Allen Lab Section *In Situ* Hybridization Protocol

(McMahon Lab Protocol modified by Ben Allen)

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This protocol has been revised based on a series of protocols by Jason Bielagus, Mark Wijgerde, Jill and Andy McMahon, Jing Yu, Todd Valerius, and Josh Mugford.

I. Tissue Preparation

Day 1:

1. Dissect out embryos, transfer to 4ml glass vial containing 4% PFA (EMS cat. #15710) diluted in 1X PBS.

2. Fix embryos in 4% PFA at 4˚C on a rotator for 24 hrs.

Day 2:

1. Wash embryos 3 x 5min at RT in 1X PBS.

2. Cryoprotect embryos in 1X PBS + 30% sucrose overnight at 4˚C on rocker.

Day 3:

1. Transfer embryos through three progressive OCT washes to remove excess sucrose.

2. Freeze embryos in OCT using EtOH/dry ice bath.

3. Store embryos at -80°C until ready for sectioning.

Day 4:

1. Cut 20µm frozen sections using Microm HM550 cryostat (-15°C sample temperature, -15°C chamber temperature).

N.B.:. Be sure to use "precleaned superfrost plus" slides (VWR 48311-703). Air dry sections for at least 30min. Store at -70˚C. Use within 2 weeks.

II. *In Situ* Hybridization – Hybridization

1. Fix sections in 4% PFA / PBS for 10 min. Do not discard, save for step 5.
2. Wash sections 3 x 5min at RT with 1X PBS.
3. Treat with 10µg/ml of Proteinase K in PBS for 10 min. **(N.B.: Proteinase K strength varies from lot to lot – the timing/concentration should be adjusted every time a new stock is made).**
4. Wash sections briefly in PBS, 3x.
5. Fix in 4% PFA / PBS for 5 min.
6. Wash sections 3 x 5min at RT with 1X PBS.
7. **Acetylation:** In a glass histology jar on a stirrer, combine

	H2O	200ml
	Triethanolamine	2.66ml
	37% HCl (12M)	0.35ml
Begin stirring and add:	Acetic Anhydride	0.75ml

   Place rack of slides in jar, while stirring, for 10 min.
8. Wash sections 3 x 5min at RT with 1X PBS.
9. Wash sections 1 x 5min with 0.85% NaCl.

10. Wash sections 1 x 5min with 70% EtOH (diluted from 100% EtOH in 0.85%NaCl).
11. Wash sections 1 x 5 min with 95% EtOH.
12. Air dry sections for 10min. **(N.B.: This step is very flexible; samples can be safely air dried anywhere from 1min to 2hrs- or possibly even longer).**
13. **Hybridization:** Dilute probes with hybridization buffer such that the probe concentration is 1ng/µl. Boil the diluted probe for 5min. at 100°C in heating block. Place probe on ice for 1-2min. before adding to the slides.
14. Arrange slides horizontally in a humidified chamber (humidified with 5X SSC), remove excess solution, apply ~100µl of hybridization buffer with probe to each slide, and apply parafilm. Incubate at 70˚C overnight.

III. *In Situ* Hybridization – Washes and Antibody

Day 5:

1. Immerse slides in 5X SSC, pH 4.5 at 65˚C to allow parafilm to separate (5 min).
2. Wash slides in 1X SSC pH4.5/50% formamide at 65˚C for 30 min.
3. Wash slides 1 x 10min. with TNE at 37˚C.
4. Dilute 75µl RNase A (10mg/ml) into 150ml TNE.
5. Incubate slides in RNase A (5ug/ml) in TNE at 37˚C for 15min.
6. Wash slides 1 x 10min. with TNE at 37˚C.
7. Wash slides 1 x 20min. with 2xSSC, pH 4.5 at 65˚C.
8. Wash slides 2 x 20min. with 0.2xSSC, pH 4.5 at 65˚C.
9. Wash slides 3 x 5min. with MBST.
10. Block slides with MBST + 10% HISS + 2% BMB for 1-2 hrs at RT.
11. Dilute AP conjugated anti-DIG Ab (Roche cat. #11 093 274 910) 1:4000 in MBST + 1% HISS + 2% BMB.
12. Arrange slides horizontally in a humidified chamber (humidified with MBST), remove excess solution, apply ~200µl of antibody solution to each slide, and apply parafilm. Incubate at 4˚C overnight.

