Allen Lab Whole Mount In Situ Hybridization Protocol
(McMahon Lab Protocol modified by Ben Allen)
Updated 091709

Day 1 (embryo collection):

1. Dissect embryos in 1X PBS, removing as many extraembryonic membranes as possible.

2. To prevent probe and antibody trapping:
   Puncture amnion of E8.5 embryos
   Puncture mylencephalon, telencephalon, and make small hole in heart for E9.5 and older embryos

3. Fix embryos in 4% PFA at 4°C O/N on rocker.

N.B.: Use Electron Microscopy grade PFA (EMS catalog #15710) or make up fresh PFA.

Day 2 (pre-treatment and hybridization of embryos):

1. Wash embryos 2 x 10min at 4°C with 1X PBST.

2. Dehydrate embryos at RT with 1 x 5 min washes of each of the following:
   25% MeOH in 1X PBST
   50% MeOH in 1X PBST
   75%MeOH in 1X PBST

3. Dehydrate embryos 2 x 10min at RT with 100% MeOH. Embryos will become cloudy in 100% MeOH.

N.B. embryos can be stored at this step in 100% MeOH at -20°C for at least 6 months.

4. Rehydrate samples at RT with 1x 5min washes of each of the following:
   75% MeOH in 1X PBST
   50% MeOH in 1X PBST
   25% MeOH in 1X PBST

5. Wash samples 2 x 5 min at RT with 1X PBST.

6. Bleach samples with 6% Hydrogen Peroxide (3ml of 30% H_2O_2 + 12ml 1X PBST) for 1hr at RT.

N.B.: embryos should be white after 1hr.

7. Wash samples 3 x 5min at RT with 1X PBST.

8. Treat embryos with 10µg/ml proteinase K at RT (10µl of 10mg/ml Prot.K in 10ml 1X PBST) for the appropriate time as follows:
   2min for E7.0
   5min for E7.5
   10min for E8.5
   15-20min for E9.5
   25-30min for E10.5

N.B.: may need to increase or decrease digestion time depending on which tissue is being examined (e.g. internal structures like notochord, or superficial structure such as AER).

9. Wash embryos 1 x 5min at RT with freshly prepared 2mg/ml glycine in 1X PBST.

N.B.: embryos very fragile after Prot. K treatment before second fixation.

10. Wash embryos 3 x 5min at RT with 1X PBST.

11. Refix embryos for 20min at RT with 0.2% glutaraldehyde, 4% PFA in 1X PBST (80µl 25% glutaraldehyde in 10ml 4% PFA/1X PBT).

N.B.: embryos sticky after fixation.

12. Wash embryos 2 x 5min at RT with 1X PBST.

13. Transfer embryos to screw cap vials for subsequent probe addition.

14. Add 500µl of Prehyb. Solution to each tube, and incubate for 1hr at 70°C in Hybaid Oven.

N.B.: Can store embryos o/n at this stage at 4°C, and re-heat to 70°C the next day.

15. Prior to probe addition, thaw 20X probe, dilute to 1X in Prehyb. Solution, boil for 10min, then incubate on ice for 2min.

16. Add enough probe to cover the entire embryo(s) and incubate O/N at 70°C in Hybaid oven.

Day 3 (Post-hybridization washes and Ab incubation):

1. Prepare solution I as follows:

For 50ml:	25ml	formamide
	10ml	20X SSC, pH4.5
	5ml	10% SDS
	10ml	ddH_2O
	50ml total volume	

2. Pre-warm solution I to 70°C.

3. Remove excess probe (can be stored at -80°C, and re-used at least 5 times) and add Solution I.

4. Rinse embryos 3 times with solution I.

5. Wash embryos 2 x 30min at 70°C on rocker with solution I.

6. Prepare solution II as follows:

For 100ml:	10ml	5M NaCl
	1ml	1M Tris, pH7.5
	100µl	Tween-20
	88.9µl	ddH_2O
	100ml total volume	

