

IMMUNOFLUORESCENCE

Antibodies:

1. All antibodies, polyclonal and monoclonal, should be aliquoted and stored at -70°C. After thawing do not refreeze.
2. Dilutions of antibodies should always be made into PBS + 5 mg/ml BSA. Diluted antibodies can be stored at 4°C for several months.

Immunofluorescence:

1. Preparation of coverslips - coverslips should be acid washed before use, as follows:
Mix 50 ml dH₂O, 2 ml conc. HNO₃, 6 ml conc. HCl.
Swirl coverslips in acid for 30 min. to 1 hr.
Pour off acid and rinse coverslips thoroughly with dH₂O (15-20 changes). When pH of coverslips in H₂O is equal to that of input H₂O swirl coverslips in H₂O for 30 minutes.
Pour off dH₂O and store coverslips in 95% EtOH (make the 95% EtOH from good absolute EtOH instead of using 95% from stockroom).
2. Seeding of coverslips - with a pair of EtOH-washed forceps remove a single coverslip and flame it before placing it in a tissue culture dish. After placing several coverslips in dish, flood dish with media, then add freshly trypsinized cells at a low density (20-30% confluence is best).
3. Fixing cells before staining-the preferred method of fixation depends upon the particular antibody used for staining. Different types of fixation will preserve specific epitopes differently.
 - A. Water-soluble organic solvents: (acetone, EtOH or MeOH) These solvents fix and permeabilize cells by precipitating proteins and extracting membrane lipids. Can produce distortion of cell surface. Chill solvent to -20°C in a glass container. Rinse coverslips 2x in PBS and then immerse in cold solvent 2 minutes. Remove coverslips and promptly rinse 2-3x in PBS.
 - B. Formaldehyde -TX100 treatment: This method is better for preserving fine structures, but stands a better chance of altering or destroying epitopes. To prepare 3.7% (w/v) formaldehyde - add 3.7 g solid paraformaldehyde to dH₂O with a pellet of NaOH. Stir, heating, add 10 ml of 10xPBS and adjust to pH 7.4. Bring volume up to 100 ml with dH₂O. Use freshly prepared or store at -20°C and thaw just before use (do not refreeze). Fixation of cells - wash coverslips with PBS. Add formaldehyde solution (37°C) to cells, just enough to cover, and let stand 20 minutes at room temperature. Rinse 2x with PBS, followed by PBS plus 0.1 M glycine for

5 min.

Formaldehyde-fixed cells must be permeabilized after fixation. This can be achieved by incubating coverslips for 5 min in PBS + 0.1% TX-100 followed by PBS wash. Alternatively, cells may be permeabilized by incubating for 2 minutes in acetone at -20°C, followed by PBS wash.

4. Staining of cells:

To make a humidified chamber for staining coverslips place a paper towel in the bottom of a glass petri dish. Wet the paper towel with dH₂O, cover the dish and place in 37°C incubator. Before staining lay a piece of parafilm over the paper towel. This gives a flat surface on which to set coverslips.

To stain - with forceps dip coverslip in PBS, then wick away most of the liquid by touching the edge of the coverslip to a kimwipe: lay the coverslip (cell side up) on the parafilm and quickly apply a small amount of a suitable dilution of 1° antibody. 35 µl will cover a 12 mm coverslip. Do not allow the cells to dry out at any time. Incubate at 37°C for 15 min to an hour. To apply 2° antibody (see * for preabsorption of 2°), pick up coverslip and wash 2-3x in PBS (remember which side the cells are on!), and apply 2° antibody same as primary. Incubate 37°C 15 min. to 1 hour.

To mount coverslips - wash off 2° antibody 2-3x in PBS, then dip coverslip in dH₂O to remove all salt before mounting. Mount coverslip on a glass slide on which a small drop of Gelvatol (Monsanto) or other mounting media has been placed. If using Gelvatol dry slide with hair dryer.

*Immunofluorescence - preabsorption of 2° antibody.

Fluorescein-conjugated goat anti-mouse is bought from Cappel Worthington (Cooper-Biomedical). The anti-mouse IgG [F(ab')₂ fragment specific] fraction cat # 1211-0111 is best. 2° antibodies should be preabsorbed against the cell type being used for fluorescence to reduce background staining due to non-specific reactivity. Scrape a p60 plate of cells and pellet in an eppendorf. Fix according to procedures described above. Dilute 2° in PBS/BSA and incubate with fixed cells 4-6 hours at 4°C on shaker. After incubation clarify antibody solution by spinning in microfuge 15 min. Remove supe and store wrapped in foil at 4°C.

Antibody from Cappel comes in 2 ml and should be aliquoted and frozen at -20°C upon arrival. A 1/50 dilution is usually sufficient for staining, but this should be rechecked for each new batch of antibody purchased.

Reference: K. Wang, J. Feramisco and J. Ash (1982) *Methods in Enzymology* **85**, 514-562.