Protocol for AlexaFluor/LY Antibody Processing
(using unlabelled primary antibodies)
updated 3/7/2012 pbm

This protocol requires 2 days of processing to complete the immunocytochemistry staining.

General Instructions:

- All containers used for solutions and tissue must be clean. Typically, this means to use 50 mL conical tissue culture (sterile) tubes for the Tris-Triton or PBS mixture, and 10 mL conical (sterile) tubes for the BSA-Tris-Triton/PBS, ABC, and DAB solutions. Do not use laboratory beakers or glassware to make up these solutions. Use graduated cylinders only to measure out the larger Tris volumes (then transfer to a conical tube); use culture pipettes to measure out 2-10 mL volumes; and use the appropriate pipettors for smaller volumes. Dispose the small pipette tips in the sharps containers; dispose of the culture (2,5,10 mL) pipettes in the biohazard red bags.

- The incubations should be done in a 24 or 48well plate. Use 2, 5 or 10 mL tissue-culture (sterile) pipettes to transfer solutions being brought into the processing; and use clean, fire-polished, pasteur pipettes to withdraw solutions during solution changes.

- All solutions need to be brought to pH appropriately (e.g., ±0.05 units from the nominal value); the reactions may not proceed if the pH is off.

- All reactions are run at nominal room temperature (20 ± 3 °C), *except* for the antibody incubation, which is run at 4°C (i.e., in refrigerator or cold room).

- Times listed are approximate except for the final steps for ABC and DAB (if used), which should be adhered to closely. In particular, the wash steps can be extended (but not shortened), for a total wash time not to exceed 1.5x the nominal total value, without compromising the reactions. Always time each step.

- When making solutions containing multiple items (e.g., BSA+antibody; ABC, DAB), add each item in the specified sequence, mixing well before adding the next item. Mix by inverting and righting the conical test tube 10-15 times, slowly and gently. Do not shake or vortex solutions with proteins or antibodies. Solutions containing Triton or BSA will tend to foam, and this can denature the antibodies and the BSA, which will cause problems with the reactions (i.e, you're wasting your time). It is inevitable that some bubbles will be on the top of the solution, but major foaming should be avoided.

- Except when adding Triton to the 0.5M Tris stock, do not sonicate the solutions. When making the DAB solution ONLY, you may use the vortexer (this solution contains no protein or Triton).

- *Procedure for changing solutions and washes:* Make sure that each solution is drawn off completely. Use a fire-polished Pasteur pipette, and draw the solution off away from the slice (avoid sucking the slice into the pipette). Do not use a vacuum aspirator; use a manual pipette bulb. Tilt the plate at an angle while aspirating the solution. When all the solution is off all of the slices in the plate, tilt the plate it the other way and draw off any remaining solution in the wells. Then immediately add the next solution, using a calibrated pipette to help divide the solution evenly among the wells. Do not let the slices dry out!

- All steps are run with the plate on a shaker (IKA Schuttler), with the speed set at 200/min. Tape the plate down, and/or use rubber bands to help hold it in place. Do not use the timer on the shaker; rather set it on infinity (ccw). It is better for the shaking to continue after the time is up than it is for the shaking action to stop. Use a separate timer to keep track of the times.

A. **Choice of Primary Antibody:**

Anti-Alexa-Fluor 488 antibody (Invitrogen anti—Alexa Fluor 488 antibody, rabbit IgG fraction, 1mg/ml, Catalog # A-11094 ($273.36/0.5 ml)
Anti-Lucifer Yellow antibody (Invitrogen anti-Lucifer Yellow antibody, rabbit IgG fraction, 3mg/ml, A-5750, Invitrogen, $270.35/0.5ml)

Dilute 0.5 mL antibody as received 1:10 in 0.22 μm filtered PBS (500 μL anti-Alexa or anti LY + 4.5 mL PBS).
Date, label, and store as 100 μL or 200 μL aliquots at -70°C.
Record the Lot number, received data, and aliquot date in your data notebook, and make sure that this information appears on the box that the aliquots are stored in so that others can use them.
This is done once per lot of antibody.

