Immunostaining on glass coverslips

(From Hank Dudek, Greenberg lab, October 1995)

Solutions:

8% Paraformaldehyde, 4% sucrose in 1xPBS: for100 mL (make in hood, avoid breathing para dust).

Heat 60 mL of ddH2O to 80°C.
Add 8 gm paraformaldehyde (weigh out in hood).
Turn off heater, keep stirring until solution is uniformly chalky.
Add 1N NaOH drop by drop until solution is clear.
Add 10 mL of 10xPBS.
Add 4 gm of sucrose. Mix well.
Bring volume to 100 mL with ddH2O.
Cool.
pH to 7.4 with NaPO4 (filter if necessary).

This can be stored at 4°C for up to two months (may want to check pH after a number of weeks).

1x PBS/0.1% Triton X-100 (need large volumes for lots of washes).

Add 1 mL Triton X-100 per L of 1X PBS (may want to try adding 0.02% NaAzide - which should not affect immunofluorescence, but which will interfere with peroxidase activity).

10x PBS: for 1L

80 g NaCl
2 g KCl
11.5 g Na2HPO4.7H2O
2 g KH2PO4

Mounting solution:

Glycerol gelatin (Sigma #GG1) (can make 500 uL aliquots and store at –20°C).
Add anti-bleaching agent, p-phenylene diamine, if needed to final of 100 ug/mL (can simply dilute HD's 25 mg/mL stock 1:250 in the glycerol gelatin), also store at –20°C.

Protocol:

Day 1

- Warm 8% Para/4% sucrose fix solution to 37°C.
- Add equal volume of fix solution directly to the wells (these limits the amount of cells lost with handling).
- Fix at RT for 5-20 min (e.g., beta-gal 5 min sufficient, BrdU 15 min).
- Prepare two small trays with PBS/0.1%Triton/10mM glycine (add 7.5 mg glycine to 10 mL PBS/Triton) (each small tuperware is about 100 mL).
- Transfer coverslips to coverslip rack always maintaining same orientation, e.g., cell side forward; take from wells left to right, row by row.

- Wash in PBS/Triton/glycine 1x 5 -20 min, RT (tilt racks when submerging them, so slips more horizontal) (for BrdU staining, switch to BrdU protocol at this point).
- Transfer coverslips to 24-well plate (unless prefer to leave out the normal goat serum).
- Block in 3% BSA/0.1% Triton/1% NGS/PBS (each drop of goat serum from vectastain kit = 45 uL).
- RT 2 hrs (up to 5 hrs).
- Transfer to humidity chamber (150 mm petri dish with moist blotting paper + layer of parafilm + labelled spaces for coverslips).
- Aspirate off block and add 1ary antibody (don't let coverslip dry out) (use 50 uL for 15 mm slips, 15 uL for 12 mm slips).
- Leave at 4°C where they won't get disturbed, for 2 hrs to overnight.

Day 2

- Aspirate primary antibody (only process 12 slips at a time so they don't try out excessively).
- Transfer slips to coverslip rack.
- Wash in PBS/0.1% Triton X-100, 2 x 5 min, RT.
- Dilute secondary antibody and keep protected from light.
- Transfer to humidity chamber (new parafilm).
- Aspirate liquid and add secondary antibody in block with 1% NGS (shield chamber from light).
- RT 1-2 hrs
- Warm up mounting solution to 60°C.
- Aspirate antibody, transfer to coverslip rack (again, at most, 12 at a time).
- Wash 1x PBS/Triton with 2.5 ug/mL of Hoechst 33258 dye to stain nuclei.
- Wash 1x PBS/Triton.
- Mount 1 coverslip at a time:
    - aspirate excess liquid, blot slip on kimwipe
    - place 10 uL of mountant onto slide (5 uL for 12 mm coverslips), remove any bubbles
    - center coverslip on drop
    - let dry, no need to seal with nail polish