Fluorescence Procedures for the Actin and Tubulin Cytoskeleton in Fixed Cells

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General Strategy

We typically work with tissue culture, primary mammalian cells, and cell extracts, but the protocols can be adapted to other systems, such as whole embryos or lower eukaryotes. The cytoskeleton is very dynamic and sensitive to changes in both the chemical and mechanical environment. Optimal conditions for fluorescence of proteins of the actin and tubulin cytoskeleton are based on: preserving cell structure; properties of individual cytoskeletal proteins and any antibodies to be used; background fluorescence. Buffers, fixes and detergents can dramatically affect cell preservation, and some antibodies will only bind antigen under specific fixation conditions. There is often quite a high level of actin monomer, tubulin subunits and cytoskeletal binding proteins free in the cytoplasm, especially in tissue culture cells. This can reduce the resolution of cytoskeletal polymers and makes it difficult to analyze the detailed localization of polymer binding proteins. To overcome this cells can be briefly extracted (seconds) before fixing under conditions that stabilize actin filaments or microtubules and preserve cell structure. This selectively removes free subunits/unbound binding proteins from the cytoplasm without causing significant changes in polymeric structure.

Growing Cells for Immunofluorescence

We plate cells on glass coverslips (12mm circles or similar). We pretreat coverslips typically with poly-L-lysine (PLL) if cells are loosely adherent to glass. Polyornithine is better for some neurons. Cells can also be grown in a commercial, removable chamber attached to a plastic coverslip.

Preparing Glass Coverslips

- Acid wash coverslips. This helps cells and polyamino acids stick to glass.

1. Heat coverslips in a loosely covered glass beaker in 1M HCl at 50-60oC for 4-16h.
2. Cool.
3. Wash coverslips extensively in dH2O, then ddH20.
4. Rinse coverslips in ethanol and leave to dry between a folded sheet of whatman paper (dry as separate coverslips).
5. Keep in a sterile tissue culture dish (can store for a year).

- Coat with polyamino acid.

1. Coat coverslips in bulk in 10-15ml 1mg/ml PLL (or 500ug/ml polyornithine), rocking or rotating for a minimum of 30 minutes in a 10 or 15cm tissue culture dish.
2. Save the polyamino acid (can reuse 3-4 times).
3. Wash the coverslips in dH2O, then ddH20 at least 5 changes in each (free polyaminoacid is cytotoxic).
4. Rinse coverslips in 100% ethanol and dry those to be used immediately on one end in an open tissue culture dish in a sterile incubator.
5. When dry, add cells.
6. Dry remaining coverslips between a folded sheet of whatman paper (dry as separate coverslips).
7. Keep in a sterile tissue culture dish (can store for a year). Do step 4 before use. Can keep 10-20ml aliquots of 1mg/ml PLL and 500ug/ml polyornithine stocks at -20 deg C. High molecular weight PLL is standard (greater than 300K), but lower molecular weight PLLs can also be tried.

- Optional-- Coat polyaminoacid/acid washed/coverslips with matrix molecules. This helps the attachment of very poorly adherent cells (e.g. neurons), and increases the growth rate of other cell types (e.g. primary culture cells). Different extracellular matrix molecules can also change the morphology of certain cell types (e.g protrusion of lamellipodia or filopodia, flattening of cell bodies useful for microinjection- usually determined empirically).

1. Coat individual polyamino acid/acid washed/coverslips with a drop of specific matrix molecule (held by surface tension) from frozen stocks for a minimum of 30 minutes at room temperature or in a 37 deg C incubator, or overnight at 4 deg C. Examples: --Collagen type IV/PLL/acid washed coverslips PC12 cells (100ug/ml collagen), somites (2mg/ml collagen). --1x matrigel/PLL/acid washed coverslips Primary fibroblasts, neuroblastomas, fibromas,

amphibian motor neurons, embryonic dorsal root ganglia. --10ug/ml laminin-polyornithine acid washed coverslips Adult dorsal root ganglia.

