Roehl Lab Whole-Mount *in situ* Hybridization

Adapted from Thisse protocol (Nat Protoc. 2008;3(1):59-69)

Probe synthesis

Template:

set up 100ul PCR reaction with 20ng of plasmid (do not use more) and 1ul of each primer (from 25uM stock)

(Large scale:
Linearize 800ng of DNA in 20ul for 2hr.
Add 10ul each primer (100uM) to make 40ul stock
Use 1ul in 100ul PCR rxn)

96C (45sec) ---- 35X [94C (45 sec) 55C (30 sec) 72C (120sec) --- 72C (5minutes)

spin in Microcon YM-50 15min at 1000g
Add 400ul H2O and for 45min at 1000g
Add 20ul H2O invert and spin in new tube 1 min.
Run 1ul on a gel and quantify 1ul in spec.

Transcription mix:

Synthesis of the antisense RNA probe.

H2O + 1ug DNA	21ul
Transcription buffer (NEB)	3ul
NTP-DIG-RNA (Boehringer)	3ul
RNAsein (Promega)	1.5ul
T3/T7 RNA polymerase	1.5ul

Incubate 2h at 37°C.

18ul H2O + 2ul RNAse free DNAse (Roche) 30' at 37°C.
Spin sigmaspin for 15 sec at 750g
Remove base of sigmaspin and spin for 2min at 750g
Apply probe and spin for 4 min at 750g
Add 14ul RNAlater
Run 1ul on gel

Fixation and storage of embryos

(Can remove chorions by pronase treatment.)

1. Fix embryos in 4% PFA in PBS overnight to 2 days at 4°C.

2. Rinse in PBST (2 X 5') and dechorionate.

Bring through MeOH/PBST series 10' each step (30%, 60%, 3 X 100%).

4. Store in 100% MeOH at -20°C 1h (or store up to 1 year).

5. Reverse the MeOH series.

6. Rinse PBST 5 X 5'.

Digestion

1. Digest with Proteinase K (10 ug/ml) in PBST.

1 cell – 1 somite:	30 seconds
1-8 somite:	1'
9-18 somite:	3'
18 somite – 24 somite:	10'
24h (p5):	15'
30h (p13):	22'
36h/48h embryos:	30'
60h	40'
72h	50'

2. Refix in 4% PFA-PBS, 20' RT.

3. Rinse PBST 4 X 5´.

Prehyb:

1. Add 500ul Hyb+ and incubate at 70°C..

3. Incubate 2hr at 70°C.

4. Replace Hyb with 200ul of Hyb+ containing 1to 4ul of antisense RNA probe.

5. Hybridize overnight at 70°C.

In situ Day 2:

Washes:

1. 25% 2X SSCT/75% Hyb- at 70°C 1 X 10'.

2. 50% 2X SSCT/50% Hyb- at 70°C 1 X 10'.

3. 75% 2X SSCT/25% Hyb- at 70°C 1 X 10'.

4. 2X SSCT at 70°C 1 X 10'.

5. 0.2X SSCT at 70°C 2 x 30'.

6. Allow to cool to RT 5'.

7. 25% PBST/75% 0.2X SSCT buffer at RT 10'.

8. 50% PBST/50% 0.2X SSCT buffer at RT 10'.

9. 75% PBST/25% 0.2X SSCT buffer at RT 10'.

10. Wash with PBST buffer at RT 2 X 5'.

(Optional – refix with with 4% PFA in PBS for 20min at RT then wash well)

11. Blocking buffer 3-4h RT.

12. Incubate in antibody solution overnight with agitation at +4°C.
(anti-DIG 1:10000, 1:5000 anti-fl)

In situ Day 3:

Washes:

1. PBST at RT, brief wash.

2. PBST 6 x 15'.

3. Staining buffer 2 X 5'.

Staining:

1. Incubate embryos in staining solution at RT or 4C in the dark and monitor with a dissecting microscope.

Staining solution 1ml	3ml	5ml	15ml	25ml
NBT 50mg/ml - 4.5ul	13.5	22.5	67.5	112.5
BCIP 50 mg/ml - 3.5ul	10.5	17.5	52.5	87.5

At RT this takes 1 to 3 hrs and at 4°C leave overnight.

2. Stop the reaction by rinsing in PBST 1mM EDTA 1 X 15'.

3. Fix in 4% PFA 30'.

4. MeOH series as above and maintain in 100% MeOH 2h until blue at RT.

5. Reverse MeOH series.

6. Wash 2X in PBST 5'.

7. Bleach pigment: 30' at 37C in bleaching solution (mix every 10 min).

8. Wash 3X in PBST 5'.

9. Glycerol series (50% 10’ then keep in 75-100%).

10. Store RT in the dark (up to 5 years).

Solutions:

Blocking Buffer: 50mlPBST + 1ml sheep serum + 100mg BSA.

PFA: 2gm PFA in 50ml PBS icubate at 70C 2-3 hr until dissolved (mix often) – store 1-2 days at 4C

Proteinase K (Boehringer 1000144) Make 10mg/ml suspension in PBST –20C.

