In situ hybridizations for Ascidians

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1. Fix embryos in 10mL of fresh 4% paraformaldehyde in 0.5M NaCl, 0.1M MOPS pH 7.5, 2mM MgSO4, 1mM EGTA at room temperature for 30minutes — O
2. Transfer to 25% EtOH and wash — O
3. Transfer to 50% EtOH and wash — O
4. Transfer to 75% EtOH and wash — O
5. Transfer to 100% EtOH and wash — O
6. Store in 100% EtOH at -20°C — O

** Use nutator for timed washes and rocks unless otherwise stated below
**10X PBS is 80g NaCl, 2g KCl, 14.4g Na2HPO4, and 2.4g KH2PO4 per 1L (pH to 7.4 then bring up to volume). Use 50mL 10X PBS and 5mL of 10%Tween80 to make 0.5L 1X PBT containing 0.1% Tween.

7. Wash with ethanol/PBT (75:25), by inverting 4-5x to lift pellet — O
8. Wash with ethanol/PBT (50:50), by inverting 4-5x to lift pellet — O
9. Wash with ethanol/PBT (25:75), by inverting 4-5x to lift pellet — O
10. Rock for 2 minutes in PBT (0.1% Tween-80 in 1x PBS), REPEAT x3 — OOO
11. Rock in 2ug/ml Proteinase K in PBT for 15 min at room temp (10,000X Stock, 1 µl per 10 mls) — O
12. Rock in 2mg/ml Glycine in PBT at room temp x2 (5 min.) — OO
13. Rock in PBT, REPEAT x5 (wash quickly) — OOOOO
14. Post-fix for 25 minutes in PBT + 2% paraformaldehyde — O
15. Rock for 2 minutes in PBT, REPEAT x5 — OOOOO
16. Wash in 100mM triethanolamine (pH 8.0) for 10 minutes 400 µl in 30 mls + 10 µl conc. HCL — O
17. Wash in 100mM triethanolamine+0.25% acetic anhydride, 5min, x2 — OO
18. Wash in PBT for 5 minutes, REPEAT x2 — OO
19. Rock for 5 minutes in PBT/hybridization solution (50:50) — O
20. Rock for 5 minutes in hybridization solution — O
    Separate embryos into separate Hyb rxn tubes if necessary
21. Pre-hybridize for 1.5 hours at 55°C in hybridization solution invert every 30 minutes — O
22. Prepare RNA antisense probe by adding 1µL (or less) of probe stock to 50µL of hybridization solution, heat at 80°C for 3 minutes and place on ice — O
23. Remove as much hybridization solution from the embryos as possible — O
24. Add hybridization solution containing probe and hybridize at least 48 hours (Fri. night -Mon. morning) at 55°C with occasional flicking to mix the probe — O

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*(start this step early to allow embryos to pre-absorb Ab for 2-3 hours)

25. Wash 2 X 20 minutes in hybridization buffer at hybridization temperature — OO
26. Wash 20 minutes in 75% hyb. buffer / 25% 2XSSC at hybridization temperature — O
27. Wash 20 minutes in 50% hyb. buffer / 50% 2XSSC at hybridization temperature — O
28. Wash 20 minutes in 25% hyb. buffer / 75% 2XSSC at hybridization temperature — O
29. Wash 20 minutes in 100% 2X SSC at hybridization temperature — O

30. Wash 2 X 20 minutes in 0.05X SSC at hybridization temperature OO
31. Wash 1 X 5 minutes in 50% 0.05X SSC / 50% Maleic Acid buffer (100mM Maleic Acid, 150mM NaCl, pH to 7.5 with NaOH) at room temperature (maleic acid idea from MQM) O
32. Wash 2 X 5 minutes in 100% Maleic Acid buffer at room temperature OO
33. *Pre-absorb αdig-antibody on <u>unprobed</u> embryos in 1mL of Maleic Acid buff. w/ 1X block (1% Roche Blocking Reagent). O

