**In situ Hybridization - Ozpolat Lab Protocol**

Animals were fixed in 4% paraformaldehyde at 4C overnight, transferred stepwise into 100% methanol, and kept at -20C until the start of the in situ procedure.

**Day 1**

1. Rehydrate samples through a methanol/DEPC-treated PBSt series (on ice).
2. Digest in 0.1mg/mL Proteinase K (Ambion AM2548) for 5 minutes at room temperature
3. Briefly (<1 minute) wash in a glycine (.2mg/mL) DEPC-PBSt solution and refix in 4% paraformaldehyde for 30 minutes on ice.
4. Wash with DEPC-PBSt five times, 5 minutes each.
5. Step samples into the hybridization solution (33%, 66%, 100%) and incubate in a 65C water bath for 4 hours.
6. Dilute dig-labeled RNA probes (Ambion AM1310, Sigma/Roche 11277073910) to 2ng/uL in 100% hybridization solution. Denature at 85C for 10 minutes.
7. Incubate samples in the probe solution at 65C overnight (approximately 16 hours).

**Day 2**

1. SSC Formamide washes:
   1. Probe solution was then removed and replaced with 4X formamide wash solution. Repeat 4X SSC wash twice
   2. two 2X SSC washes at 65C
   3. three 0.2X SSC washes at 65C
2. Wash with 0.2X SSC made without formamide three times (20 minutes each) at room temperature.
3. Wash for five minutes in PBSt.
4. Block samples for two hours at room temperature in 5% sheep serum/PBSt. Block primary antibody at 4C.
5. Replace with anti-dig primary antibody (1:5000) (Roche 11093274910) in 5% sheep serum and incubated overnight at 4C on Nutator.

**Day 3**

1. Wash samples eight times in PBSt (20 minutes each time)
2. Two 5-minute washes in AP buffer without MgCl2
3. One 5-minute wash in AP buffer with MgCl2
4. Transfer into NBT/BCIP staining buffer (ThermoFisher 34042). Protect from light and monitor periodically for color change. Include sense probe controls to account for background staining. Keeping most samples in NBT/BCIP overnight at 4C typically provides enough time for the signal to develop with minimal background in most Platynereis samples.

**Day 4**

1. Wash samples twice with stop buffer to end the reaction.
2. Wash at least three times in PBSt (30 minutes each) on ice and on a nutator.
3. Transfer samples into 75% glycerol/25% PBSt stepwise and store (protected from light) at -20C.

**Materials/Reagents**

NBT/BCIP Solution: ThermoFisher 34042

Anti-dig antibody: Roche 11093274910 (from Sigma)

**In situ solutions**

\* Solutions should be autoclaved to sterilize  
+ Solutions should be made with RNase/DNase-free water

\*10X PBS

18.6 mM NaH2PO4.H20 (2.56g/Liter)

84.1 mM Na2HPO4.2H2O (14.97g/Liter)

1,750 mM NaCl (102.2g/Liter)

Mix phosphates in 800mL dH20. Check to ensure pH is near 7.4 (within .4), adjust pH to 7.4 with HCl or NaOH. Add NaCl and remaining water. Make sure that you are dealing with the correct phosphate stock powders (monohydrate vs. dihydrate etc).

\*PTw

100mL 10x PBS stock

895mL dH20

5mL 20% Tween-20

Dilute 10x PBS to 1x. DEPC treat, autoclave and cool. Add tween.

+ Heparin

Make a stock of 50 mg/ml in H2O, store at -20°C

+ Hybridization Mix

50% formamide (Fluka, ultra pure)  
5X SSC  
50 µg/ml heparin  
0.1%Tween20  
5 mg/ml torula RNA  
  
store at -20°C.

For 50 ml of Hyb. Mix:

Formamide 100 % 25 ml  
SSC 20 x 12.5 ml  
Heparin 50 mg/m l50 µl  
Torula-RNA (Sigma) solid 250 mg  
Tween20 10 % 500 µl  
H2O add to 50 ml

\*20x SSC pH 7.0

175.3g NaCl  
88.2g NaCitrate  
1L dH20  
  
Mix salts and dH20.  
pH to 7.0, DEPC treat and autoclave.

\*20x SSC pH 4.5

175.3g NaCl  
88.2g NaCitrate  
1L dH20  
  
Mix salts and dH20.  
pH to 4.5, DEPC treat and autoclave.

+Formamide Wash Solutions

4X SSC wash (50ml)  
Formamide 25ml  
20X SSC pH 7 10ml  
20% Tween-20    250µl  
H2O 14.75ml

2X SSC wash (50ml)

Formamide 25ml  
20X SSC pH 7 5ml  
20% Tween-20    250µl  
H2O 19.75ml

0.2X SSC wash (50ml)

Formamide 25ml  
20X SSC pH 7 500µl  
20% Tween-20    250µl  
H2O 24.25ml

\*1 M NaCl (500mL)

29.22g NaCl  
500mL dH20  
  
Autoclave to sterilize.

\*1M MgCl2 (250mL)

50.75g MgCl2 (if hexahydrate only)  
250mL dH20  
  
Mix and autoclave to sterilize

\*1M Tris pH 9.5 (500mL)

60.57g Tris  
500mL dH20  
  
Mix and autoclave to sterilize

+5X AP Buffer (200ml)

1M Tris pH 9.5 100ml  
5M NaCl 20ml  
H2O 80ml  
  
Store at room temperature.

+1X AP Buffer (50ml)

5X AP Buffer 10ml  
H2O 39.75ml  
20% Tween-20 250µl

+1X AP Buffer with MgCl2 (50ml)

5X AP Buffer 10ml  
H2O 37.25ml  
1M MgCl2 2.5ml  
20% Tween-20 250µl

+STOP Buffer (50ml)

2M Tris pH 7.5 2.5ml  
5M NaCl 1ml  
1M MgCl2 2.5ml  
20% Tween-20 250µl  
H2O to 50mL.  
  
Store solution at 4C.