20 x SSC 0.8 litre: 140.2g NaCl[3M] 65.8g NaCitrate dihydrate[0.3M] pH 7.0)

1 M Tris pH9.5 0.8litre: 95g tris base, use over 3ml HCl to pH.

4M NaCl 0.8litre: 187g NaCl

Bleaching solution: 10% H2O2/0.5 XSSC/5% Formamide

Heparin at 50 mg/ml (Sigma H3393) –20C.

400ml 2X Maleic buffer: 13.9g maleic acid, 4.67g NaCl, 8g NaOH pellets – check pH then adjust to then pH 7.5.

50ml staining buffer: 5ml tris pH9.5 1M, 2.5ml MgCl2 1M, 1.66ml NaCl 4M,, 250ul 20% Tween-20.

NBT stock: 50 mg Nitro Blue Tetrazolium in 0.7 ml of anhydrous Dimethyl-formamide + 0.3 ml H2O 4C.

BCIP stock: 50 mg of 5-Bromo 4-Chloro3Indolyl Phosphate in 1ml anhydrous Dimethyl-formamide 4C.

Hyb+: in 50ml falcon: 25ml formamide, 25mg tRNA, 12.5ml 20X SSC, 250ul 20% Tween-20, 50ul heparin stock, 460ul 1M Citric acid and fill with H2O.

To kill primary antibody: 3 X 5' in acidic glycine (0.1M). 50ml acidic glycine: 0.375g glycine, pH to 2.2 with concentrated HCl (about 450ul). Store at -20^oC.

Fast red staining: use tris pH8.2 – one tablet in 4ml mix with p1000 in 15ml falcon, mix with 4ml of a 1:100 dilution of Naphtol stock, filter, use immediately.

Naphtol AS-MX Phosphate, Na salt: 50mg in 1ml DMSO –20C.

Holger Green: Use HRP 2^oantibody and Wash in PBST. 1ml: 1.5ul fl and 2ul 3% H_2O_2 in PBST. Incubate in the dark for 30min then 4 X 10min in PBST.

Use 1% H2O2 (33ul/1ml) in MeOH (30min RT) to inactivate HRP antibody.

tRNA: Aliquot 800ul phenol (pH5.5) into into 18 X 2ml eppendorf tubes. Dissolve tRNA (Sigma R7876-2.5KU) in 14.5ml H2O (RNA) and add 800ul into each of the eppendorfs. Cap and shake vigorously for 5min. Spin hard at RT for 8min. Remove supernatent almost completely to tubes containing 800ml CHCl3. Repeat shake and spin for 4min. Remove supernatent almost completely to tubes containing 90ul 5M NH4OAc (RNA) + 800ul isopropanol (RNA). Freeze 15 min – then spin hard at 4C for 30 min. Wash 1X with 70%EtOH (RNA) – spinning down to remove all of the wash. Dry for no more than 5min then resuspend in 2ml H2O (RNA). Take out 1ul and mix well in 2ml H2O and spec. Freeze in aliquots at -70C.

SIGMA R6625-25G (Ribonucleic acid from torula yeast Type VI)
or ROCHE 109 223 (Yeast RNA)
300mg in 20ml 4X SSC
10min at 68C
1X phenol/CHCl3
Spin at 4.5K rpm in swinging bucket
1X CHCl3
2.5ml NaOAc +25ml Isopropanol
Spin 30min
Resuspend in 4ml
Spec 1ul in 2ml (around 40ug/ml)

Aliquot 6 X 625ul (each makes 50 ml)

Black DAB - use 1% saturated $(NH_4)_2Ni_2$(I,II)$(SO4)_2$ in DAB mix. Store at RT.

BM purple: after washes rinse in 100mM Tris pH8.2 then BMpurple.

AB complex: Use biotinylated 2^oantibody(1:200). Make AB comlex 30min before use (8ul each in 1ml PBST). Incubate embryos in AB for 45min. Wash 3X in PBST.

To block endogenous AP:
1) after proK wash embryos breifly in H_2O, then 1hr acetic anhydride. 2.5ul acetic anhydride and 1ml 0.1M triethanolamine (pH7), mix vigorously.
2) use 1mM levamosol while staining

To block endogenous peroxidase:
1) 10min RT in 1ml 100%MeOH with 10ul 30%H2O2
2) 2% H2O2 in PBST 10min RT

TCA: 2% TCA is PBS 3hrs at RT, wash 3X, 4min in 0.25% Trypsin at 4C, wash 5X. 10% TCA in H2O is stored at 4C.

ERK staining: Cells were fixed in 4% formaldehyde (fresh) for 20 min. After washes, the cells were permeabilized with 0.5% Triton X-100 in phosphate-buffered saline for 5 min, then 1hr acetic anhydride. 2.5ul acetic anhydride and 1ml 0.1M triethanolamine (pH7), mix vigorously.

1litre PBS: 1.48g $NaHPO_4$, 0.43g KH_2PO_4, 7.2 g NaCl pH to 7.2

1litre 10X PBS: 80g NaCl, 2g KCl, 14.4g Na_2HPO4-$7H_2O$ (9.6g Na_2HPO_4-$2H_2O$), 2.4g KH_2PO_4, bring up to 800ml, pH to 7.4 with HCl, then up to 1l.

400ml 2X Maleic buffer: 13.9g maleic acid, 4.67g NaCl, 8g NaOH pellets then pH 7.5. Block with 2% blocking reagent.