Whole Mount Mouse Embryo in situ Protocol

<u>Day O</u>

1. Dissect embryos in PBS

2. Fix embryos in 4% paraformaldehyde at room temperature or 4C.

3. Dehydrate the embryos into 100% methanol and store at –20C. (optional)

4. (optional) Prior to use, bleach the embryos in 5:1 methanol: 30% hydrogen peroxide or 5:1 PFA:30% hydrogen peroxide for 5-6 hours at room temperature, followed by several washes in methanol or PFA. The embryos can then be stored in methanol at –20C, or in PFA at 4C indefinitely.

<u>Day 1</u>

- Each wash, unless indicated, is with at least 2ml of solution
- Use an inverter mixer for all washes

1. If dehydrated, rehydrate the embryos through graded methanol series
   a. 75% x 5min @4C
   b. 50% x 5min @4C
   c. 25% x 15min @4C (or until embryos sink*)
   d. transfer samples to a 2ml eppendorf tube (Rnase, Dnase free microfuge tube)

2. Wash three times in PBS
   a. PBS x 5min
   b. PBS x 5min
   c. PBS x 5min

3. Digest with 10ug/ml (2µl of 10mg/ml stock) of Proteinase K x 30 min @ RT*
   a. Actual PK digestion time depends upon the age of the embryo. The following is a rough estimate of times:

      E8.5 = 6 min
      E9.5 = 10 min
      E10.5 = 13 min
      E11.5 = 15 min
      E12.5 = 17 min
      E14.5 = 20 min
      E16.5 = 30 min
      E18.5 = 40 min

*Deproteination is a critical step. Under-digestion will result in poor probe penetration. Over-digestion will result in a loss of structural integrity.

4. Wash in PBS
   a. PBS x 1min
   b. PBS x 5min
   c. PBS x 5min
   d. PBS x 5min

5. Discard wash solution*
   *Pay special attention to discard as much of the PBT solution as possible.

6. Add 1.8 ml of pre-hybridization mix and allow the samples to incubate at the correct hybridization temp in a rotating hybridization oven for at least 1 hour.
   a. As one hour nears, prepare the salmon sperm DNA and riboprobe as follows:
      -prepare 200ul of salmon sperm DNA per 2ml sample; denature at 90C for 10 min and place on ice until ready to use.

7. After at least 1 hour of prehybridization, add 200ul of denatured ssDNA to each sample, and about 100ηg riboprobe to each sample. Hybridize overnight.

Day 2

1. Remove hybridization mix, replace with 2.0 ml 2X SSC x 10min @ hyb temp.
   a. Wash with 2X SSC x 10min @ hyb temp
   b. Wash with 2X SSC x 10min @ hyb temp
   c. Wash with 2X SSC x 10min @ hyb temp
   d. Incubate in 2X SSC x 60min @ 70C*

   *70C kills any endogenous alkaline phosphatase activity. This is perhaps the most significant step towards reducing background. Breaking the 70C wash into two 30-minute sections may minimize damage to the tissue.

2. Wash with RNase A Buffer x 5 min @ RT

3. Discard, add RNase A Buffer + 2.0 ul of RNAse A Enzyme (5mg/ml, 83U/mg) x 60min @37C

4. Discard, Wash four times with 1X Wash (BM)
   a. 1X wash solution, x 10min @RT
   b. 1X wash solution, x 10min @RT
   c. 1X wash solution, x 60min @70C

5. Incubate in block buffer on inverter x 1 hr @ RT

   a. At this time, prepare 2.0ml of block buffer per sample and add 1µl Anti-digoxigenin antibody per sample (1:2000) to this master mix, invert briefly, and let sit at 4C until use.

6. After 1 hour, discard block buffer and add 2.0ml of pre-absorbed block buffer + antibody to samples and incubate overnight @ RT on an inverter.

DAY 3

1. Discard block solution.
2. Wash with 1X wash buffer and wash every hour throughout the day, for a minimum of 5 hours.
3. Wash with 1X was buffer overnight at RT.

DAY 4

1. Rinse with detection buffer for 10 min @ RT.

2. Detect with BM Purple at RT until desired signal strength is obtained (with longer probes you may be able to detect overnight).

3. Store Samples in 4% PFA at 4C.

Solutions

RNase Buffer:
0.5M NaCl (29mg/ml)
10mM Tris-HCl (pH 7.5)

Hybridization Mix:
50% Formamide by volume
50% 2 X SSC by volume
6% dextran sulfate by mass

SSC = saline – sodium citrate