Whole Mount *In Situ* Hybridization with mRNA Probes

Optimized for *Aurelia* jellyfish, modified from Nakanishi *et al.* (2010) Evol Dev. 12(4):404-15.
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Antisense probe(s)	Sense probe(s)

Fixation:

☐ 1. Anesthetize animals in 7.3% MgCl2
☐ 2. Fix animals in 4% formaldehyde for 1 h @ room temperature (RT).
☐ 3. Acclimate from 7.3% MgCl2 to MeOH (25%, 50%, 75%, 100%) using 5 min washes
☐ 4. Store animals at -10°C

Rehydration:

5 minutes each wash each @ RT:
☐ 5. 60% MeOH 40% PBSTr
☐ 6. 30% MeOH 70% PBSTr
☐ 7. 100% PBSTr

8. Wash 3x in PBSTr over a 2 hour period at RT: (1) ☐ (2) ☐ (3) ☐

Digestion – only perform if probe appears to have difficulty penetrating sample:

☐ 9. Digest the samples with Proteinase K. For ephyrae, use 0.05 mg/ml Proteinase K in PTw (1× PBS, 0.05% Tween-20) for 10 min at room temperature without shaking. For embryos, larvae and newly settled polyps, use 0.01 mg/ml Proteinase K in PTw for 5 min at room temperature without shaking.

10. Wash the samples twice with 1 ml of 2 mg ml–1 glycine in PTw for 5 min each with gentle rocking at RT: (1) ☐ (2) ☐

Protein Acetylation:

5 minutes each wash each @ RT:
☐ 11. TEA solution
☐ 12. TEA solution
☐ 13. TEA solution plus 0.3% acetic anhydride
☐ 14. TEA solution plus 0.6% acetic anhydride

Refixation:

15. Wash 2x 5 minutes in PBSTr: (1) ☐ (2) ☐

☐ 16. Refix for 1hour with 4% formaldehyde in PBSTr at RT.

17. Wash 5x 5 minutes in PBSTr at RT: (1) ☐ (2) ☐ (3) ☐ (4) ☐ (5) ☐

☐ 18. Put aside several animals for pre-absorption with antibody.

Prehybridization:

☐ 19. Prehybridize in Hybridization Buffer (HB) **without SDS** for 10 min at RT.

- *save this "prehyb solution" for later use*

☐ 20. Prehybridize in HB **with SDS** for 1 hour at 55°C.

- *preheat additional Hybridization Buffer solution to 55°C for next steps*

☐ 21. Prehybridize in HB without SDS for 0-24 hours at 55°C.
Increased time can significantly decrease background staining

Probe Hybridization:

☐ 22. Shock probe @ 80°C for 2 minutes. Place on ice for 10 min. Heat to 55°C.

☐ 23. Wash once with prehyb without SDS at 55°C. Save prehyb at RT or -20°C.

☐ 24. Add probe (1μg/ml), hybridize 1-2 days (approx. 40-72 hours) at 55°C.

Washes:

☐ 25. Remove probe and keep for reuse.

☐ 26. Wash in HB **without SDS** at 55°C for 10 min.

☐ 27. Wash in HB **with SDS** at 55°C for 40 min.

All additional washes for 30 min:
☐ 28. 75% HB, 25% 2x SSC
☐ 29. 50% HB, 50% 2x SSC
☐ 30. 25% HB, 75% 2x SSC
☐ 31. 100% 2x SSC

32. Wash 3x in 0.05X SSC at 55°C for 20 min each: (1) ☐ (2) ☐ (3) ☐

Washes at RT (10 min each)
☐ 33. 75% 0.05X SSC, 25% 2x TNT
☐ 34. 50% 0.05X SSC, 50% 2x TNT
☐ 35. 25% 0.05X SSC, 75% 2x TNT
36. 2x 100% TNT (10 min each): (1) ☐ (2) ☐

☐ 37. Block in TNB for 1 hour.

☐ 38. Add anti-digoxygenin "POD" antibody (for FISH, 1:250) or anti-digoxygenin "AP" antibody (for color reaction, 1:1000-2000) and any additional primary antibodies overnight at 4°C.

Fluorescence Amplification (for POD samples):

☐ 39A. Wash in TNT for 2 hours at RT.

☐ 40A. Incubate in 300 µl of TSA Plus Fluorescein Evaluation Kit (PerkinElmer; Cat No./ID: NEL741E001KT) for 20 min at RT.

☐ 41A. Wash in PBSTr for 1 hour at RT.

☐ 42A Block in 3% NGS for 1 hour at RT.

☐ 43A. Add secondary antibodies, and incubate overnight at 4°C.

☐ 44A. Wash in PBSTr for 2 hours at RT.

☐ 45A. Mount on glass slides.

OR

Color Reaction Amplification (for AP samples):

☐ 39B. Wash in TNT 10 times (or more) for 20-30 minutes.

40B. Wash 2x in Color Reaction Buffer for 10 minutes each: 1) ☐ 2) ☐

☐ 41B. Develop in AP substrate solution (make fresh) at RT in dark. Monitor color development. (Can also develop slower at 4 degrees). This might take 30 min to two weeks.

☐ 42B. Stop reaction by washing 5 x with PTw. Mount in 70% glycerol in PTw.

RECIPIES

PBSTr (50mL)
150 µl Triton X-100 in 50 mL PBS

PBt / PBtw (50mL)
50 µl Tween-20 in 50mL PBS

TEA Buffer (50mL)
500 µl Triethanolamine
approx. 270-300 µl HCl (until pH ≈ 7.5)
to 50 mL with PBSTr

Hybridization Buffer (40mL)
20 ml of formamide
10 ml of 20× SSC (pH. 4.5)
100 µl of 20 mg/ml heparin
100 µl (0.05%) Tween-20
1% SDS (only in HB + SDS solution)
200 µl of 10 mg/ml sheared salmon sperm DNA
7.5 ml of H2O

TNT Buffer (50mL)
5 ml of 1M Tris-HCl (pH 7.5)
7.5 ml of 1M NaCl
25 µl of Tween-20

Color Reaction Buffer (50mL)
5 ml of 1M Tris, pH = 9.5
5 ml of 1M NaCl
2.5 ml of 1M MgCl2
100 µl (0.05%) Tween-20
To 50 mL in H2O

AP substrate solution
Color Reaction Buffer plus:
4.5 µl/ml NBT (nitro-blue tetrazolium)
3.5 µl/ml BCIP (5-bromo-4-chloro-3'-indolyphosphate)