

Immunofluorescent Staining of cells for microscopy

- In a dry 24-well dish lay down cover-slips
For poorly adherent cells use laminin coated coverslips (BD Biocoat Poly-D-Lysine/Laminin coating 12mm diam BD# 354087; also available through fisher)
For regularly adherent cells Use #1 coverslips (Fisherbrand cat #12-545-80 12mm circle 1 thickness = 0.13-0.17mm The 1.5 are 0.16-0.19mm, the ideal optics are for 0.17mm, the cat # for 1.5 is #12-545-81, use 1, not 1.5) (No need to acid treat) Keep the coverslips in a beaker with 100% EtOH and right before using them separate them in the tissue culture hood on a clean paper towel and allow them to dry
- Seed cells at about 1×10^5 cells per well. And allow them to attach overnight.
- **Alternative protocol For cells that will be transfected** day 1) seed 24 well (see protocol for transfection). Day 2) transfect, wait 6 hours, trypsin treat and split into 4 wells for microscopy. Day 3) fix and Stain. Alternatively Day 1) transfect the same day as seed, wait 6 hours and split. Day 2) fix and stain. Have a mock transfected control as the lipofectamine makes the cells auto-fluoresce more.
- Remove medium by aspiration and wash 1x in PBS
- Fix by adding 300 μ l of 1-2% paraformaldehyde/PBS for 10-20 min RmT°. (Buy 16% paraformaldehyde solution from Electron Microscope Sciences Cat #15710 keep at Rm T°, dilute to 4% in PBS and keep at -20°C. Dilute in PBS right before using). 10 min with 2% is what was recommended QFM class
- An alternative fix is EtOH with glycine/PBS (For 250ml of solution mix: 175ml EtOH; 18.75ml of 200mM Glycine/PBS; and 56.25 ml of PBS. Keep solution at -20°C). For this fix, do 5 min or more. at -20°C. Glycine quenches free aldehyde groups reducing autofluorescence
- Wash in PBS/200 mM Glycine, 0.02% Sodium azide (Make the solution and filter sterilize, it has good shelf-life) 3x over 5-10 min.
- For the first round try without blocking, otherwise use normal serum from the same species as the 2ary antibody. So for example if you are going to use goat anti-mouse FITC conjugated use Normal Goat Serum, preferably purified. Use at around 6 mg/ml in PBS/0.1% Brij 58 (see below).
- For any step that involves an antibody spin the stock vials first to remove any precipitates, then make dilutions.
- Alternative Blocking buffer from the Microscopy class is 1xPBS, 2% BSA (fraction V). To block add 5% Normal goat serum in blocking buffer for 30 minutes. The antibodies are diluted in the blocking buffer and also the washes.
- If using more than one color, match your secondary species, all donkey or all goat
- Add either no primary antibody, or diluted 1ary Ab; usually start at around 5 μ g/ml diluted in PBS/0.1% Brij 58 (Make a 1% solution of Brij in PBS by warming up to 37°C, then use this to make the solution, also filter sterilize the solution and keep both stock brij and solution for about 2 months). Place at RmT° for 1 hour or 4°C overnight (less background).
- Wash 3x in PBS/0.1% Brij 58 over 5-10 min.
- Add PBS/0.1%Brij only or 2ary Antibody, prefer Molecular Probes Alexa Fluor 488 (Green) or 568 (red) (Start at 10 μ g/ml), if not possible use Jackson's FITC or if not possible TRITC at 1:200 in PBS/0.1% Brij 58. Place at 4°C for 1 hour in the DARK!!! Or rotate covered from light 1hr room temperature
- Wash 3x in over 5-10 min.
- Date and number a glass microscope slide. Place one small dot of Fluoromount (Southern biotechnologies)

- Alternatively you can use a mounting medium that has DAPI stain (for nuclei) in it. It is also good to prevent bleaching. Prolong Gold Antifade with DAPI (Molecular Probes Cat# 36931)
- With a syringe needle shaped as a hook dislodge the laminin coverslip from the bottom of the wells and leave it “standing” on its side.
- With serrated curved tweezers grab the cover slip, lightly blot it on paper towels (allow only the narrow part of the cover to touch the towel).
- Place the cover cell-side down on top of the drop of Prolong gold. Repeat for all covers.
- With the tweezers lightly press the top of the coverslips to eliminate residual prolong gold; only press vertically, no lateral movements.
- Using folded paper towels lightly blot all the coverslips with one vertical motion.
- Place flat inside a dark drawer and let it harden overnight. If you are in a hurry to view them, this can be sped up by placing at 37°C
- After they harden store in the dark at 4°C or at -20°C. Until ready to view by microscopy.