2. Wash 5x with calcium and magnesium free PBS, then 1x with culture media.
3. Plate cells. Coverslips must be coated fresh before plating cells. Washed, coated, coverslips can be stored for a maximum of one day in the cold room.

Reagents and Buffers

BRB80 is good for microtubules and 'cytoskeleton buffer' is good for both actin filaments and microtubules. The optional inclusion of sucrose keeps the cells isoosmotic which also helps preservation.

Brinkley Buffer 1980 (BRB80)
80mM PIPES pH 6.8
1mM MgCl2
1mM EGTA
Store at 4 deg C; (we generally make and store as a 5X stock)

Cytoskeleton Buffer (CB) with sucrose (CBS)
10mM MES pH 6.1
138mM KCl
3mM MgCl
2mM EGTA
Store at 4 deg C
Add sucrose fresh on the day of use of the buffer to final 0.32 M from 4 deg C stock (78% is 7X).

Detergents
In general Triton-X-100 (TX) is best, but it can sometimes be too harsh for delicate cells. We standardly use TX at a concentration of 0.1-0.5%, but gentler detergents such as saponin can also be substituted. TWEEN is often used in whole embryos. Make solutions with detergent fresh on the day of use.

TBS (For all steps after fixing.)
0.15 M NaCl
0.02 M Tris-Cl pH 7.4
Can make 10X stocks. Keep at room temperature or 4iC. PBS can be

substituted.

Antibody Diluting Solution("AbDil"; used to dilute antibody stocks and to preblock cells.)
TBS-0.1% TX
2% BSA
0.1% Azide
Store at 4 deg C

Mounting Media: We have had great success with mounting our coverslips in:
0.5 % p-phenylenediamine (Free Base; purchased from Sigma)
in 20 mM Tris, pH 8.8, 90 % glycerol

We prepare this by adding the p-phenylenediamine to the tris/glycerol and dissolving it by bubbling nitrogen through the tube for 3-4 hours. The mounting medium is stored at -20 deg C. It will turn brown over time and we generally discard once it turns dark brown before making a fresh batch. In our experience, this recipe results in greatest photostability for all the fluorophores we use - rhodamine, fluorescein, Cy5 and DNA binding dyes.

Adding Solutions, Washing and Blocking

Mechanical manipulations should be kept to a minimum without compromising the quality of the final image. For cells on coverslips, aspirate solutions gently from the side of the dish or coverslip with one hand and add new solutions gently to the other side with your other hand. Never drop solutions directly onto the cells and do not allow cells to dry out. Rinsing cells before fixing does not make much difference. Residual serum proteins from the cell growth media may also help to 'buffer' cells during fixation. Washing off excessive antibodies is crucial for good staining. The block step minimizes background staining.

Secondary Antibodies

We normally purchase our fluorescently labelled secondary antibodies from Jackson Laboratories. We especially like their anti-IgG antibodies raised in donkey-- these are very clean. We follow their directions for reconstitution and storage. For a working solution, we dilute antibodies (usually 1:50 or 1:200-- you'll have to determine what works for you)

in AbDil and store this at 4 deg C. If you notice high bakground, filter through a 0.2 um syringe filter or spin in a microfuge.

Procedures

For antibodies that have unknown properties on fixed cells it is best to start with one fixing condition that preserves native structure (e.g. formaldehyde or glutaraldehyde) and one fixing condition that denatures proteins (e.g. methanol or acetone). Simultaneous fixing and permeabilizing also works well for some antibodies. Generally for actin filaments and the actin cytoskeleton we prefer methanol over acetone fixation, and formaldehyde over glutaraldehyde fixation. Glutaraldehyde requires a reducing step that can mechanically dislodge any delicate actin-containing structures (e.g. filopodia, lamellipodia, retraction fibers, growth cones). Fluorescent-phalloidin is commonly used to stain actin filaments which only binds native actin.

