Whole mount RNA *in situ* hybridization

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1. *Xenia* polyps were relaxed in Ca^{2+} free sea water (described above) for 30 min and fixed in 4% PFA in Ca^{2+} free sea water overnight at 4ºC.
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 $Prehyb^{+}$ (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 $Prehyb^{+}$ 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 $MgCl_2$, 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™ BCIP®/NBT tablet (Sigma, B5655) in 10 ml H_2O)) 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.