Whole Mount In Situ Hybridization – Short Worksheet

Fixation of embryos - long term storage:
Fix embryos in MEMFA for 1-2 hr:
1xMEMFA: 1/10 vol 10xMEMFA salt
1/10 vol formaldehyde
8/10 vol water
Dehydrate in MeOH (Change MeOH several times)
Embryos can now be stored long term at –20°C

BEGIN IN SITU
DAY 1: ~3 hours needed until 6 hr. prehyb step
Reagents:
Thaw paraformaldehyde 20% (10-20 ml)
Set water bath to 60°C (shaker)
Make 1 Liter 1xPBS+0.1% Tween-20 (PTw)
Thaw Proteinase K 10mg/ml (50-100 µl)
Methanol
0.1M Triethanolamine pH 7-8 (~100-200 ml)
Acetic Anhydride (125-250 µl)
Hyb Buffer (50-100 ml)

Rehydration: (PTw=1xPBS+0.1%Tween-20)
5 minutes each wash each RT (room temperature)
  1) Methanol (MeOH)
  2) 75% MeOH 25% H2O
  3) 50% MeOH 50% H2O
  4) 25% MeOH 75% PTw
  5) 100% PTw

3x 5 minutes in PTw RT

Proteinase K ~5 min. RT
(100µl 10mg/ml PK/100ml PTw)
Rinse 2x 5 minutes in 0.1M triethanolamine pH 7-8 RT

Add 12.5µl acetic anhydride/5mls: 5 minutes (125µl in 50 ml/250µl in 100 ml) - in triethanolamine RT
Repeat acetic anhydride addition: 5 minutes RT
Wash 2x 5 minutes in PTw RT

2) Refix for 20 minutes with 4% paraformaldehyde in PTw (10 ml 20% PF into 40 ml PTw) RT

Wash 5x 5 minutes in PTw (wash off excess paraformaldehyde) RT

Day 2: ~4 hours
Reagents:
2xSSC (100 ml 20xSSC into 900 ml H2O)
Thaw RNAse A (20 mg/ml)/RNAse T1 (10 mg/ml)
(Both at 1000x)
Thaw MAB + 10% BMB Blocking agent (5-10 ml)
2xSSC with RNase A 20 µg/ml, RNAse T1 10 µg/ml
0.2xSSC (100 ml 2xSSC into 900 ml H2O)
MAB pH 7.5

Remove probe and keep for reuse
Replace probe with hyb. buffer, Incubate 60°C for 3 minutes
Wash 3 minutes 2xSSC, 60°C
Wash 3 minutes 2xSSC, 60°C

Washes: 3x 20 minutes 2xSSC, 60°C

Wash 30 minutes (37°C) 2x SSC with RNase A 20 µg/ml, RNAse T1 10 µg/ml
Wash once in 2x SSC 10 minutes room temp.
Wash twice in 0.2x SSC 30 minutes, 60°C.

Wash twice in MAB, 10-15 minutes, RT.

Wash in MAB + 2% BMB Blocking Reagent (10 ml 10% BMB/1xMAB to 50 ml with MAB) 1 hr or more, RT.

Replace with MAB + 2% BMB Blocking Reagent + Ab (1/3000 dilution of the anti-digoxigenin AP antibody) overnight at 4°C (or 4 hrs at RT.) 0.2 µL/0.5 mL -SAVE antibody - 0.5 mL into a probe test tube works also!!!
**DAY 2.5-3 (5.5 hours)**

**Reagents:**
- Thaw Alkaline Phosphatase buffer (with 0.005 M levamisol and 0.1% Tween) (100-200 ml)
- MAB - Maleic Acid Buffer pH 7.5

Wash embryos at least 5 times, 1 hour each with MAB RT

1)  
2)  
3)  
4)  
5)  

Wash twice, 5 minutes each at room temperature with Alkaline phosphatase buffer (make sure levamisol 0.005 M and 0.1% Tween have been added) RT

1)  
2)  

- Replace last wash with BM purple

**Stopping Color Reaction:**

**Reagents:**
- MAB (maleic acid buffer)
- Bouin's Fixative (70 ml saturated Picric acid/5 ml glacial acetic acid/25 ml formaldehyde)
- 1xSSC (1 liter)
- EtOH
- Bleach Solution (1% H$_2$O$_2$, 5% formamide, 0.5x SSC)
- 20xSSC
- formamide
- 30% peroxide

Stop chromogenic reaction with quick wash in MAB RT

Fix overnight in Bouin's RT

Multiple 10 minute washes in 70% buffered EtOH RT

Rehydrate stepwise into 1X SSC: RT

- 5 min 75% buffered EtOH: 25% 1X SSC
- 5 min 50%-50%
- 5 min 25%-75%
- 5 min 100% 1xSSC
- 5 min once more in 1xSSC

Bleach embryos in bleaching soln. (~1-2 hours for 50 mls: 1.25 ml 20xSSC 2.5 ml formamide 2 ml 30% peroxide 45 ml distilled water Use light box. RT

Wash twice for 5 min in 1xSSC RT

Take pictures of uncleared or clear embryos in BB:BA clearing agent (benzyl benzoate/benzyl alcohol).