For microtubules and the tubulin cytoskeleton the choice of fixative depends on whether the object of the experiment is to visualize microtubules alone or to visualize microtubules in addition to your favorite antigen. For microtubules alone, glutaraldehyde fixation after a brief extraction is preferable. For visualizing your favorite antigen with microtubules methanol seems to be the fixative of choice. Formaldehyde does not preserve microtubules very well; however, sometimes it is necessary to use formaldehyde and accept the poor microtubule morphology. In our lab, excellent microtubule co-immunufluorescence has been performed using straight methanol fixation for > 5 different antibodies.

Actin Cytoskeleton

Methanol fixation

1. Fix in -20oC methanol for 1-2.5 minutes
2. Rinse in TBS
3. Permeabilize in TBS-0.5% TX for 10 minutes
4. Rinse in TBS-0.1% TX (3 changes in 3-5 minutes is adequate)

5. Block in Abdil for 10 minutes
6. Add primary antibody diluted in Abdil for 1-1.5 hours
7. Wash in TBS-0.1%TX (5 changes over 15-30 minutes is fine, but longer does not harm)
8. Add secondary antibody for about 45 minutes
9. Wash in TBS-0.1% TX
10. Incubate in 1-10ug/ml DAPI or Hoesht in Abdil to stain nuclei if required for 10 minutes
11. Wash in TBS-0.1% TX
12. Rinse in TBS
13. Drain, mount, seal
14. When sealed add water to the top of the coverslip, then aspirate (removes salts).

Formaldehyde Fixation

1. Fix in 4% formaldehyde (16% stock EM grade) in CBS for 20 minutes
2. Rinse in TBS
3. Permeabilize as for methanol fixation
4. Procede as for methanol fixation

Can substitute 1-2% glutaraldehyde for formaldehyde. Quench the reaction with sodium borohydride (do this 3x 1 minute, each time use freshly dissolved borohydride-just a pinch in a 1ml tube in TBS, you will get lots of bubbles). Rinse off reducing agent in TBS (3 changes in 3-5 minutes is adequate).

Staining Actin Filaments with Fluorescent-Phalloidin

1. Fix in 4% formaldehyde (16% stock EM grade) in CBS for 20 minutes
2. Rinse in TBS
3. Permeabilize in TBS-0.5% TX for 10 minutes
4. Rinse in TBS-0.1% TX (3 changes in 3-5 minutes is adequate)
5. Block in Abdil for 10 minutes
6. Incubate in fluorescent-phalloidin (1ug/ml from 1mg/ml frozen stock in DMSO) for 20 minutes in Abdil. Do not incubate for longer than 20 minutes; highly fluorescent compounds such as fluorescent-phalloidin are usually sticky and will increase background staining with longer incubations.
7. Wash in TBS-0.1% TX
8. Incubate in 1-10ug/ml DAPI or Hoesht in Abdil to stain nuclei if required for 10 minutes
9. Wash in TBS-0.1% TX
10. Rinse in TBS
11. Drain, mount, seal

12. When sealed add water to the top of the coverslip, then aspirate.

Double label experiments

In general the best fluorescence is obtained by sequentially incubating in the individual antibodies (primary, secondary, primary, secondary). It is important to titrate the concentration of antibodies or fluorescent probes. This is because if one of the stains is very weak and the other strong, any bleed through between fluorescence channels during observation makes it almost impossible to assess colocalization. (Bleed through can be minimized with the appropriate choice of bandpass excitation and emission filters. A filter that blocks the first color can also be inserted into the light path when viewing the second color). Single label controls should be initially included to confirm the general localization of test antigens. For double label experiments that include one antibody and fluorescent-phalloidin, incubate in fluorescent-phalloidin for 20 minutes in Abdil after washing off the secondary antibody.

