In situ* hybridization for *Crepidula fornicata

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- Altered for this project with help from Kimberly Perry (University of Illinois), Dede Lyons (Duke University), Antje Fischer (Marine Biological Laboratory), and Molly Phillips (Marine Biological Laboratory)

To visualize gene expression in Crepidula embryos

Probes were synthesized by the following steps:

- Extract total RNA
- Make cDNA with the SMARTer PCR cDNA Synthesis Kit (Clontech)
- Clone genes by PCR, then amplify with a T7 overhang
- Create probes by transcription reaction, using TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific or Fermentas)

Recipes:

10X PBS

Ingredients:

- 18.6 mM NaH_2PO_4-H_2O (2.56g/L)
- 84.1 mM $NaHPO_4$-$2H_2O$ (14.97g/L)
- 1,750 mM NaCl (102.2g/L)
- DEPC-treated H_2O

Recipe:

- 800 mL bottle of DEPC-treated H_2O
- Add 2.05g NaH_2PO_4-H_2O and 11.98g $NaHPO_4$-$2H_2O$
- Check to ensure pH is near 7.4 (within 0.4)
- Adjust pH to 7.4 with HCl or NaOH
- Add 81.76g NaCl

PTw

Ingredients:

- 50 mL 10X PBS
- 895 mL DEPC-H_2O
- 5 mL 20% Tween-20

Recipe:

- Dilute 10x PBS to 1x
- Autoclave and cool
- Add Tween

PBT

Ingredients:

- 50 mL 10X PBS
- 445 mL DEPC-H_2O
- 5 mL 20% Triton X-100

- 0.5g BSA

Recipe:

- Mix PBS and water
- Add BSA (stored in 4°C fridge)
- Filter sterilize
- Add Triton X-100

Hybridization Buffer

Ingredients:

- 10 mL 20X SSC pH 4.5
- 7.2 mL DEPC-H_2O
- 2 mL 20% SDS
- 0.5 mL 20% Tween-20
- 0.2 mL Salmon Sperm DNA (10mg/mL)
- 0.1 mL Heparin (20mg/mL)
- 20 mL Formamide

Recipe:

- Mix all ingredients except formamide
- Add formamide under fume hood
- Store at -20°C when not using buffer at hybe temp
    - Buffer will precipitate in freezer
    - Precipitate should disappear when heated

Roche Blocking Buffer: 10X Stock

Ingredients:

- 5g Blocking buffer powder
- 50mL Maleic Acid Buffer

Recipe:

- Heat and shake to dissolve
- Autoclave to sterilize
- Store in fridge

AP Buffer

Ingredients:

- 36.25 mL DEPC-H_2O
- 5 mL 1M NaCl
- 5 mL 1M Tris, pH 9.5
- 2.5 mL 1M $MgCl_2$
- 1.25 mL 20% Tween-20

Recipe:

- Add all ingredients, then take pH
- Adjust to ~9.5
- Keep at RT for use the same day (make daily)

AP Substrate Solution

Ingredients:

- AP Buffer
- NBT (p-Nitro-Blue tetrazolium chloride)
- BCIP (5-Bromo-4-chloro-3-indolyl phosphate)

Recipe:

- Add 3.3µL NBT and 3.3µL BCIP to 1mL AP buffer

Protocol:

General Notes:

- Use RNAse-free equipment and solutions through hybridization step.
- All washes are 1 mL, 5 min. at RT in **24-well plates** while shaking gently, unless otherwise stated. (It is critical that washes are done in well plates; embryos will fall apart in tubes.)
- Hybridization temp: 65°C.
- Additional notes are italicized.

