Whole mount *in situ* hybridization

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Solutions are mixed and prepared in clean, autoclaved (121°C for 45 minutes) glassware or in new RNAse/DNAse free polypropylene tubes (Corning® 50mL PP Centrifuge Tubes #430921, 2mL Eppendorf® Safe-Lock microcentrifuge tubes #022363352).

Dissection of embryos

1. Prepare 3 tubes for each embryo,
   i) one with 1ml 4% paraformaldehyde (PFA) / 1X PBS for the embryo,
   ii) one with 300µl 25mM NaOH to genotype the embryo.
   iii) one with 500µl DEPC PBS for the extra tissue collected.
2. Dissect the 12.5 dpc embryos in ice-cold 1X PBS.
3. Immediately fix the bottom half of the embryo in 4% PFA / 1X PBS overnight at 4°C.
4. Stick a forelimb to the side of the 25mM NaOH tube. Put remainder of embryo in tube with 500µl DEPC PBS.

Preparation of embryos

1. Wash embryo 2X with 1X PBS for 5 minutes at room temperature on a platform shaker.
2. Dehydrate in a 25%, 50%, 75% & 100% methanol / 1X PBS series. Incubate in each solution for 10 minutes, on ice using a platform shaker.
3. Store embryos in 100% methanol at -20°C until ready to use.

Day 1: Pre-hybridization

Approximately 2ml of solution is used per embryo, per step.

1. Rehydrate embryos in a 75%, 50% & 25% methanol / DEPC treated PBT series. Incubate in each solution for 10 minutes, on ice using a platform shaker.
2. All Day 1 steps from here until the pre-hybridization step are carried out at room temperature on a platform shaker.
3. Pre-warm the pre-hybridization solution in a 65°C oven.
4. Wash embryos 2X in DEPC treated PBT for 5 minutes.
5. Bleach in 4% H_2O_2 for 1 hour.
6. Wash 2X in DEPC treated PBT for 5 minutes.
7. Treat embryo with Proteinase K at a concentration of 10µg/ml for 10 minutes.
8. Wash 2X in DEPC treated PBT for 10 minutes.
9. Incubate in postfix for 20 minutes.
10. Wash 2X in DEPC treated PBT for 5 minutes.

11. Incubate the embryos in the pre-warmed pre-hybridization buffer overnight at 65°C in a hybridization oven with shaking.

Day 2: Hybridization buffer with probe

Approximately 2ml of solution is used per embryo, per step.

1. Pre-warm fresh pre-hybridization buffer to 65°C.
2. Thaw probes on ice.
3. Add probe to 2ml hybridization buffer. Probe concentration: 0.1µg/ml
4. Incubate the embryo in the hybridization/probe buffer for 3 days at 65°C in a hybridization oven with shaking.

Day 5: Stringency washes, blocking & anti-digoxigenin immunological staining

Approximately 2ml of solution is used per embryo, per step.

1. Wash 3X in 2X SSC with CHAPS for 30 minutes at 65°C with shaking.
2. Wash 3X in 0.2X SSC with CHAPS for 30 minutes at 65°C with shaking.
3. Wash 2X in KTBT for 10 minutes at room temperature with shaking.
4. Pre-block in KTBT/GS for 3 hours at 4°C on a platform with shaking.
5. At the same time, prepare blocking solution by adding anti-digoxigenin antibody (1:3000 dilution) and incubate at 4°C for 3 hours.
6. Block in pre-absorbed antibody at 4°C overnight.

Day 6: Washes

Approximately 2ml of solution is used per embryo, per step.

1. Wash 4X in KTBT for 1 hour at room temperature on a platform shaker.
2. Wash in KTBT overnight at 4°C on a platform shaker.

Day 7: Colour development

Approximately 2ml of solution is used per embryo, per step.