Extraction then Fixation

Extract in CBS with 0.1% TX100 and 1ug/ml phalloidin for 30-60 seconds. Immediately add fix of choice (do not wash after extracting). Proceed as above. For a first round of experiments always compare staining to control cells that were not extracted. If you are planning a double label experiment with fluorescent-phalloidin and wish to extract before fixing- do not substitute fluorescent-phalloidin for phalloidin in the extraction for the following reasons:

- The extraction time is too short for good intensity of fluorescence.
- The extraction is so short that phalloidin does not saturate all the binding sites, so that when you incubate with fluorescent-phalloidin later in the procedure you still get good intensity of fluorescence.
- It is too expensive.

Tubulin Cytoskeleton

Glutaraldehyde Fixation: (Microtubules alone)

1. Extract cells in Microtubule Stabilizing Buffer (MTSB) + 0.5 % TX-100 for 30 seconds.

MTSB = BRB80 + 4 mM EGTA

1. Add glutaraldehyde to 0.5 % final. (I generally add from a 50% stock to the container with the coverslip and mix it in gently but rapidly) - Fix for 10'.
2. During fixation, make 0.1% NaBH4 (sodium borohydride) in PBS. This is used to quench unreacted glutaraldehyde which is very fluorescent if not reduced.
3. After fixation, quench for 7'. CAUTION! The borohydride will bubble vigorously and may cause coverslips to float and flip occasionally (see comment below)
4. Rinse well in PBS and process for tubulin immunofluorescence.
5. Block in AbDil for 10'.
6. Anti-tubulin for 20' - 30' (We use DM1alpha)
7. Wash 4x TBST (TBST = TBS + 0.1% Triton X-100)
8. Secondary for 20' - 30'
9. Wash 4X TBST.
10. Wash once with TBST + 1 ug/ml Hoechst. A rapid rinse will be sufficient.
11. Drain, mount and seal.

COMMENT : The most troublesome aspect of this procedure is the borohydride quenching. Please try this on a test basis before wasting valuable antibody/cells! I tape a razor blade onto the frosted part of a microscope slide (dull side facing out) and this blunt edge is placed onto a porcelain coversip holder to physically block the coverslips from floating up when transferred to the quench. However, after a few tries, this is no longer a problem and the microtubules are beautifully preserved by this method. Some cellular structures may get dislodged by the borohydride although this method has been used successfully for microtubule immunofluorescence in neurons which tend to be fairly fragile. Glutaraldehyde fixation does not preserve other antigens very well and methanol appears to be the best compromise between preservation of microtubules and maintaining antigenicity of other proteins.

Methanol Fixation: (for co-microtubule immunofluorescence)

1. Fix cells in -20 deg C methanol for 3'.
2. Rehydrate in TBST 3 x 5'.
3. Process for immunofluoresence as above.

(NOTE: One can extract cells in MTSB + 0.5% TX-100 for 30 seconds before fixing in methanol. Extraction can often generate artifactual localizations - especially for motor proteins where it has been documented that after extraction one often sees colocalization with microtubules which is not present in straight methanol fixation. This colocalization of motors with microtubules is abolished by addition of ATP to the extraction buffer suggesting that the observed colocalization is artifactually generated by rigor binding of motors to microtubules during the extraction.)

NOTES: General comments on double label immunofluorescence are given above in the section on actin fixation. The one problem with methanol fixation is its destruction of chromosome morphology. Methanol tends to 'puff' up mitotic chromosomes which are best preserved by formaldehyde. For centromeric antigens, we often use formaldehyde fixation and accept the poor microtubule morphology. Autoimmune sera to centromeric components, however, often require methanol fixation and then we have to accept poor chromosome morphology. As always, the conditions will need to be optimized depending on the nature of your antibody. To maximize the chances for success, for a newly generated antibody we always try methanol and formaldehyde fixation (3% formaldehyde for 15') with and without MTSB + 0.5% TX-100 extraction and compare the observed staining with all four conditions. The optimal condition for the antibody is then used for double label immunofluorescence with microtubules. Microtubule structure is poor and very variable with formaldehyde but sometimes formaldehyde ends up being necessary. Mixed formaldehyde/methanol fixative recipes have been described but we have never tried them.