7. Pre-warm solution II to 70°C.

8. Wash embryos 1 x 10min at 70°C on rocker with 1:1 mix of solution I: solution II.

9. Wash embryos 3 x 5min at RT with solution II.

10. Dilute RNAseA (Sigma cat. #R6513; 10mg/ml) to 100µg/ml in solution II (150µl RNAse A into 15ml solution II).

11. Wash embryos 1 x 60min at 37°C on rocker with 100µg/ml RNAse A in solution II.

12. Rinse embryos 1 time with solution II.

13. Prepare solution III as follows:

For 50ml:	25ml	formamide

5ml 20X SSC, pH4.5
1ml 10% SDS
19ml ddH_2O
50ml total volume

14. Wash embryos 1 x 5min at RT with solution III.

15. Wash embryos 2 x 30min at 65°C on rocker with solution III.
N.B.: Prepare MBST solutions during these washes.

16. Prepare MBST as follows:
For 100ml: 20ml 10X MBS, pH7.5
0.2ml Tween-20
179.8ml ddH_2O
200ml total volume

17. Prepare 10% HISS + 2% (w/v) BMB in MBST as follows:
For 15ml: 2ml 100% HISS
400mg BMB
18ml MBST, pH7.5
20ml total volume
Heat MBST + BMB mix **prior** to HISS addition in order to dissolve BMB (~30min at 65°C).

18. Wash embryos 3 x 5min at RT with MBST.

19. Pre-block embryos for 3-4hrs at 37°C in MBST + 10% HISS + 2% BMB.

20. Dilute AP conjugated anti-DIG Ab (Roche cat. #11 093 274 910) 1:4000 in MBST + 1% HISS + 2% BMB.

21. Remove blocking serum from embryos, and add Ab mix to embryos.

22. Incubate embryos on rocker at 4°C O/N.

Day 4 (Post-Ab washes):

1. Wash embryos 3 x 5min at RT with MBST.

2. Wash embryos 8 x 1hr at RT with MBST.

3. Wash embryos 1 x O/N at 4°C with MBST.
N.B.: Repeating steps 1-4 (i.e. a second day of washing) will greatly decrease the background without affecting the signal intensity.

Day 5 (In situ detection):

1. Remove BM Purple (Roche cat. #11442074001) from 4°C cooler; let sit at RT for at least 1hr.

2. Prepare NTMT + 2mM Levamisole as follows:
2ml 5M NaCl
5ml 2M Tris, pH9.5
5ml 1M $MgCL_2$

0.1ml Tween-20
0.2ml Levamisole (Sigma cat. #L9756; make up fresh- dissolve 0.12g in 0.5ml of ddH_2O).
87.7ml ddH_2O
100ml total volume

3. Wash embryos 3 x 5min at RT with NTMT + Levamisole.

4. Add 1ml BM Purple to each vial.

5. Cover vials with aluminum foil; incubate on rocker at R/T from 2hrs to O/N.

6. To stop BM Purple colorimetric reaction, wash embryos 3 x 5min at RT with 1X PBST, pH4.5.

7. Fix embryos for 1hr at RT with 4% PFA, 0.1% glutaraldehyde in 1X PBST.

8. Wash embryos 3 x 5min with 1X PBST.

9. Clear embryos with 50% glycerol in 1X PBST and 80% glycerol in 1X PBST.
   N.B.: To completely clear older embryos (E10.5 and older):
   1. Wash embryos 2 x 5min with 1X PBST.
   2. Dehydrate embryos at RT with 1 x 10 min washes of each of the following:
      25% MeOH in 1X PBST
      50% MeOH in 1X PBST
      75%MeOH in 1X PBST
   3. Dehydrate embryos 2 x 10min at RT with 100% MeOH.
   4. Clear embryos in 1:1 Benzyl Alcohol:Benzyl Benzoate solution (use glass dishes!). Embryos will clear immediately, but also are quite fragile. Return to MeOH for storage or through MeOH series and into 1X PBST.

10. Store embryos at 4°C.