Maryna Lesoway
Host Lab: Jon Henry lab
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***In situ* Hybridization for Crepidula embryos**
(Can also be used for *Cerebratulus* (nemertean) and anemone embryos)

Rationale and Background:
Visualization of mRNA localization in embryos of *C. fornicata*.

Protocol:

NB: Wear gloves and use RNAse-free equipment and solutions through the hybridization step. All washes are 500 ul for 5 minutes at room temperature (RT) on a rocker table unless otherwise stated. Be very gentle when changing rinses on delicate embryos, particularly early stages to avoid damage to samples.

DAY 1

Pretreatment
- Transfer fixed embryos in methanol to a sterile 24 well culture dish.
- Rehydrate through: 60% MeOH:40% PTw
  30% MeOH:70% PTw
  4x PTw washes
- Digest with Proteinase-K (0.01 mg/ml in PTw) for 5 minutes (embryos) to 20 minutes (hardier specimens). Do not place on shaker.
- Stop digestion with 2 washes of 2mg/ml glycine in PTw.
- Wash with 1% triethanolamine in PTw and add 1.5ul acetic anhydride. After 5 minutes, add 1.5ul more of acetic anhdride. The acetic anhydride will not readily mix into the triethylamine. You will have to swirl and rock the solution to ensure that it mixes. This will take 1-2 minutes.
- Wash 2x in PTw.
- Refix in 3.7% formaldehyde in PTw for 1 hour at room temperature.
- Wash 5x in PTw

Prehybridization
- Remove as much liquid as possible and add 500ul of hybridization (hybe) buffer. Incubate 10 minutes at room temperature.
- Remove liquid and add 500ul of hybridization buffer. Place at hybridization temperature (65°C) at least 1-4 hours or overnight.

Hybridization
- Dilute the DIG-labeled probe to a final concentration of 10 - 0.05ng/ul (usually 1.0ng/ul) in hybridization solution. The DIG-labeled probe should be stored as a 50ng/ul stock solution in hybridization buffer at -20°C. Denature the probe at 80-95°C max for 10 minutes.

Alternately, denature diluted probe at hybridization temperature for 30 minutes.

- Remove prehybridization and add probe to each well of samples. Hybridize overnight or the weekend. Be sure to keep embryos in a moist chamber for long hybridization times.

DAY 2

- Prewarm hybridization buffer to hybridization temperature.
- Remove probe. This can be re-used 4-5 times. Store at -20°C.
- Wash 1x for 10 minutes, 1x for 40 minutes with hybridization buffer at hybridization temperature.

Washes

- 30 minutes 75% hybe + 25% 2X SSC at hybe temp
- 30 minutes 50% hybe + 50% 2X SSC at hybe temp
- 30 minutes 25% hybe + 75% 2X SSC at hybe temp
- 30 minutes 100% 2X SSSC at hybe temp

(Gloves are no longer required at this point)

- 3x 20 minutes in 0.05X SSC at hybe temp

- 10 minutes in 75% 0.05X SSC + 25% PTw at RT
- 10 minutes in 50% 0.05X SSC + 50% PTw at RT
- 10 minutes in 25% 0.05X SSC + 75% PTw at RT
- 10 minutes in 100% PTw at RT

Visualization of Probe

- Wash 5x with PBT at RT. (This may be unnecessary)
- Block in Boehringer-Mannheim Blocking (BMB) buffer, diluted to 1X with maleic acid buffer (MAB) 1 hour at room temperature on rocker (or overnight at 4°C).
- Incubate with Boehringer-Mannheim anti-DIG/AP (diluted in blocking buffer to 1:5000) at 4°C overnight on rocker. Alternately, incubate 1-4 hours at room temperature.

DAY 3

- Wash 10x (or more) for 20-30 minutes in PBT.
- Optional: for untested probes (i.e. not certain about developing time), leave overnight in PBT and begin development the next day. You will be less likely to overdevelop, but you have to be able to handle the suspense. And the extra day.
- Wash 3x for 10 minutes in AP buffer (prepared immediately prior to use).
  Note: Embryos tend to stick a lot.

- Develop in AP substrate solution (make fresh) at RT in the dark. Alternately, use BM Purple (Roche) to develop. Monitor colour development. You can also develop more slowly at 4°C. Development may take from hours to days, depending on the probe and stage being tested.
- Stop the development reaction by washing 5x with PTw.
  Be careful when developing that you allow the stain to develop fully - underdevelopment will be difficult to image, while overdevelopment will result in loss of signal.
- Mount in 70% glycerol in PTw with plasticine feet to support cover slip.

Solutions:

Hybridization Buffer (40 ml)	Add	Final Concentration
Formamide	20 ml	50%
20X SSC (pH 4.5)	10 ml	5X
20mg/ml heparin	0.1 ml	50 ug/ml
20% Tween-20	0.5 ml	0.1%
20 % SDS	2.0 ml	1%
10 mg/ml SS DNA	0.2 ml	100 ug/ml
dH_20	7.5 ml	to 40 ml

10X PBS (1L)
18.6 mM NaH_2PO_4 (2.56g NaH_2PO_4-H_2O per liter dH_2O)
84.1 mM Na_2HPO_4 (11.94g Na_2HPO_4-H_2O per litre dH_20)
1.75M NaCl (102.2g NaCl per liter dH_2O)
Mix phosphates in about 800 mL of dH_2O for a 1.0L volume. Check pH. It should be 7.4 ± 0.4. If more than 0.4 off, start over. Otherwise, adjust pH to 7.4 with NaOH or HCl. Add the NaCl and dH_2O to 1L.

PTw
1X PBS + 0.1%Tween-20 detergent
(100mL 10X PBS, 895mL dH_2O, DEPC treat/ autoclave; when cool, add 5mL 20% Tween)

PBT
1X PBS + 0.2% Triton X-100 + 0.1% BSA (store at 4°C)
(to 990mL 1X PBS, add 10mL 20% Triton X-100, then add 1g BSA and filter sterilize)

20X SSC
0.3M Na citrate + 3M NaCl
(for 1L, add 175.3g NaCl to 88.2g Na citrate, pH to 7.0 and autoclave)

Alkaline Phosphatase (AP) Buffer (50 ml)		Final Concentration
1M NaCl	5.0 mL	100mM
1M MgCl2	2.5 mL	50mM
1M Tris Base, pH 9.5	5.0 mL	100mM
20% Tween-20	1.25 mL	0.5%

dH_2O 36.25 mL

Prepare just prior to use. The solution will become cloudy after a few hours and will no longer work for the enzymatic reaction.

AP Substrate Solution

To AP Buffer, add 6.6µL/mL NBT (stock: 50 mg/mL in 70% dimethyl formamide + 30% water), and 3.3 µL/mL BCIP (stock: 50 mg/mL in dimethyl formamide). Keep this solution dark.

Product Information

Product	Vendor	Catalogue Number
anti-DIG/AP	Boehringer-Mannheim	1 093 274
BCIP	Amersham Pharmacia Biotech	US 12390
Blocking Buffer	Boehringer-Mannheim	1 096 176
NBT	Amersham Pharmacia Biotech	US 19535
Proteinase-K	GibcoBRL	25530-049
Salmon Testes gDNA	Sigma	D 9156