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.
  o 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

<table>
<thead>
<tr>
<th>Staining buffer for wash</th>
<th>Staining Buffer + NBT/BCIP</th>
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<tbody>
<tr>
<td>MgCl2 (1M)</td>
<td>2.5 ml</td>
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<tr>
<td>NaCl (5M)</td>
<td>1 ml</td>
</tr>
<tr>
<td>Tris (1M) pH 9.5</td>
<td>5 ml</td>
</tr>
<tr>
<td>10% Tween 20</td>
<td>500 ul</td>
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<tr>
<td>MiliQ</td>
<td>41 ml</td>
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<tr>
<td>Total</td>
<td>50ml</td>
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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).