Insitu Protocol *(modified RSG 09/11/19)*

Reagents:

AP Buffer (50 mL):

5mL of 1M Tris HCl pH 9.5 [100mM]
2.5 mL of 1M MgCl2 [50mM]
1.25 mL of 4M NaCl [100mM]
50 uL Tween 20 [0.1%]
Vt -50ml with MilliQ H20

1X PBS (1L):

100 mL 10X PBS (Duelbecco's Phosphate Buffered Saline) (Sigma D1408)
900 mL MilliQ H2O

2x SSC (1L):

100 mL 20X SSC (Sigma S6639)
900 mL MilliQ H2O

0.2X SSC (1L):

10 mL 20X SSC
990 mL MilliQ H2O

75% Methanol in PBS (500 mL):

375 mL Methanol (Fisher A433F)
125 mL 1X PBS

50% Methanol in PBS (500 ml):

250 mL Methanol
250 mL 1X PBS

25% Methanol in PBS (500 ml):

125 mL Methanol
375 mL 1X PBS

Tris HCl 9.5 1M (1L):

121.1 g Tris HCl (Sigma T3253)
900 mL H20

Titrate with HCl until pH~ 9.5

Other reagents:

-BM Purple, Roche 11442074001
-Anti-DIG antibody, Roche 11093274910
-DIG ntp mix (10x), Roche 11277073910

0.1% Tween 20 in 1X PBS (1X PBT) (1 L)):

1 mL 100% Tween 20 (Sigma P2287)
999 mL 1X PBS

Hyb Buffer (50% Formamide)

100 mL Formamide (Sigma F9037)
50 mL 20X SSC
1 mL 20% Tween 20
10 mg Heparin Sodium Salt (50 ug/mL)
100 mg tRNA

Alpha Blocking Buffer (Good for 2 weeks):

50 mL 1X PBT
1 mL 100% Sheep Serum
100 mg Bovine Serum Albumin (Sigma A2153)

4% PFA (5 mL):

541 uL 37% Formaldehyde (Sigma 252549)
4460 uL 1X PBS

Post-Bleaching Solution (15 mL):

13.275 mL MilliQ H2O
375 uL 20X SSC
750 uL Formamide
600 uL 30% H2O2 (Sigma 216763)

Take care in handling H2O2, it is very caustic.
Prepare solution in this order.

Proteinase K (10 μg/mL)

1 uL Proteinase K 10 mg/mL
999 uL 1X PBS

In situ Hybridization Protocol:

Day 1:

Fixation of embryos/ larval fish

- Collect fish in Eppendorf tubes, if > 5dpf, add Tricaine and let sit for ~ 15 minutes (otherwise, the fish may become contorted), be sure to lay the tube flat so that the fish do not become contorted in the bottom of the vial
- Fix tissue in 4% PFA over night @4degrees

Day 2:

Bleaching (this can also be done after coloring with post-bleaching solution)

- Place fixed embryos in tubes (either open or with a hole poked in the top) or in glass vials with loose caps.
- Incubate embryos with 3% H2O2/0.5% KOH medium (prepared fresh) at room temperature until pigmentation disappears.
  - This will take ~30mins-1hour. Check progress with scope
  - *Be careful the solution is bubbly and caustic! If caps are not loose, pressure will build and may splatter solution on you.*
- Wash embryos in 1x PBS for 5 min to stop bleaching reaction.

Dehydrate to 100% Methanol (MeOH)

- Replace solution with 25% Methanol ~5 min
- Replace with 50% Methanol ~5 min
- Replace with 75 % Methanol ~5 min
- Replace with 100% Methanol ~ 5 min
- Replace with fresh 100% Methanol ~5 min

Leave at -20 Degree Freezer O/N or until use

Day 3:

Rehydrate fish

- Replace with 75% MeOH ~ 5 min ❑
- Replace with 50% MeOH ~ 5 min ❑
- Replace with 25% MeOH ~ 5min ❑
- Replace with 1X PBT 4 washes ~ 5 min each ❑ ❑ ❑ ❑