Day 1

1. Transfer samples, stored in methanol, from tubes to a 24-well plate
2. Use ~1mL for each wash, and rehydrate through:
    a. 60% MeOH/40% PTw
    b. 30% MeOH/70% PTw
    c. 4x 5 minutes in PTw
    - *Make fresh aliquot of glycine for step 4: 2 mg/mL in PTw*
3. Digest with Proteinase-K (.01 mg/ml in PTw – make fresh) for no more than 5 minutes at RT without rocking
    → If not doing a Pro-K digestion, keep samples in PTw and move on to step 5*
    - *While samples incubate in Proteinase K, make 1% triethanolamine in PTw*
4. Remove Proteinase K and wash with 2x 2 mg/mL glycine in PTw
    - *Be sure to end Proteinase K reaction quickly with glycine*
5. Add 3 µl acetic anhydride to every 1 mL 1% triethanolamine, vortex thoroughly, and add to embryos immediately for 5 minutes
6. Take remaining 1% triethanolamine, add 3µl more acetic anhydride to every 1 mL, vortex thoroughly, and add immediately to embryos for 5 minutes – *do not remove solution from step 5 from wells!*
7. Wash briefly in PTw, then wash 2× 5 min in PTw
    - *Make 4% PFA in PTw on ice, then let thaw*
8. Refix in 4% PFA in PTw for 1 hour at RT – *do not add PFA cold!*
    - *During refix, remove hybridization buffer from -20°C freezer and warm up in oven until there is no more precipitate and/or make more if necessary*
9. Wash 5x in PTw
10. Remove as much liquid as possible without letting the embryos become dry, add 1mL hybe buffer, incubate for 10 minutes at RT.
11. Remove liquid – add 500µl hybe buffer.
12. Place at hybe temp, 65°C, for approximately 3 hours
13. Dilute probe to a final concentration of 2 ng/µl in hybe buffer

- *Dig-labeled probe stored as a 100 ng/µl stock in hybe buffer at -20°C*
- *10 µl probe (100 ng/µl) with 490 µl hybe buffer*

14. Denature probe at 80-90°C max for 10 minutes
15. Heat shock probes on ice!
16. Remove prehybe and add 500µl probe to each well
    - *Work quickly to keep embryos warm in well plate!*
17. Hybridize overnight at 65°C

*We were not able to confirm whether *Crepidula* embryos will stain without a Pro-K digestion. This is worth investigating because the Pro-K digestion is extremely harsh for young *Crepidula* embryos (pre-gastrulation).

Day 2

- *Make sure hybe buffer is prewarmed*
- *Keep incubator at hybe temp and set up digital dry bath at hybe temp, 65°C*
- *Make 2x SSC from 20x stock SSC pH 7 – 1 mL 20X SSC pH 7 in 9 mL DEPC-H_2O*
- *Make three SSC solutions that will be used in step 21 and put them in incubator so that they warm up to hybridization temperature*

18. Remove probe and recapture for storage at -20°C – *can be reused 4-5 times*
19. Wash 1x for 10 minutes with hybe buffer at hybe temp (500 uL)
20. Wash 1x for 40 minutes with hybe buffer at hybe temp (500 uL)
21. Wash (~1mL) using the following steps at hybridization temp:
    a. 30 min in 75% hybe + 25% 2X SSC at hybe temp
    b. 30 min in 50% hybe + 50% 2X SSC at hybe temp
    c. 30 min in 25% hybe + 75% 2X SSC at hybe temp
    - *Incubate 2X SSC at hybe temp for next wash*
22. SSC washes (~1mL) at hybridization temp:
    a. 30 min in 100% 2X SSC at hybe temp
    - *Make 0.2X SSC: 1 mL 2X SSC in 9 mL DEPC-H_2O*
    - *Incubate some at hybe temp for next washes*
    b. 3x 20 min in 0.2X SSC at hybe temp
    - *During 0.2X SSC washes, make three SSC solutions that will be used in step 23 and keep solutions at room temp*
23. Wash (~1mL) using the following steps at room temp:
    a. 10 min in 75% 0.2X SSC + 25% PTw
    b. 10 min in 50% 0.2X SSC + 50% PTw
    c. 10 min in 25% 0.2X SSC + 75% PTw at RT
24. Wash 10 min in 100% PTw at RT
    - *During PTw washes, make 1X blocking buffer (diluted in MAB) to be used for steps 26-28*
25. Wash 3x for 5 minutes with PTw at RT
26. Incubate in 500 uL 1X Boehringer-Mannheim/Roche blocking buffer for 1 hour at RT while shaking
27. Make solution with blocking buffer and anti-Dig/AP (diluted to 1:5000 in 1X blocking buffer) and incubate for the same hour at RT while shaking

28. Remove blocking buffer from embryo tubes, add 250uL per tube anti-Dig/AP, and incubate at 4°C overnight or over the weekend while shaking

Day 3

29. Wash 10x for 20-30 minutes in PBT at RT (while shaking)
    - *Make AP buffer*
30. Wash (~1 mL) 3x for 10 minutes in AP buffer at RT
    - *Make AP substrate solution*
31. Remove remaining AP buffer, then add 1 mL AP substrate solution and cover plate with foil
32. Monitor color development, changing substrate solution frequently
33. Stop reaction by washing 5x with PTw
34. Remove PTw, add glycerol, store in 4°C fridge