1. Wash 2X in NTMT at room temperature, shaking, for 15 minutes.
2. Transfer embryos to 24-well plate.
3. Add NBT/BCIP colour (enough to cover the embryo) at room temperature, cover with foil and place on a platform shaker.
4. Check colour at 10 minutes under the microscope. Re-check periodically until colour development is perfect and record the time of development.
5. Wash 6X with KTBT for 15 minutes at room temperature on a platform shaker.
6. Fix in 4% PFA / 1X PBS overnight at 4°C.
7. Transfer to 1X PBS for storage at 4°C.

8. Photograph as soon as possible in petri dishes with 1% agarose as background.

Solutions – make enough for 2ml per embryo for each step in the protocol

1X PBS (1l)
100ml 10X PBS (Fisher Scientific #BP3994)
900ml ddH_20
Autoclave at 121°C for 45 minutes.

DEPC treated 1X PBS (1l)
100ml 10X PBS (Fisher Scientific #BP3994)
900µl DEPC (Research Products International Corp #D43060-25.0)
Add ddH_20 to make 1l.
Let sit overnight.
Autoclave at 121°C for 45 minutes.

DEPC treated PBT (1l)
1l DEPC treated 1X PBS
1000µl Triton X-100 (MP Biomedicals #194854)

4% PFA / 1X PBS (1l)
100ml 10X PBS (Fisher Scientific #BP3994)
40g PFA (Sigma #P6148)
Add ddH_20 to make 1l.
pH to 7.2.
Vacuum filter.
Store 40ml aliquots at -20°C.

2X SSC (1l)
100ml 20X SSC (Fisher Scientific #BP13254)
900ml ultrapure H_20

0.2X SSC (1l)
10ml 20X SSC (Fisher Scientific #BP13254)
990ml ultrapure H_20

10% CHAPS (10ml)
10ml ddH_20
1g CHAPS (Sigma #C3023

KTBT (TBST) 500ml
25ml Tris HCl pH 7.5
15ml 5M NaCl
1.5ml 2M KCl
5ml Tween 20 (Fisher Scientific #BP337-100)
Make up to 500ml with ddH_20

2X SSC with CHAPS (50ml)
50ml 2X SSC
500µl CHAPS (Sigma #C3023)

0.2X SSC with CHAPS (50ml)
50ml 0.2X SSC
500µl CHAPS (Sigma #C3023)

Proteinase K (10µg/ml) 10ml
10ml DEPC treated PBT
10µl Proteinase K at 10mg/ml (Fisher Scientific # BP1700)

Postfix (20ml)
20ml 4% PFA
160µl gluteraldehyde (Fisher Scientific #O2957-1)

Pre-hybridization buffer (1l)
20g blocking powder (Roche #11096176001)
1g CHAPS (Sigma #C3023)
0.5g heparin (Sigma #H3149)
500ml DI formamide (American Bioanalytical #AB00600)
250ml 20X SSC (Fisher Scientific #BP13254)
5ml tRNA (200mg/ml) (Sigma #R6625)
10ml 0.5M EDTA
Make up to 1l with DEPC treated H_20.
1ml Triton X-100 (MP Biomedicals #194854)
Dissolve overnight at 65°C.
Store in 50ml aliquots at -80°C.

Block (KTBT/GS) 20ml
16ml KTBT
4ml goat serum (Sigma #G6767)

Polyclonal Sheep anti-Digoxin, Digoxigenin antibody (1:3000) 9ml
9ml KTBT/GS
3 µl anti-DIG (Roche #11093274910)

Hybridization buffer with probe
2ml pre-hybridization buffer
1-2 µl probe (Probe concentration: 0.1µg/ml)

NTMT (50ml)
5ml 1M Tris HCl pH 9.5

1ml 5M NaCl
2.5ml 1M $MgCl_2$
500µl 1% Triton X-100 (MP Biomedicals #194854)
Make up to 50ml with ddH_20.

Colour (NBT/BCIP) 1ml
2.25µl NBT (75mg/ml in 70% DMF / ddH20) (Roche #11585029001)
3.5µl BCIP (50mg/ml in 100% DMF) (Roche #11585002001)
Make up to 1ml with ddH20.