh prior to use by dissolving the appropriate amount of EM grade paraformaldehyde in PBS in a Pyrex bottle on a hot plate with a stir bar. Paraformaldehyde goes into solution at 70^0C. Do not tighten the bottle cap so as to avoid build-up of pressure. Place the dissolved solution on ice to cool down and check pH.

[e]Alternative fixation procedures include methanol at -20°C for 5 min or 3.7% paraformaldehyde, 5% acetic acid for 20 min (4). Since both of these methods also permeabilize membranes, skip to step 5.

[f]It is most convenient to use a vacuum suction device to remove the solution from the 6-well plate and to simultaneously add the new solution using a 10ml pipette. It is crucial that the cells on the coverslip never dry out.

[g]We recommend Dumont GG forceps (Electron Microscopy Sciences).

[h]Nuclei can be counterstained for easy visualization by use of DAPI or Hoechst 33342. After two washes as described in step 14, incubate coverslips for 5 min in 2 ml 500 ng/ml DAPI or Hoechst 33342 and wash twice more as described in step 14. DAPI and Hoechst give a blue stain.

[i]Vectashield (Vector Laboratories) or mounting medium from Molecular Probes is recommended.

[j]A good indication that all excessive mounting medium has been removed is if the coverslip cannot be moved on the coverslide when gently pushed with the filter paper.

- Suspension cells can be centrifuged onto a coverslip in a Cytospin centrifuge and then processed as described. Alternatively, cells can be labeled in suspension as described above. Start out with about 3 x10^6 cells and at each step, spin the cells for 2 min at 3 000 xg and carefully remove the supernatant. Antibody incubations are done in a volume of 100ul on a rocking platform. After the last wash re-suspend the cells directly in 50 ul mounting medium and spot 15 ul on a microscopy slide and cover with an empty coverslip.

- This protocol can be used for detection of multiple proteins. Generally results are not compromised by the simultaneous incubation of multiple primary or secondary antibodies. If detection of mutiple proteins is done step-wise it is recommended that steps 7-16 are carried out for the first antibody and then repeated for the second antibody. In either case it is essential to ensure that the primary antibodies used are from different species (polyclonal from rabbit and monoclonal from mouse are commonly used) and that the secondary antibodies do not cross-react between these species. When doing multiple labels the recommended sequence of fluorophores is fluorescin, Texas Red, Cye 5, AMCA. Note that Cye 5 emits in the infrared part of the spectrum and requires the use of an appropriate cooled CCD camera for detection. Indirect immunofluorescence can be combined with in situ hybridization methods to detect RNA or DNA.