IV. Detection

Day 6:

1. Immerse slides in MBST to separate parafilm from the slides.
2. Wash the slides 3 x 5min in MBST.
3. Wash the slides 3 x 5min in NTMT, pH9.5.
4. Arrange slides horizontally in a humidified chamber (humidified with MBST), remove excess solution, apply ~200µl of BM purple to each slide, and apply parafilm. Incubation times will vary depending on the strength of the probe from a few hours to a few days.
5. To mount slides:
    1. Wash slides 3 x 5min with 1X PBST, pH4.5.
    2. Fix slides in 4% PFA + 0.2% glutaraldehyde for 30 min. at RT.
    3. Wash slides 3 x 5min. with 1X PBS.

4. Rinse slides 1x briefly in 70% EtOH and dry at 60˚C for ~10 min.
5. Mount coverslips on slides with Glycergel (DAKO cat. #C0563; pre-heated to 60°C).

Solutions, materials and other notes:

Hybridization Buffer (5xSSC)
50% formamide (Fisher BP 227 100)
5X SSC pH 4.5 (use citric acid to adjust pH)
50µg/ml yeast tRNA (Gibco 15401-011)
1% SDS
50µg/ml Heparin (Sigma H8514)

Proteinase K
(Roche 161 519). Dilute stock to10mg/ml with water. Store at –20˚C.

20X SSC (pH 4.5)
3M NaCl
0.3M Sodium Citrate
Adjust pH to 4.5 with citric acid (~1.8g/100ml)

TNE (10mM Tris, pH7.5, 500mM NaCl, 1mM EDTA)
For 500ml:

5ml	1M Tris pH7.5
50ml	5M NaCl
1ml	500mM EDTA
444ml	ddH_2O
500ml total volume	

BM
2% Blocking Reagent (Boehringer Mannheim 1096 176) . Solution must be heated to 65˚C and agitated for BR to go into solution.

BMB
2% BM plus 20% heat inactivated sheep serum (HISS).

Antibody solution
2% BM plus 1% HISS plus anti-DIG antibody (Roche 1093274) diluted 1:4000.

10X MBS (1M Maleic Acid, 1.5M NaCl, pH7.5)
For 1L:

116.07g	Maleic Acid
87.66g	NaCl
700ml	ddH_2O

pH to 7.5 with NaOH pellets – add 10g of pellets at a time, up to 70g. Add 1g of pellets at a time, taking frequent pH measurements. 75-80g of NaOH pellets will be needed to attain the proper pH. If the pH rises above 7.5, the solution can be adjusted downward with 12M HCl.

Q.S. to 1L with ddH_2O

N.B.: In general, this solution will take a couple hours to prepare, and should be monitored carefully, since the reaction is exothermic.

1X MBST
Dilute 10X MBS to 1X with ddH_2O and add 0.1% Tween20.

NTMT (100mM NaCl, 100mM Tris pH 9.5, 50mM $MgCl_2$, 2mM Levamisole, 0.1% Tween-20)
For 500ml:

10ml 5M NaCl
25ml 2M Tris, pH9.5
25ml 1M $MgCl_2$
1ml 1M Levamisole (prepare fresh: dissolve .24g in 1ml of ddH_2O)
0.5ml Tween-20
438.5ml ddH_2O
500ml total volume

Notes

For a humidified chamber, use either a plastic box with pipettes glued to the bottom (upon which the slides will rest, above the solution in the bottom), or a 100-slide slide box. To properly humidify the chamber, the solution in the bottom need not be too deep, but it needs to entirely cover the bottom of the box.

Be sure to keep the sections from drying out. Do try to remove as much of the previous solution from a slide before applying a new solution, but take care not to let the tissue sections on the slide become dry. Drying out the sections increases background.