Easy Intracellular Immufluorescence Microscopy Protocol

Materials

Cells
Coverslips (1 per sample analyzed)
Dishes for plating - 12, 24, 6-well dishes are OK or petri dishes, depending on the # of samples desired
12-well dish or staining jar/ coverslip holder for washes
150cm2 petri dish for staining
Primary antibodies
Fluorophore-conjugated 2˚ antibodies
Fluoromount G or equivalent
Slides
Phosphate Buffered Saline (PBS), room temperature
PBS/ 2% formaldehyde - this should be made relatively fresh (good for ~ a week or two) - remember that stock formaldehyde is 37%, so this is a 1:18.5 dilution!

Preparation - cells

Adherent cells: Cells should be plated on coverslips and should be about 50% confluent at time of analysis. Depending on the experiment, cells can be grouped - one coverslip will fit in each well of a 12- or 24-well plate (hard to get out of a 24-well plate); up to 5 coverslips will fit in each well of a 6-well plate; and up to 12 coverslips will fit in a 60mm petri dish.

- HeLa or NRK cells - dilute a confluent (**NOT OVERCONFLUENT!!!**) flask 1:10 per unit area for appropriate density 2 days after plating

Non-adherent cells: This will depend on the cell type. They can be adhered to coverslips using poly-L-lysine, gelatin, or commercial reagents designed for adherence. Alternatively, they can be spun down onto slides after staining using a cytospin centrifuge. Density will have to be pre-determined based on the fraction of cells that become adherent under the appropriate conditions.

Protocol

1. Fix cells for 15-30 minutes in PBS/ 2% formaldehyde - 1ml per well in a 12-24well dish, 3ml per well in a 6ml dish.

-Fixative should be at ambient temperature or 37˚C to avoid disrupting microtubule architechture of cells.

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Alternative fixations:

- 2% formaldehyde with 0.1% glutaraldehyde in PBS - better fixative, particularly for cytosolic proteins, but may destroy more antigenic epitopes
- Cold methanol fixation - plunge coverslips into methanol at -20˚C for 2 minutes; can add acetone at -20˚C for an additional 2 minutes - this procedure maintains some epitopes that are destroyed by formaldehyde fixation, but may destroy others and preserves cell architechture less well. Also - do not need to use detergent (saponin) in the subsequent incubations

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2. Wash coverslips 2X 5-10' with PBS

- Be gentle - try to dribble PBS down the sides of the wells so as not to disturb the cells on the coverslips
- The last wash can stay of forever if you want to defer staining to another day - just add azide to 0.02% and store coverslips at 4°C.
- **REMEMBER - FORMALDEHYDE IS NASTY!! - ALL MANIPULATIONS WITH FORMALDEHYDE SHOULD BE DONE IN THE FUME HOOD, AND WASTE SHOULD BE DISPOSED OF AS CHEMICAL WASTE!!!**

3. Prepare antibody dilutions. Generally, a good serum or ascites can be diluted 1:200-1:1000, sometimes more, sometimes less. They should be titrated for optimal use. If appropriate specific 2° antibodies are available, it is possible to incubate with two or three different antibodies at the same time - *e.g.* a mouse monoclonal antibody and a rabbit polyclonal antiserum, or an IgG and an IgM antibody. For intracellular immunofluorescence, antibodies should contain:

- PBS (for serum or ascites dilutions; hybridoma supernatant can be used straight or diluted to a low degree)
- 0.2% saponin (for permeabilization - some folks use 0.1% Triton X-100 or NP-40, but these are more disruptive and should only be used if saponin does not work)
- 0.1% BSA (not necessary for hybridoma supernant)
- if desired for storage, 0.02% sodium azide

4. Prepare a 150mm dish with a layer of parafilm at the bottom, smoothed out to avoid any wrinkles. Place a wet kimwipe in one corner of the dish - this will provide humidity and ensure that your coverslips do not dry out. Label the parafilm with the sample numbers for your experiment, and place a drop of 20μl of 1° antibody solution under each label. Now, drain the coverslip of PBS by touching the edge to a kimwipe, and place each coverslip face down onto the drop of antibody.

Keep the dish covered, and incubate 1 hr, room temperature.

-You may accelerate the incubation by placing the dish at 37°C. Often 20-30' is sufficient, even at room temperature.

5. Dip each coverslip in a beaker of PBS for a quick rinse, and then wash by placing the coverslip in either a 24well plate or a slide dish/coverslip holder filled with PBS for 10' at room temp.

6. Prepare 2° antibodies. For double staining, you can use FITC-conjugated antibodies to one 1° antibody and either Texas Red or LRSC-conjugated antibodies to the other. AMCA can be used as a third colour with a microscope containing a UV filter, or cells can be counterstained at the end with DAPI or Hoechst dye for visualization of DNA. Antibodies should be diluted in PBS with saponin and BSA as described above.

7. Place 20μl of 2° antibody on fresh parafilm in the 150mm dish. Drain each coverslip and place it onto the drop of antibody.

Keep the dish covered, and incubate 30-45' at room temperature.

8. Dip and wash the coverslips as described above.

9. Mount each coverslip on a slide using a drop of Fluoromount G or other suitable mounting medium. Drain excess Fluoromount by inverting the slide on a bunch of kimwipes and pressing firmly. Allow Flouromount to dry at least 5 minutes, and then observe under the microscope.