## Whole mount RNA in situ hybridization

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- 1. *Xenia* polyps were relaxed in Ca<sup>2+</sup> free sea water (described above) for 30 min and fixed in 4% PFA in Ca<sup>2+</sup> free sea water overnight at 4°C.
- 2. Fixed polyps were washed with PBST (0.1% Tween 20 in PBS) twice for 10 min each, and then incubated in 100% methanol at -20°C overnight.
- 3. Wash the polyps sequentially in 75%, 50%, and 25% methanol for 5 min each and then washed in PBST for 10 min.
- 4. Digest the polyps with 50 µg/ml proteinase K in PBST for 20 min followed by washing in PBST briefly.
- 5. Post-fixed the polyps were in 4% PFA at room temperature for 20 min.
- 6. Wash the polyps with PBST for 10 min, twice.
- 7. Pre-hybridization in Prehyb<sup>+</sup> (50% Formamide, 5XSSC, 50 µg/ml Heparin, 2.5% Tween 20, 50 µg/ml SSDNA (Sigma, D1626)) (For gastrodermis probe, add SDS(2% final concentration) to help penetrate) at 68°C for 2 h.
- 8. Replace the Prehyb<sup>+</sup> with probe and incubation overnight at 68°C.
- 9. Remove the probe and save the probe under -20. The probe can be re-used multiple times.
- 10. Wash polyps sequentially in 2X SSC (0.3 M NaCl and 0.03 M sodium citrate) containing 50% formamide for 20 min twice, 2X SSC containing 25% formamide for 20 min, 2X SSC for 20 min twice, and 0.2X SSC for 30 min 3 times each, all at 68°C.
- 11. Wash polyps with PBST at room temperature for 10 min
- 12. Incubate polyps in DIG blocking buffer (1% ISH blocking reagent (Roche, 11096176001) in maleic acid buffer (0.1M maleic acid, 0.15 M NaCl, pH 7.5) for 1 hour at room temperature

- 13. Replace the DIG blocking buffer with anti-DIG antibody (Anti-Digoxigenin-AP (Roche, 11093274910), 1:5000 dilution in DIG blocking buffer). Incubate overnight at 4°C.
- 14. Remove antibody and wash with PBST for 10 min at room temperature. Repeat twice.
- 15. Wash with 9.5T buffer (100 mM Tris-HCl pH9.5, 50 mM MgCl<sub>2</sub>, 100 mM NaCl, 0.1% Tween 20) for 10 min at room temperature. Repeat twice.
- 16. Remove 9.5T and develop signal by incubation in BCIP/NBT buffer (1 SIGMAFAST<sup>TM</sup> BCIP®/NBT tablet (Sigma, B5655) in 10 ml H<sub>2</sub>O)) at 4°C until brown-purplish colors were sufficiently dark. The developing time was depend on the gene expression, and can vary from hours to days.
- 17. Wash the polpys with PBST for 10 min, twice.
- 18. Post-fixed in 4% PFA overnight at 4°C followed by washing in PBST twice for 10 min each.
- 19. Optional step (convert brownish color to blue): Wash in methanol for 3 h at room temperature. The tissues were kept in PBS and imaged using SMZ1500 microscope (Nikon) under Ring Light System (Fiber-Lite).
- 20. For cross section, the whole mount sample was processed for cryosection.