Immunocytochemistry for Confocal Microscopy
III. Immuno Run

1. **Rinse:** The sections are processed in small plastic microbeakers (Fisher Scientific). In a standard run, 2-6 sections are used per well. Each well will be for a particular primary antibody (or antibody combination). If sections were fixed in paraformaldehyde, first rinse in cold (4ºC) 1X PBS.
   - 3 15min rinses
   - 1 1hr. rinse.

2. **Block:** Block the sections using serum made from the same species as the secondary antibodies. We use Normal Donkey Serum (NDS; 1:20) made in cold 1X PBTA (i.e. 25 µl NDS in 475 µl 1X PBTA) and add 500 µl for each well. Incubate 2 hrs to overnight at 4ºC on a rotator.

3. **Primary Antibodies:** Make all antibody dilutions in cold (4ºC) 1X PBTA. Make 500 µl total solution for each well. Remove Blocking solution and add primary antibody solution. Incubate overnight at 4ºC on a rotator.

4. **Rinse** Remove primary antibodies (and save for re-use if the antibodies are valuable; store most at 4ºC). Rinse with cold (4ºC) 1X PBTA on a rotator.
   - 3 15min rinses
   - 1 1hr. rinse.

5. **Secondary Antibodies:** Dilute secondary antibodies (i.e. donkey anti-mouse-Cy3; "DAM"-Cy3) to 1:200 in cold (4ºC) 1X PBTA. Strepavadin probes are diluted 1:100. Remove final rise and add diluted secondary antibodies. Incubate overnight 4ºC on a rotator. (We purchase all the secondary antibodies from Jackson Labs).

6. **Rinse:** Remove (and discard) secondary antibodies from cups/wells and
   - 3 15min rinses
   - 1 1hr. rinse.

7. **Mounting on Slides:**
   Lift sections GENTLY out of well with a spatula.

   Push the section off the spatula and onto a glass slide using a razor blade (blunt side works well). Place up to 4 sections per slide, side by side.

   Wick away excess PBTA with a Kimwipe and place a drop of *n-propyl-gallate* on the sections (use a pastuer pipette).

   Place a coverslip (thickness=0 mm) onto the sections. If bubbles cover any of the tissue, the cover slip can be lifted with a razor and spatula (but be slow and careful). Wick away excess or add a small amount (if not enough) of *n-propyl-gallate* at edge of cover slip. It's important not to have too much *n-propyl-gallate* or the sections will begin to detach from the slide.

   Seal cover slip with nail polish. Store slides in the dark at 4^0C.