Immunofluorescence – cryosections (B. Björk 3/15/12)

Tissue Preparation:

1. Sacrifice pregnant females carrying embryos of the desired developmental stage as determined by observation of a post-copulatory plug as embryonic day (E) 0.5.

2. Perform Caesarian section to remove uterine horns containing embryos, and place them in ice-cold PBS-0.1% Tween (PBSw). Dissect embryos carefully, taking care while removing them from their yolk sac. SAVE yolk sacs from each embryo, rinse in cold PBSw and store at -20ºC for genomic DNA isolation and genotyping PCR.

3. Separate heads from bodies for E12.5 – E15.5 and fix in 4% Paraformaldehyde (PFA) 30 min. to overnight at 4ºC.

4. Wash 3 times in cold PBS/0.1% Tween (PBSw)

5. Incubate embryos in 30% Sucrose/PBSw overnight at 4ºC.

6. Incubate embryos in 1:1 (OCT freezing medium: 30% Sucrose/PBSw) until embryos drop to the bottom of tube/well.

7. Embed embryos in OCT over dry ice, and store @ -80ºC.

8. Cut 10 µM sections through embryos using a cryostat, and dry sections for 30 min. on slide dryer.

Immunolabeling:

1. Antigen retrieval, **IF NECESSARY**, in 10 mM Sodium Citrate, pH 6/0.5% Tween from 1 M stock solution (pH with 30-40 drops Concentrated HCl in 1 L 10 mM solution to achieve pH 6).
   a. Pre-warm Na Citrate in a 250 ml slide staining reservoir placed in a secondary container of water for 3 min. in a microwave.
   b. Place rack of slides in the pre-warmed Na Citrate solution and heat on high for 10 min. (periodically add additional Na Citrate solution to account for loss of volume due to evaporation.
   c. Cool on bench (approximately 20 min.); mid-way through replace half volume with PBS.

2. If antigen retrieval is not necessary, then remove slides from the -80ºC freezer and allow them to dry completely.

3. Outline sections to be incubated with the same primary antibodies with a hydrophobic barrier pen.
4. Block sections in Primary antibody Blocking solution for 30 min. at room temperature (or longer at 4°C)

| Heat-inactivated goat serum (5%) | 2.5 ml |
| 20% Triton-X100 (0.1%)            | 250 μl |
| PBS                               | 47.25 ml |
| T.V.                              | 50 ml |

5. Wash slides briefly in 1X PBS

6. Incubate sections with Primary antibody (diluted to appropriate concentration in Primary antibody Blocking solution) overnight at 4°C in a tightly closed humid container.

7. Wash three times in PBS for 5 min. each at room temperature.

8. Incubate sections with Secondary antibody (diluted to appropriate concentration in Secondary Blocking Solution) for 1-2 hours at room temperature.

| Heat-inactivated goat serum (1%) | 2.5 ml |
| 20% Triton-X100 (0.1%)           | 250 μl |
| PBS                               | 47.25 ml |
| T.V.                              | 50 ml |

9. Wash three times in PBS for 5 min. each at room temperature.

10. Mount sections in VectaShield containing DAPI under glass coverslips.