Whole Mount microRNA ISH Protocol (Shorter Version)

Adapted from Nieto, Patel and Wilkinson (1996)

Embryo Processing

1. Embryo collection:
   Collect embryos in ice-cold **saline (chick salt).** Rinse several times with ice-cold saline.

2. Prepare embryos for fixation as follows:
   For HH (Hamburger & Hamilton) stages 11 and below proceed to step 3.
   For HH stages 12-18, open the extraembryonic and pericardial membrane.
   For HH stages 19 and above, remove the extraembryonic and vitellin membranes and open the pericardial membrane.

3. Fix embryos as follows:
   For HH stages 18 and below, spread embryos flat by gently adding fresh ice-cold **4% paraformaldehyde (PFA)** drop wise. After all embryos have been flattened in **PFA**, carefully remove this saline/PFA mixture and add fresh **PFA** drop wise.

   For HH stages 19 and above, since membranes have been removed, place up to 30 embryos drop wise into a 15 ml conical tube of fresh **PFA**. After 5 minutes of gentle mixing, replace this solution with fresh **PFA**. Fix overnight at 4˚C (or 2 hours at RT).

4. Trim embryos as follows:
   Transfer embryos into a clean 100 mm petri dish containing **PBT** and trim off excess membrane.

   For HH stages 15 and above, major cavities are poked. This may include any or all of the following: forebrain, hindbrain, eye, neural tube, and allantois.

5. Dehydrate embryos:
   Group embryos by stage and transfer into 6 well plate containing mesh baskets. Dehydrate through a **graded MeOH series,** by lifting mesh well insert into the next MeOH dilution. Typical time between changes is 5 minutes.

6. Freeze embryos:
   Transfer embryos into a scintillation vial containing 100% MeOH. Freeze in at least 10 ml of 100% MeOH at –20˚C for at least 1 hour but less than a week.

7. Rehydrate embryos:
   Rehydrate embryos through a **graded MeOH series** in a scintillation vial. Time between changes is 5-15 minutes or until embryos sink. This is followed by a change in **PBT**.

8. Digest embryos:
   Using **Proteinase K** in a 20 ml volume as follows:

Days of incubation	HH stages	treatment
1	7 and below	none
2	8 to 13	10 min in 10 μg/ml Proteinase K in PBS
3	14 to 18	20 min in 10 μg/ml Proteinase K in PBS
4	19 to 24	20 min in 20 μg/ml Proteinase K in PBS

9. Rinse once for 5 minutes with 20 ml of **PBT**.

10. Post-fixation of embryos:
    For 20 minute in at least 10 ml of **4% PFA with 0.2% gluteraldehyde**.

11. Rinse twice in **PBT** for 5 minutes each.

12. Thoroughly remove **PBT** and add **prehyb** with sufficient volume to allow embryos to sink. Switch to fresh prehyb. Go to step 13 or (optional) store at –20˚C until ready to use for ISH.

Prehybridization and Hybridization

13. Prehybridation:
    Incubate embryos in **prehyb** at the annealing temperature for 2 hours.

14. Hybridization:
    Add 1 µl of a 5 µM DIG-labeled LNA **working stock** per ml of **prehyb** (5nM final concentration, see page 7 of 8 of the long protocol for the dilution directions) and incubate at the annealing temperature overnight.

Post-hybridization Washes

15. Transfer embryos into 6 well plate containing mesh baskets.
    Wash 3x20 min with prewarmed **2x SSC with 0.1% CHAPS** at the annealing temperature.

16. Wash 3x20 min with prewarmed **0.2x SSC with 0.1% CHAPS** at the annealing temperature.

17. Rinse 2x10 min at room temperature with **KTBTw**.

Antibody

18. Transfer embryos into a 24 well plate. Block embryos in 20% sheep serum in **KTBTw** for 2-3 hours or longer at 4˚C.

19. Preabsorb the anti-DIG-AP Fab fragment (Roche cat # 11 093 274 910) with 3mg **chick embryo powder**/µl concentrated antibody in 0.5 ml 20% sheep serum in **KTBTw**.

20. Incubate with 1:4000 dilution of anti-DIG-AP Fab fragment in 20% sheep serum in **KTBTw** overnight at 4˚C.

21. Transfer back into 6 well baskets to allow for increased wash volumes. Wash 5x1 hour in **KTBTw** on a shaker or nutator at room temperature. (May continue overnight at 4˚C).

Color Reaction

22. Rinse 2x15 minutes in fresh **NTMTw.**

23. Add **color reagent** and incubate in the dark at room temperature. React until signal to background is optimized.

24. Stop color reaction with 2 brief rinses in **KTBTw**, then **PBS**.

Photography

25. Dehydrate embryos in a **graded MEOH** series. (optional)

26. Rehydrate embryos in a **graded MEOH** series, followed by a change in **PBT** for photography.

Solutions: see the full length protocol