    (# of samples)(0.25µl αdig-AP Ab)= how many µl Ab to be preabsorbed.
    At step 34, aspirate pre-absorbed Ab from settled embryos, and fill to final volume with Maleic Acid block. (# of Samples)(0.5 mL)=final volume w/ antibody at 1:2000 dilution.
34. Block samples in 1mL Maleic Acid block for 1 hour at RT O
35. Wash overnight at RT in 0.5mL Maleic Acid block w/ pre-absorbed Ab at a final dilution of 1:2000 (Rock or leave tube on its side) O

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36. Rock 10X 20 minutes in 800 µL of PBT OOOOO OOOOO
37. Make up AP staining buffer (AP) and rinse quickly O
    AP (100mM NaCl, 50mM MgCl2, 100mM Tris, pH 9.5, 0.1% Tween-80)
38. Rock 2X 5 minutes in AP staining buffer OO
39. Drain and add 400 µL of AP/NBT/BCIP stain O
    Immediately before staining, add 9 µL of NBT (75mg/mL in 70% DMF) and 7 µL of BCIP (50mg/mL in DMF) per 1mL of AP staining buffer and mix well.
40. Gently pipet embryos into a staining dish and keep in dark for staining O
41. To stop staining, transfer embryos to an eppendorf tube containing 800uL of PBT and rock for 5 minutes O

<u>To Store in Glycerol @ 4C:</u>

42. Rock for 5 min in PBT. OOO
43. Remove PBT and add 500µL of 50% glycerol…store in eppie @ 4C O

OR

<u>To Permanently Mount:</u>

42. Rock for 5 minutes in PBT/EtOH (50:50) O
43. Dehydrate by rocking for 5 minutes in 25% ethanol O
44. Dehydrate by rocking for 5 minutes in 55% ethanol O
45. Dehydrate by rocking for 5 minutes in 75% ethanol O
46. Dehydrate by rocking for 5 minutes in 100% ethanol, 6X OOOOOO
47. Wash quickly in ethanol/xylene (50:50) and remove with pipet O
48. Wash quickly in 100% xylene and remove all but 100 µL O
49. Add 18-20 drops of Permount (400uL). Suck up embryos first, then ermount. Place on slide, add coverslip and dry flat overnight O

Digoxigenin-UTP labeling of RNA probes

5x transcription buffer	2uL (0.4M Tris, pH 7.5; 0.06M MgCl2; 0.1M NaCl; 0.02M Spermidine-HCl)
10x dig U NTP mix	1uL (10mM ATP,10mM GTP,10mM CTP,6mM UTP, 4mM dig UTP (Boehringer)
50mM DTT	1uL
RNase inhibitor (50U/uL)	1uL
Linearized DNA (1ug)	
T7 or T3 RNA polymerase	1uL
DepC H2O	to 10 uL

-incubate at Room Temp. for 2 to 4 hours
Begin optional steps:
-add 1uL of T7 or T3 RNA polymerase
-incubate at room temperature for 2 hours
End optional steps
May remove 1 ul probe for gel analysis here or after RQDNAse step
-Add 1uL of RQ-DNAseI
-incubate at 37C for 15minutes
-add 40uL H_2O
-add 5uL **4M LiCl**
-add 1uL **20mg/mL tRNA** (phenol/chloroform extracted)
-add 150uL ethanol
-mix and freeze (-20^{o}C) for at least 15 minutes (or overnight if using it tomorrow)
Store probe in EtOH at -80°C until use.

Probe Stock: (to be stored @-80C in Hyb solution)
-spin probe/ethanol at max speed for 20 minutes at 4^{o}C
-wash pellet in 70% ethanol
-air dry pellet and dissolve in 150uL hybridization solution (low pH idea from MQM):

Component	Final Conc.	To make 40 ml add:
Formamide	50%	20 ml of 100%
SSC (pH 4.5)	5X	10 ml of 20X SSC
Heparin	50 ug/ml	100 ul of 20mg/ml
SSDNA	100 ug/ml	400ul of 10mg/ml
SDS	0.05%	200 ul of 10% SDS
Tween-80	0.1%	40 ul of 100%

Bring to volume with DEPC water.

Use 1-4 µl probe stock in 50uL hybridization reaction for10-20uL of settled embryos