Protocol for Immunofluorescence: For Determining the Localization of PP2A (by Johann Jackson)

Preparation of Cells and Tissues

Cells for staining are usually prepared from one of three sources: adherent cells, suspension cells, or whole tissues. Adherent cells normally are prepared for cell staining by growing on a suitable support. Suspension cells can be fixed directly or can be attached to a solid support by centrifugation, chemical cross-linking, or simple dehydration.

Adherent Cells:

Adherent cells are easily prepared for cell staining by growing on a suitable microscope slide, coverslip, or plastic tissue culture dish. For high-resolution work and for studies that will be photographed, cells should be grown on cover slips or slides. For lower-resolution work or for large-scale screening procedures, the cells can be grown on tissue culture dishes.

Growing Adherent Cells on Coverslips

Sterilization of Cover Slips

Under the hood

1) Supplies: Bunsen burner, two forceps, #1 hematology coverslips, and a falcon tube of 70% ethanol

2) Place Bunsen burner on blue flame and dip forceps in ethanol and wave through fire for 3 seconds. Allow flame on forceps to disappear and wave the forceps through the flame repeatedly until forceps are dry. (Warning: Be careful not to leave forceps in fire for over a 3 second period, because will cause rusting.)

3) With forceps pick up a coverslip and dip it in ethanol and wave through flame, *Briefly!!* (Note: If coverslip left too long in flame it will crack, bend, or break into pieces). Allow coverslip to cool (3 seconds) and repeat process until there is no ethanol left on coverslip. After coverslip is dry place in p100 dish. (Note: Always plate of three coverslips over the amount required for the experiment-- due to overlapping of coverslips during incubation.)

4) Split cells to coverslip p100 plate and incubate overnight. (Note: you want to have a 50-60% confluence for the coverslip plate.)

5) Once incubation period is over, view plate under microscope to determine if the cells are adherent.

6) If cells are adherent move on to fixation of the cells.
**Fixation** (Method of F. Solomon & co-workers)

1) Make solutions

   a) 4% Paraformaldehyde (note: wear mask when making because it is dangerous to inhale)
      
      in a 250 ml beaker or bottle
      add 100 ml of 1X PBS
      4g of paraformaldehyde powder
      *heat on hot plate in fume hood until powder goes into solution or right before solution boils (100°C). Allow to cool and store 10 ml or other appropriately sized aliquots in 15ml falcon tubes at -20 °C for later use.

   b) 0.5% NP-40 in PBS
      
      in 25 ml flask
      add 0.5 ml of 10% NP-40
      add 9.5 ml of 1X PBS

2) Fixing Cells  Under the hood

   A) Remove media from coverslip plate with Pasteur pipette.

   B) Pour 10ml (one falcon tube per plate) of 4% paraformaldehyde into p100 with coverslips and let stand for 20 min. at room temperature

   C) Remove paraformaldehyde and add 0.5% NP-40 in PBS and let stand for 10 min. at room temperature.

   D) Remove all of 0.5% NP-40 from dish, and WASH plates three times with 8 ml (or enough to cover the coverslips) of 4 °C PBS.

   E) Leave last PBS wash on plate and proceed to Blocking.

**Blocking**

1) Make Solutions

   10% Donkey Serum in PBS (40 μl per cover slip)
   
   a) Make up appropriate amount in eppendorf tubes and spin down dilution on high in 4 °C centrifuge for 5 min.

   EX: For 4 cover slips you need 160 μl. You want to make one over so your need 200 μl.
   10% Donkey Serum : 1/10 * 200 = 20 μl
   PBS: 200 - 20 = 180 μl
2) Prepare dish
   A) Cut parafilm in the shape of the bottom of a p100 and place in the dish
   B) On the outside of the bottom of the dish -- draw a pie grid and label
       appropriately, according to cell type (if variant), dilution of primary Ab, and
dilution of secondary Ab: i.e. Zips
       10 µg 4B7
       1:1000 RHD
       (RHD stands for rhodamine)

3) Remove coverslips from the p100 with forceps containing PBS. Blot dry bottom of
   coverslip using a Kim wipe and drain top of coverslip. Note: Be careful not to
   break coverslip and not to allow the top of coverslip to touch the Kim wipe.

4) Place coverslips in new dish on appropriate division. (Be careful not to flip
    coverslips onto side with cells because will be destroyed). After all coverslips have
    been placed on dish move on to step 5.

5) Add 40 µl of 10 % Donkey Serum to each coverslip and incubate at room
    temperature for 20 min.

6) Wash each coverslip in 1X PBS twice for 10 sec each wash. (useful to use 24
    microwell plate for washes, using each well for one wash). Blot dry again on
    Kim wipes and place on new parafilm p100 plate with labeled grid.

7) After washes begin Primary Ab staining.

**Primary Antibody Staining**

Making Solutions

   a) 3% BSA/PBS (Note: only used for Ab dilutions)
      Stir bar in jar
      3 g of BSA (Bovine Serum Albumin) + 100 µl of nonsterile 1X PBS
      Stir mixture for 30 min. to make sure BSA fully dissolves

1) Determine dilutions experimentally using 3% BSA/PBS to dilute antibody in
   eppendorf tubes.

2) Prepare enough for appropriate # of coverslips (40 µl each coverslip) and spin
   down dilution at full speed in 4 °C centrifuge for 5 min.
   **Note**: controls only receive 3% BSA/PBS for this incubation

3) Place 40 µl on coverslip and incubate for 30 min. at room temperature.

4) Wash coverslips in 3 times in 1X 4°C PBS using 3 new wells in the microwell
    plate. Blot dry as before on Kim wipes.

5) Place in new dish with parafilm for Secondary Staining.
Secondary Antibody Staining

1) Determine the type of secondary Ab needed which is determine by the compatibility to the primary Ab. Determine the dilutions (using 3% BSA/PBS) experimentally spin down dilutions in 4 °C centrifuge on high for 5 min. **MAKE SURE YOUR STOCK SOLUTION AND DILUTIONS ARE KEPT COLD AND OUT OF THE LIGHT UNTIL USED.** (helpful to cover centrifuge tube with foil before making dilutions.)

2) Place 40 µl appropriate dilution on coverslip (As of 8/11/95 dilutions used were 1:1000 for both Rhodamine and FITC obtained from Jackson Immuno Research)

3) Incubate in the dark for 30 min. at room temperature (cover p100 with foil).

4) Wash coverslips 5 times (5 microwells) in 4 °C PBS, blot dry, and prepare to mount.

Mounting

1) Label glass slide with date, cell type, fixing solution, dilution of primary Ab used, and dilution of secondary antibody used.

2) After blotting dry the coverslips, place a drop of fluoromount solution on glass slide using a Pasteur pipette and bulb.

3) Place coverslip with forceps (cells down) on the edge of the fluid and allow to drop. (Note: If bubble present under coverslip tap gentle on back of coverslip until removed. Make sure not to press down onto coverslip, just allow the bubble to migrate out.)

4) Allow to dry overnight on flat surface at 4 °C in the dark.

Fluorescent Microscope

View slides under microscope. Compare the control to the experimental slides. Be careful of bleaching slides, especially of fluorosein.