Fluorescence in situ hybridization for whole-mount larval brains

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[modified from Leslie Vosshall's alkaline phosphatase protocol and Michael Pankratz's multichannel fluorescence protocol]

All steps done in RNAse-free 1.5 ml Eppendorf tubes (preferably with safe-lock)

Day 1

1. Dissect tissue in RNAse-free PBS on ice/cold stage and transfer to a 1.5 ml tube containing Fixative solution on ice.
2. Fix for **1 hr** at RT.
3. Wash **5/5/10/20 min** with PBTw.
4. Wash stepwise 10 min 30% MeOH in PBTw, 10 min 70% MeOH in PBTw, then 100% MeOH.
5. Store sample -20 C in MeOH overnight or longer.

Day 2

6. Transfer stepwise back to PBTw (5 min 70% MeOH, 5 min 30% MeOH)
7. Wash **5/10/15/30/60 min** in PBTw. Meanwhile, prewarm HybeB solution, Hybe solution, and riboprobe to 65 C.
8. Add 0.25 ml PBTw and 0.25 ml HybeB, mix, allow brains to sink.
9. Wash 10 min in 0.30 ml HybeB at 65 C, allow brain to sink.
10. Add 0.25 ml HybeB and 0.25 ml Hybe, mix, allow brains to sink.
11. Wash 15 min in 0.30 ml Hybe at 65 C, allow brains to sink.
12. Discard supernatant, pre-hybridize 1 hour in 0.30 ml Hybe at 65 C.
13. Discard most of supernatant, add 5 ul riboprobe.
14. Hybridize overnight 65 C.

Day 3

15. Add 0.30 ml Hybe, allow brains to sink
16. Discard supernatant, post-hybridize 1 hour in Hybe solution at 65 C.
17. Wash 15 min in 0.25 ml HybeB + 0.25 ml Hybe at 65 C, allow brain to sink.
18. Wash 15 min in 0.30 ml HybeB at 65 C, allow brain to sink.
19. Wash 15 min in 0.25 ml HybeB + 0.25 ml PBTw at RT, allow brain to sink.
20. Wash extensively in PBTw (5/10/15/30/60/60 min) at RT.
21. Block in blocking solution (PBTw+5% goat serum) for 1 hour.
22. Incubate overnight at 4 C in blocking solution with anti-DIG POD coupled antibody.

Day 4

23. Wash extensively in PBTw (5/10/15/30/60 min).
24. Discard supernatant, add 0.15 ml 1X Plus Amplification Diluent (PerkinElmer), allow brains to sink.
25. Incubate in 0.85 ml 1X Plus Amplification Diluent for 15 min, then add 7.5 ul TSA Plus Fluorophore Amplification Reagent (PerkinElmer), mix carefully, incubate in the dark 7-10 min at RT. All steps done in the dark/ low light from here on.
26. Discard supernatant, wash 5/10/15/20 min PBTw.
27. Mount in mounting medium (Vectashield with DAPI)

DIG RNA riboprobe synthesis

Linearize plasmid
Calculate amount of plasmid needed to achieve equivalent of 1 µg insert DNA. [ex. Insert is 0.5 kb in 4.5 kb total plasmid at 0.5 µg/µl. So take 18 µl (9 µg of the whole thing), which gives 1 µg insert]

Digest with appropriate enzyme in a final volume of 50 ul:
X µl plasmid DNA
3 µl enzyme
5 µl 10X Buffer
0.5 µl 100X BSA (if required)
add H2O to 50 ul

Check 1 µl of digest on a gel in comparison to 50-100 ng the uncut plasmid

Purify template DNA
Following QIAquick protocol for PCR with modification:
- final step: elute DNA using 30 µl of RNAse-free water (not EB buffer)
- after adding the RNase-free water wait for 10 minutes before final centrifugation.
-recover in a RNase-free Eppendorf tube.

Check 0.5 µl of the purified product on a gel.

Synthesize riboprobe
10.5 µl template in H2O
4 µl Promega 5X transcription buffer
2 µl BMB 10X DIG RNA labeling mix
1 µl 0.1M DTT
0.5 µl Promega rRNAsin
2 µl RNA polymerase (SP6, T3, or T7)

Mix gently, spin briefly, incubate at 37 C for 2 hours.

Purification of riboprobe using G50 ProbeQuant columns.
- Resuspend the resin by vortexing the column;
- Crack off the bottom of the column;
- Place the column in a RNase-free tube and spin for 1 min at **0.8 rcf**;
- Discard flow-through and repeat spin in the same tube.
- Transfer the column to a new RNase-free Eppendorf tube and add the riboprobe. Centrifuge at **0.8 rcf** for 2 min.
- Bring volume to 101 ul with RNase-free water.
- Check 2 µl of probe on a gel

*do not hydrolyze probe for this protocol.
- Add 200 µl in situ ***Hybe Buffer*** + 1 µl RNase inhibitor (RNAsin).
- Denature RNA probe (100 C for 5 min, Ice 3 min, Store at -20 or proceed to hybridization at 65 C).

Reagents

10% stock of Tween-20 (in PBS)
Mix 1.5 ml 100% tween-20 with 13.5 ml PBS

PBTw
0.5% (v/v) Tween-20 in 1X PBS

HybeB
50% (v/v) Formamide, 5X SSC

Hybe solution: use the pre-made one in stock (-20C reagent freezer).

Block Solution (10 ml)

1	ml	Heat-inactivated normal sheep goat serum
200	ul	10% BSA
8.8	m	PBTw

Amplification reagent and 1X plus amplification diluent from TSA Plus Fluorescene Kit (PerkinElmer, Inc.)

Making Fixative to use within 2-3 days.

1. Make RNase- free glassware and stir bars by adding a stir bar to a 50-100 ml beaker, adding 1 ml of 10 N NaOH [over the sink], filling bottle half full with ultrapure water, and swishing well to coat inside of bottle.
2. Carefully dump out contents of bottle and rinse well with more ultrapure water. Rinse stir bar and reserve on a clean paper towel on the side.
3. Fill bottle with 40 ml of DEPC water and microwave on high for 30 sec until water is boiling.
4. Meanwhile, weigh out 2 g of paraformaldehyde (in bucket in the fridge, use gloves and facemask).
5. Place boiled water on a stir plate in a hood, add RNase free stir bar. Carefully add 2 g paraformaldehyde powder.
6. Add 5 ul of 10N NaOH.
7. Stir until paraformaldehyde is completely dissolved and solution has cooled to ~40-50C.
8. Add 5 ml 10X PBS (homemade).
9. Add 2.5 ml of 10% Tween-20 in PBS (final will be 0.5% tween)
10. Add 0.05 g sodium deoxycholate (top shelf, chemical room)
11. Using disposable RNase free volumetric cylinder or disposable 50 ml tube, measure out fixative into a sterile filtration apparatus. If volume is less than 50 ml, add ultrapure RNase free water to a final volume of 50 ml (Check that pH=7.0)
12. Turn on vacuum and filter fixative. Store at 4C and use within 2-3 days