Fixing and Storing Zebrafish Embryos

1. Transfer dechorionated embryos into a small Wheaton vial.
2. Add 2 ml of 4% paraformaldehyde (PFA) and fix overnight at 4°C.
3. Wash embryos by aspirating the PFA and immediately adding PBSTw using a squeeze bottle. Remove wash by aspiration. Perform two "quick washes": remove PBSTw right after adding it. Then 3 washes for 5 minutes.
4. Remove final PBSTw wash by aspiration. Add enough 100% methanol to fill the entire Wheaton vial. Cap and mix gently. Aspirate methanol and replace with fresh 100% methanol. Incubate at –20°C for at least 30 min.

    • *embryos can be stored indefinitely in 100% methanol at –20°C*

5. Aspirate methanol and replace with 75% MeOH/PBSTw. Incubate for 5 min. at R.T.
6. Aspirate and replace with 50% MeOH/PBSTw, wash 5 min. at R.T.
7. Aspirate and replace with 25% MeOH/PBSTw, wash 5 min. at R.T.
8. Aspirate and wash 2 x 5 min with PBSTw
9. Add proteinase K at 10 μg/ml in PBSTw and digest according to stage of embryo:

Stage	conc (μg/ml)	time (minutes)
1-2ss		
4-5ss	10	1
10-11ss	10	2
14-15ss	10	2.5
19-20ss	10	5
25s	10	9
24 hr	10	15
26 hr	30	6
28 hr	30	8
30 hr	30	10
33 hr	30	13
36 hr	30	16
37 hr	30	17
40 hr	50	12
41 hr	50	13
45 hr	50	15
47 hr	50	17
50 hr	50	19
56 hr	50	22

10. Quickly aspirate proteinase K and add 4% PFA; incubate at R.T. for 20 minutes
11. Wash with PBSTw 2 x 1 min.; with the first wash transfer each group of embryos into a well of a 6-well plate; wash 3 x 5 min. at R.T. with gentle agitation. Washes can be carefully aspirated using a pasteur pipet attached to a vacuum flask
12. Transfer embryos to 2.0ml eppendorf tubes in PBSTw using a Pasteur pipet

Hybridization

1. Remove excess PBSTw and add 500µl of HB4; incubate at 65°C with agitation for 1-2 hr.

    - *the best way to remove the excess PBSTw is to vacuum aspirate the majority of liquid then use a P200 pipet to remove the remaining liquid – get as much liquid off as possible*

2. Dilute probe 1:10 in HB4 (final volume: 100µl) for each sample to be hybridized and heat to 80°C for 5-10 min.; cool on ice

    - *if the riboprobe synthesis reaction gave a low yield of probe, the amount used for hybridization should be increased*

3. Remove HB4 and replace with probe/HB4 mix

    - *as in step 1, first vacuum aspirate most of the volume to remove the HB4 then get remaining liquid with a P200*

4. Incubate overnight at 70°C with gentle agitation
5. Wash embryos 2 x 30 min. in 2x SSCTw/50% formamide at 65°C
6. Wash 15 min. in 2xSSCTw at 65°C
7. Wash 2 x 30 min. in 0.2xSSCTw at 65°C

Staining

1. Block for at least 1 hour with PBSTw/5%sheep serum
2. Incubate embryos in 100µl of preabsorbed sheep anti-DIG Fab fragments at 1:2000 in PBSTw for 2 hours at R.T. with shaking
3. Wash 6x at R.T. with PBSTw; time for incubation is variable; start with first wash at 5 min., second at 10 min., etc. second to last wash can be overnight at 4C. The final wash can be up to 1 hour, the following day.
4. Wash 2x 5 min. with staining buffer (SB) (do not shake)
5. Stain in staining solution (SS) for up to 48 hours. (In the dark!!!!!!!!!!) Also, check after a hour to see how much stain has been absorbed.

In situ hybridization reagents and ordering information

10x PBS

PBSTw:
1x PBS
0.1% Tween-20

20 mg/ml proteinase K
Add 5 ml of milli-Q water to 5 mg vial of proteinase K
Vortex briefly to dissolve
Dispense into 10 µl aliquots and store at -20°C

4% paraformaldehyde
2 g paraformaldehyde
45 ml ddH_2O
Heat to 55°C under fume hood
Add 5 ml 10x PBS
Cool to 4°C and add 4µl of 1N NaOH
store in 40 ml aliquots at –20°C

HB4
50% formamide
5 x SSC
50µg/ml heparin
0.1%Tween-20
5mg/ml torula RNA

2xSSCTw
2xSSC
0.1% Tween-20

0.2xSSCTw
0.2xSSC
0.1% Tween-20

Sheep serum
heat inactivate at 55°C for 30 min
store at –20°C

Staining buffer (SB)
100 mM NaCl
50 mM $MgCl_2$
100mMTris, pH 9.5
0.1% Tween-20

Staining solution (SS)
make up 1 ml per sample:
3.5 µl NBT
3.5 µl BCIP
SB to 1 ml