Proteinase K treatment

- Replace PBT with 10 μg/mL Proteinase K
  - <5 dpf, no proteinase K
  - 5-7 dpf, 15 minutes proteinase K
  - 7 dpf or more, 30 minutes proteinase K
- Stop proteinase K with 2 quick PBT washes then 2 EDTA washes [0.5M] 5mins each then fix with 4% PFA ~ 20 - 60 min
- Wash with PBT 4 washes ~ 5 mins each ❑ ❑ ❑ ❑

Prehybridization treatment and probe addition

- Replace with Hyb Buffer (50% Formamide) at 70 degrees for ~2-5 hours
- Replace with antisense DIG labeled RNA Probe (150 ng) O/N at 70 degrees

Make sure the water bath has sufficient water to make it through the night with evaporation

Day 4:

- *Collect antisense DIG labeled RNA probe for later use, be sure to label the collection for number of uses. Do not let samples touch the air, it's better to sacrifice some probe.*
- *This is a long day, start early!*

Stringency washes for removing excess probe (all solutions and washes must be at 70 degrees)

- Add Hyb Buffer (50% Formamide) 10 min at 70 degrees ❑
- Replace with 75% Hyb Buffer 10 min at 70 degrees ❑
- Replace with 50% Hyb Buffer 10 min at 70 degrees ❑
- Replace with 25% Hyb Buffer 10 min at 70 degrees ❑
- Replace with 2X SSC 10 min at 70 degrees ❑
- 2 washes with 0.2X SSC 30 min at 70 degrees ❑ ❑

Rehydration in PBT (perform all washes at room temperature)

- Replace with 75% 0.2X SSC ~ 10 min at RT
- Replace with 50% 0.2X SSC ~ 10 min at RT
- Replace with 25% 0.2X SSC ~ 10 min at RT
- 4 washes with PBT ~ 10 min at RT ❑ ❑ ❑ ❑

Antibody block/incubation

- Replace PBT with alpha antibody blocking buffer for ~ 2-3 hours at RT
- Replace with 1:10,000 anti-DIG antibody (in alpha antibody blocking buffer) O/N at 4 degrees, gentle rocking.

  Make sure to be gentle when changing solutions, do not use a micropipette, use a transfer pipette (thin tip), and squeeze solution onto the wall of the tube.

Day 5:

- Discard anti-DIG antibody
- Replace with PBT ~ 10 min
- Transfer fish from Eppendorf tube to a glass vial or plastic wells **gently** with a glass pipette
- Replace with fresh PBT ~ 10 min
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- Replace with fresh PBT ~10 min
- Replace with AP Buffer ~ 10 min. Take BM purple out of fridge to warm to RT.
- Replace with fresh AP Buffer ~ 10 min
- Replace with fresh AP Buffer ~ 10 min
- Discard AP Buffer, add ~ 700 uL BM Purple (shake BM Purple vigorously prior to adding to fish), allow **gentle** agitation
- Cover the fish in saran wrap and aluminum foil, ensure no entry of light
- Check fish staining (a purple stain within the fish at various places) about every 30 minutes
    - *AP buffer can be used to wash sample and/or dilute BM Purple if needed*.

Before you leave, ensure the fish are well covered and place in fridge at 4 degrees O/N, in the morning, replace BM Purple solution

Day 6/7:

- Once stained, remove the BM purple and wash with PBT 4 times ❑ ❑ ❑ ❑
- Wash with 0.5M EDTA pH 8, 30 min
- Re-fix in 4% PFA, 1 hr to solidify stain
- If samples were not pre-bleached, bleach with post-bleaching solution
    - Make fresh solution and add to vial 1/3 full. Leave cap on loosely.
    - Leave O/N @ RT, protected from light. <u>Or</u> can leave out in the light during day, check bleaching every few hours.
    - Wash 4x in PBT, 5 mins each to stop bleaching ❑ ❑ ❑ ❑
- Replace with 25% Glycerol (cut with 1X PBS), invert vial that contains fish 2 times **gently**
- After ~ 30 min of sitting, replace with 50% Glycerol
- For imaging, use 80% glycerol
- Save in 100% glycerol indefinitely at 4 degrees

The glycerol with obliterate the fish if placed on a rocker, do not allow ANY agitation of the fish. Place on desk.