Xenopus WHOLE-MOUNT IMMUNO-HISTOCHEMISTRY protocol

General information:

1) Everything happens in scint vials, with max volume of changes.
2) Be very careful when changing solutions not to suck up the sections or small samples – always be sure you can see all the samples in the tube before putting in the suction.
3) Each vial is about 25 ml volume, so calculate how much total solution you will need based on that, multiplied on the number of vials you have.
4) Change the tip on the suction, in case someone else used it for some nasty chemical.
5) AlkPhos is the best color, but secondary AP-conjugated antibodies from Jackson have some background. Always include "no-primary" and "no-secondary" controls.

Protocol:

0. Fixation:

- very cold methanol or formaldehyde (MEMFA)
- for cytoskeleton, use:
  3% formaldehyde
  0.2% glutaraldehyde
  0.2% triton X-100
  10 mM EGTA

1. Preparation, depending on intended detection method:

- For fluorescent, need to:
  a. Dehydrate with 1X 10' 50% MeOH, 1X 10' 100% MeOH, then transfer to fresh 100% MeOH. Use immediately or store at -20C indefinitely. NOTE: for samples for paraffin sectioning use EtOH instead. Rehydrate to PBTr.
  b. Bleach for at least 1 hour in 1% H_2O_2 in bleaching buffer (5% formamide, 0.5X SSC) on a nutator with bright light. In most cases you can bleach for longer or with higher H_2O_2 concentration if your embryos are very pigmented. *Always* do this step, even for albinos; cut off the heads if possible, because they'll accumulate bubbles. Better to do longer time and not increase peroxide (otherwise too many bubbles).
- For alkaline phosphatase, need to deactivate endogenous enzyme (comes on at gastrulation or so – don't need it for cleavage stages): heat in mock hybe solution for 3 hours at 69 °C.
- For HRP, Bleach for at least 1 hour in 1% H_2O_2 in bleaching buffer (5% formamide, 0.5X SSC) on a nutator with bright light. Don't need to do this on blastomere-stage sections, but definitely do need this for later embryos.
- For β-gal, there's no background!
- For fluorescent, need to consider autofluorescence:
  a. Older frog embryos have autofluorescence in FITC channel
  b. Glutaraldehyde has fluorescence on all wavelengths, so gel/alb embedded blocks will have bright signal all around

2. Permeabilize in PBTr for 30 minutes rocking at room temperature.

3. Make blocking solution: PBT + 10% heat-inactivated goat serum. Block samples at RT for 1 hour rocking sideways.

4. Incubate in primary antibody in fresh block solution overnight at 4 °C rocking upright. Mike will add primaries, so just leave him the block solution at 4 °C.

5. Wash 6X (fill vial each time) with PBT/1h @/RT sideways rocking.

6. Make blocking solution: PBT + 10% heat-inactivated goat serum. Block samples at RT for 1 hour rocking sideways.

7. Incubate in appropriate secondary antibody overnight at 4°
   - alk. phos. conjugated goat anti-rabbit (or anti-mouse or whatever) Ig
     - from Jackson ImmunoLabs at 1:1500 or 1:2000, or
     - from Rockland (611-105-122) at 1:500 to 1:1000
   - alexafluor-conjugated at 1:500, or
   - HRP (Jackson), at 1:250 for DAB detection, or better, 1:750 for TrueBlue detection, and 1:2000 for TMB-H detection
   - β-gal-conjugated from SouthernBiotech at (1:500?)

8. Wash 6X with PBT as in step 4. Last wash should include 0.05 g Levamisole for every 100 ml of PBT, for vials intended for alk-phos detection.

9. Chromogenic reaction:
   a. AP: wash 2X for 5 min in chromogenic solution (without substrate). Replace last wash with chromogenic solution + 1 μl NBT solution/ml + 3.5μl BCIP solution/ml. Put it into cardboard boxes (dark) immediately, and watch the chromogenic reaction periodically; stop it when necessary. Don't use the ready-made stuff from Rockland/Moss.
   b. HRP: add direct TMB-H (http://www.mosssubstrates.com/). Don't dilute TMB-H – use it straight. This is better than the old way using DAB (brown, toxic). Reaction is not light sensitive! Stop it in 18 MΩ water, and post-fix with @ (Methanol will remove background but eventually also remove signal). Reaction will be complete in < 10 minutes usually; aqua color is often background - real signal is dark purple.

Notes:

1) background in older embryos is from secondary antibody, not endogenous alk-phos or primary antibody. Can reduce amount of secondary.
2) for fluorescent detection,
   - 1st antibody – use 555 (beautiful red, TRITC)
   - 2nd antibody – you have a choice: 647 is best, but can't see it on Nikon (use confocal or Olympus); or, 488, which is green (FITC) and is only good if signal is blazing.

3) if using HRP antibodies, use TrueBlue or TMB-H instead of DAB for a blue product.
4) stain DNA if desired with Hoechst dye or TO-PRO-3 (depending on wavelength desired) 5', then wash with PBST a few times.
5) Suggested dilutions:
   - DHSB primaries – 1:20 to 1:50
   - Secondary antibodies
      - DAB – 1:250 HRP secondaries (Jackson)
      - True Blue – 1:750 HRP secondaries (Jackson)
      - TMB-H – 1:@ HRP

Solutions:

1. PBT: PBS + 2 mg/ml BSA + 0.1% Tween-20 (you will need a lot of PBT, from 1st day).
2. PBTr: PBS + 2 mg/ml BSA + 0.1% Triton X-100 (only need a little, on 1st day).
3. Chromogenic reaction solution:

	<u>per 100 ml (**make fresh on last day**)</u>
100 mM Tris (pH 9.5)	10 ml of 1 M stock
50 mM $MgCl_2$	5 ml of 1M stock
100 mM NaCl	2 ml of 5 M stock
0.05% Tween 20	0.5 ml of 10% Tween-20
5 mM levamisole	0.12 g of levamisole powder

NBT stocks: nitro blue tetrazolium; 75 mg/ml in 70% dimethylformamide.
BCIP: 5-bromo-4-chloro-indolyl-phosphate; 50 mg/ml in 100% dimethylformamide for toluidine salt (or in H_20 for sodium salt).
Store both of those stocks at -80 °C.