D. C**hoice of Secondary Antibody:**

Goat anti-Rabbit: Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) *Highly Cross-Adsorbed* *2 mg/mL* (also comes in other wavelengths) 2mg/ml
Cross-absorbed against a mouse, rat, goat, etc. to minimize reaction to primary sites in the tissue, presumable to improve signal-to-noise ratio. 0.5 ml = $182.00 for 1 mg
DyLight goat anti-Rabbit from Vector Labs DI-1488-1.5 $120/1.5 mg (

Which secondary is used depends on the goals of the staining - if you are identifying cells already stained, and there is significant background fluorescence when using 488 illumination, then a longer wavelength secondary would be advisable.

Other dyes: Seta/Setau - these would have to be conjugated to the appropriate unlabeled IgG to work. They reportedly have a very large 2P cross section, but few hard numbers are given.

Follow the manufacturer's instructions regarding dilution of antibody and freeze-thaw cycles (some say "do not aliquot", in which case you must be very careful with the Ab).

Solutions You Will Need:

PBS, pH 7.4. Be sure the pH is correct and that the solution is clear (no flocculent debris). Filtered solution is preferred.

Note: Use 0.3-0.5 ml per well (section) for floating sections in 48-well plates for reactions. The volumes used should be calculated for this target amount.

Day 1:

1% Na-borohydride in PBS (1 ml/section).

Blocking Solution (0.5ml per section):

1. PBS
2. 10% horse serum (50 µl per section)
3. 0.2%BSA (by weight): (1 mg per section)
4. 0.5% TritonX-100 (2.5 µl per section)

Primary Antibody: (0.5 ml per section)

1. PBS
2. 1% horse serum (5 µl per section)
3. 0.2%BSA (1 mg/section)
4. 0.5% TritonX-100 (2.5 µl per section)
5. 1:500 dilution from the stock of primary antibody (0.25 µl per section)

   Add in the order shown, mixing the blocking serum well before adding the antibody. Invert tube slowly and gently about 10-20 times at each stage to make sure that the solution is well mixed.

NOTE: The Triton is very viscous, so when pipetting make sure that you wait for the full volume to be loaded and ejected from the pipette. This will take a few seconds. It helps to solubilize the Triton by using the sonicator for a few minutes and gently (so as not to make too much foam) gently inverting and righting the tube.

- Wash slices in 2x changes of PBS, 5 min each.
- Incubate with 1% Na-borohydride in PBS for 10 minutes. This reduced background.
- Wash slices 4X changes of PBS, 5 min each.
- Incubate with Blocking solution for 20 minutes.
- Wash 2X with PBS, 5 min each.
- Incubate with Primary antibody, 1hr on shaker, and then overnight at 4°C (refrigerator). If the sections are thick (e.g., brain slices, unsectioned), then 24-48 hours on the shaker in the coldroom may be necessary.

Day 2:

Secondary Antibody:

PBS

1:500 dilution secondary of choice (Alexa-Fluor 488 or 546 goat anti rabbit etc.). Secondary will be 1 μl per section at this concentration. If secondary is at 2 mg/ml (in 0.5 ml total stock); staining concentration of 1-10 ug/ml = 1:200 to 1:2000 dilution. Adjust this calculation for stated IgG concentration of secondary as supplied by the manufacturer.

0.5% Triton-X100 (2.5 μl per section).

- Wash section 4X in PBS, 5 min each.
- Incubate 2-4 hours at room temperature in the secondary (depending on section thickness, again)
- Wash 6X for 5 min each in PBS (to be sure unbound secondary is washed out).
- Transfer section to slide, blot away excess liquid. Mount in VectaShield or other non-fluorescent mounting medium, with coverslip.

Protocol follows from Wolfart et al., J. Neurosci 21:3443, 2001 and Liss et al., EMBO, 2001.