**Whole-mount IHC protocol for zebrafish embryos (three-day protocol)**

**Day 1: Fixation**

(pre-chill the Methanol at -20°C, prepare solutions for day 2 and 3)

1. Collect embryos (in our case 3 dpf – 5 dpf)
2. Fix embryos at RT for 2 hours in 4%PFA (rotation)
3. Wash embryos with PBS at RT x 3
4. Store embryos in pre-chilled 100%Methanol in the freezer (-20°C) overnight. (Embryos could be stored at 20°C over a few months)

**Day 2: Staining**

(heat up the heatingblock at 70°C, get ice and pre-chill the dH2O, pre-chill Acetone at -20°C)

1. Rehydrate embryos through a series wash of Methanol/PBST
2. (100% -> 75% -> 25% -> 0% MeOH/PBST, 5 minutes for each step)
3. Wash the embryos PBST for 5 minutes 3 times
4. Wash the embryos with Tris buffer for 5 minutes
5. Equilibrate the embryos in Tris buffer at 70°C for 15 minutes
6. Wash the embryos with PBST for 5 minutes twice
7. Rinse the embryos quickly with dH2O twice on the ice
8. Penetrate the embryos with pre-chilled Acetone (-20°C) at -20°C for 20 minutes
9. Remove the Acetone and rinse the embryos quickly with dH2O twice and three times for 5 minutes
10. Block the embryos with fresh prepared 10%NGS/2%FCS/PBT for 4 hours at 4°C
11. Incubate the embryos with the primary ABs (concentration varies with different ABs), diluted in 2%NGS/2%FCS/PBT at 4°C overnight

**Day 3: Staining**

1. Wash the embryos with PBT for 30 minutes 4 times
2. Incubate the embryos with the secondary ABs (concentration varies with different ABs), diluted in 2%NGS/2%BSA/PBT for 3 hours at RT (in the dark)
3. Wash the embryos with PBST for 5 minutes five times
4. Mount the Embryos using Prolong gold /diamond mounting medium (abcam) and let them dry at 4°C overnight in the dark before further usage

**Solutions**

0.1M phosphate buffer: (dissolved in 800ml ddH2O:

* 26.81g Na2HPO47 (268.0611 g/mol)
* 13.79g NaH2PO4 (137.9 g/mol)
* filled up with ddH2O until 1000 ml

PBT:

* 420ml 0.1M phosphate buffer
* 80ml 5% Triton X100

PBS pH7.4: (dissolved in 800ml ddH2O)

* 8g of NaCl
* 0.2g of KCl
* 1.44g of Na2HPO4
* 0.24g of KH2PO4
* filled up with ddH2O until 1000ml

PBST:

* 500ml PBS
* 1,67ml 30% Tween20 solution

Tris-HCL buffer:

* 150mM Tris-HCL pH 9.0
* (1,8g Tris-HCL (157.6 g/mol) diluted in100ml dH2O)

10%NGS/2%FCS/PBT:

* 1,5ml Normal goat serum (NGS)
* 0,3ml FCS (Fetal calf serum)
* PBT up to 15ml

2%NGS/2%FCS/PBT:

* 0,3ml NGS
* 0,3ml BSA
* PBT up to 15ml