Whole Mount In Situ Hybridization – 5dpf embryos

Paloma I Meneses Giles 11/2018 (modified after Megan Senecal)

Fixation:

- Fix embryos with 4% paraformaldehyde (PFA) overnight at 4°C. Always use fresh, defrosted 4% PFA. **Store samples in 4% PFA at 4˚C.**
- Transfer about 40 embryos into 2ml tubes and dehydrate in a MeOH series (33% MeOH in PBST for 5 minutes, 66% MeOH in PBST for 5 minutes and 100% MeOH for 5 minutes)
    - Use MeOH instead of EtOH because MeOH reduces background.
- Store embryos at -20°C in 100% MeOH overnight. **Ideally, dehydrate embryos the day before.** Staining is not very consistent if embryos used for ISH have been stored in MeOH for more than a week.
    - MeOH series is necessary for permeabilization of embryos.

Day 1

Rehydration:

- Rehydrate embryos by reversing the MeOH series (66% MeOH in PBST for 5 minutes and 33% MeOH in PBST for 5 minutes).
- Wash 3x 5 minutes in 100% PBST.

Proteinase and Post Fixation:

- Always treat embryos over 24hpf with ProK! (Proteinase K). It will improve probe penetration.
- Digest 5dpf embryos in **ProK** (~19mg/ml current stock) diluted **1:2000** in PBST **2-3min**. Do not rock the embryos during this process. **Invert the tubes gently every other min during the digestion.**
    - Embryos digested in 1:2000 ProK for up to 10 min stain ok.
- Rinse briefly in PBST.
- Wash 3x 5 minutes in PBST.
- Fix with 4% PFA for 30 minutes.
- Wash 3x 5 minutes in PBST.

Prehybridization:

- Prehybridize in 200uL of HYB+ buffer at 55-68°C for at least 30min if needed but ideally 2hrs and up to 2 days. **Usually 6 to 8 h.**

Hybridization:

- Use 200uL of diluted probe (dilution depends on preference, usually **10-20ng/ul** probe concentration used).
- Incubate overnight at 55-68°C.

Day 2

Probe Removal:

- Remove probe carefully and save.
    - Probes can be used several times, typically a probe in HYB+ is stable for at least half a year.
- Pre-warm B-50%Formamide/50%2xSSCT. Add ~250ul to each sample once the probe has been removed.
- Remove fish from 2mL tubes and place them in in situ baskets.
- **AT THIS POINT PUT THE EMBRYOS INTO THE MACHINE OR CONTINUE BY HAND.**
- Incubate 2x15 min 500 ul 50%Formamide/50%2xSSC.

- Incubate 5 min 500 ul Hyb wash.
- Incubate 3x15 min 500 ul Hyb wash.
- Incubate 15 min 500 ul 50% Hyb wash/50%SSC.
- Incubate 15 min 500 ul 25% Hyb wash/75% 2xSSC.
- Incubate 15 min 500 ul 2xSSCCh.
- Incubate 2x30 min 500 ul 0.2xSSCCh.
- Incubate 30 min 500 ul PBSTCh.
- Incubate 2x15 min 500 ul MAT.

- Block for at **least 2 hours** at room temp with MAB-block (MABT).
- Add Fab-AP at a **4000 fold** dilution in the MAB-block (MABT) and shake for 4 hours at room temp or overnight at 4°C.

Day 3

- Replace Fab-AP antibody solution with MAB (always make fresh).
- **AT THIS POINT PUT THE EMBRYOS INTO THE MACHINE OR CONTINUE BY HAND.**
- Incubate 8x 20 min 500 ul MAB.
- Wash 3 x 5 minutes in staining buffer (always make fresh).

Detection:

- Remove fish from in situ baskets and put them in a 24 well plate.
- Stain embryos by adding **1ml** of staining buffer with **2.5ul NBT** and **3.5ul BCIP** (from Promega) to each well. Keep in the dark.
    - **Prepare staining solution for all samples needed. Add NBT and mix before adding BCIP in the proportions listed above.** (BM purple (Roche) looks fuzzier).
- Check ISH color development every couple of hours. Replace with fresh staining solution+NBT+BCIP when needed.
- To stop reaction, completely remove staining solution, wash 3x PBST and replace with FRESH 4% PFA.
- Store in 2ml tubes in 4% PFA forever after imaging.

Staining Buffer - 30 samples

30 wells x 500 ul = 15000ul
3x x 15000 ul = 45000ul
5ml extra
30 well x 1ml = 30ml +NBT/BCIP

	Staining buffer for wash	**Staining Buffer + NBT/BCIP**
MgCl2 (1M)	2.5 ml	1.5 ml
NaCl (5M)	1 ml	600 ul
Tris (1M) pH 9.5	5 ml	3 ml
10% Tween 20	500 ul	300 ul
MiliQ	41 ml	24.6 ml
Total	**50ml**	**30ml**
		+ 75ul NBT
		+ 105 BCIP

30 samples:

50% Formamide/50%2xSSC.
30 wells x 500 ul = 15000ul
2 washes + transfer = 3x
3x x 15000 ul = 45000ul
5ml extra
Total + 50ml (25ml Formamide + 25ml 2xSSC).

Hyb wash (50% Formamide/5x SSC/0.25% CHAPS).
Total = 100ml (50ml Formamide + 25ml 20xSSC + 5ml 5% CHAPS +20ml MiliQ).

50% Hyb wash/50%SSC.
30 wells x 500ul = 15000ul
Total = 18ml (9ml Hyb wash + 9ml 2xSSC).

25% Hyb wash/75% 2xSSC.
30 wells x 500ul = 15000ul
Total = 18ml (4.5ml Hyb wash + 13.5ml 2xSSC).

2xSSCCh (2xSSC/0.25% CHAPS).
30 wells x 500ul = 15000ul
Total = 18ml (17.1ml 2xSSC + 0.9ml 5%CHAPS).

0.2xSSCh (0.2xSSC/0.25% CHAPS).
30 wells x 500ul = 15000ul
2x washes = 2x
2 x 15000ul = 30000ul
5ml extra
Total = 35ml (3.5ml 2xSSC + 1.75ml 5%CHAPS + 29.75ml MiliQ).

PBSTCh (0.25% CHAPS/PBST).
30 wells x 500ul = 15000ul
Total = 20ml (19ml PBST + 1ml 5%CHAPS).

MAT (100mM maleic acid/50uM NaCl/0.1% Tween 20/adjust pH=7.5).
30 wells x 500ul = 15000ul
2x 15000ul = 30000ul
+5ml extra
+15ml MABT
+20ml MABT + 1˚ Ab
Total = 70ml (7ml 1M maleic acid + 0.7ml 5M NaCl + 0.7ml 10% Tween 20 + 61.6ml MiliQ).

MABT (MAT/1mg/ml BSA/10% NGS).
Total = 35ml (35ml MAT + 35mg BSA). Remove 3.5ml. Add 3.5ml NGS.

MABT (MAT/1mg/ml BSA/10% NGS/1:4000 anti-Dig AP Fab antibody).
Total = 20ml (20ml MABT+BSA+10%NGS + 5ul antibody).

MAB (100uM maleic acid/50mM NaCl, adjust pH=7.5).
30 wells x 500ul = 15000ul
8x + transfer = 9x
9 x 15000ul = 135ml
+15ml (bottom)
+5ml extra
Total = 155 ml (15.5ml 1M maleic acid + 1.55ml 5M NaCl + 137.95ml MiliQ).