**Updated 7/21/22 by Patricia Graham**

**Drosophila in situ with NBT/BCIP and INT/BCIP**

Use at least 10X the volume of sample for each rinse or wash (ie. For 50ul of sample use 500ul of liquid).

Rinse = Add liquid. Allow the sample to settle. Remove liquid.

Wash = Add liquid and either rock or let sit the indicated amount of time before removing the liquid.

**Day1 (about 2.5 hours)**

1. Rinse 2X with MeOH to remove any remaining heptane from embryos and tube.
	1. *If your embryos are sticking to the tube it sometimes helps to transfer them to a new tube.*
2. Rinse 1X with 1:1 MeOH:PBST.
3. Fix in 4% PFA for 25 minutes at RT.
	1. *For Drosophila the 4% PFA can be up to one month old, but this may not be great for other organisms. It may be best to make 1-2 ml aliquots of 12% and dilute only the amount you need that week.*
4. Wash at least 3X 10 minutes with PBST or PBTT and make hybridization solutions.
	1. *For lower background you might find that using PBTT instead of PBST for all washes works better. It also seems to help make the yolk less sticky/more permeable for those embryos that are surrounded by yolk.*
	2. *For Drosophila you only usually need to use hybridization buffer (HB) and follow the simple protocol below (2 ml/sample for 0.5 ml washes).*
	3. *If you have background issues, you may want to try the more complex protocol with wash 1 and wash 2 rather than the simple protocol. For that protocol, you need 2.5 ml of hybridization buffer (HB) and 1.5 ml each wash solution per sample for 0.5 ml washes.*
	4. *For Drosophila HB can be made up to a 4 weeks ahead (ie. You can make a large batch and keep it at 4oC for about a month). If you work with another organism, you may find that making it fresh the day you plan to use it works better. You can make enough for the day you hybridize and for the washes the next day. Keeping the HB for the washes the next day on the bench overnight seems to be ok.*
5. Remove most of the PBST and heat to 90oC for five minutes to denature proteins.
	1. *This is a replacement for a proteinase K treatment that would remove proteins.*
6. Rinse 1X with 1:1 HB:PBST.
7. Incubate at least 2X 30 minutes in HB at 60oC. (Can go longer).
8. Heat probes (double check that they have different markers, eg. dig vs biotin) 5 minutes at 95oC, then ice.
	1. *With Drosophila we usually use about 50ul of probe per sample. The concentration depends on the probe and must be determined by trying a range of concentrations. If you know the concentration starting with 10 and 1 ng/ul is often good. If you do not know the concentration, starting with 1/10, 1/50 and 1/100 seems to work well.*
9. Incubate embryos overnight with probes at 60oC.

**Day 2 – detection of first color (Simple protocol 4.5 hours plus staining time and if possible inactivation time. More complex protocol 5.25 hours plus extras.)**

1. Remove and save probes (Often a strong probe can be used several times).
2. Simplest protocol (for clean probes)
	1. Wash at least 1X 20 minutes at 60oC with HB.
	2. Wash at least 1X 20 minutes at 60oC with 1:1 HB:PBST
	3. Wash at least 4X 5 minutes at 60oC with PBST
3. More complex protocol to remove background
	1. Wash embryos at least 1X 30 minutes at 60oC in HB.
	2. Wash at least 3X 10 minutes at 60oC in wash 1
	3. Wash at least 3X 10 minutes at 60oC in wash 2
	4. Wash at least 3X 15 minutes at 60oC in PBST at RT.
4. Both protocols are the same from here on. Incubate 1-2 hours in appropriate antibody for one probe (eg. Anti-dig-frag AP conjugated, 1:2000 or Anti-FL-frag AP conjugated 1:2000 or Anti-biotin AP conjugated at 1/2000 in PBST) at RT.
	1. *Another option – incubate overnight at 4oC. This may alter the signal to noise ratio.*
5. Wash at least 4 X 10 minutes in PBST
6. Wash at least 1 X 5 minutes in hybridization staining buffer (SB)
7. A) Stain in
	1. 4.5ul NBT+3.5ul BCIP in 1 ml SB. Check using the microscope every 10-15 minutes. NBT/BCIP gives a nice dark purple/brown stain and is usually the best stain to use first.
8. Stop reaction by rinsing 3X with PBST or PBTT when the signal is dark, and there is a bit of background stain in the rest of the embryo.
	1. *If you overstain – Wash at RT several times for 5-10 minutes with High Tween-salt buffer, then rinse 3X with PBST and wash 2X10 minutes with PBST. If the signal disappears too much, you can re-stain immediately or store ON at 4oC and re-stain the next day. You may want to watch during the first wash to see how quickly the background is disappearing vs. the pattern.*
9. Wash at least 3X 5 minutes with PBST.
10. Fix the stain in place
	1. **For NBT/BCIP**
		1. Rinse 1X with 1:1 PBST:MeOH
		2. Rinse 2X with MeOH
		3. Rinse 1X with EtOH
		4. Rinse 2X with MeOH
		5. Rinse 1X with 1:1 MeOH:PBST
		6. Rinse 3X with PBST
		7. Wash 3X 5 minutes with PBST
11. If there is time that day, inactivate the AP on the first Ab with one of the following possible protocols. (Can also be done on next day)
	1. Wash 3x 20 minutes at RT in 100mM glycine-HCl (pH 2.2) plus 0.1% Tween 20.
	2. Incubate the embryos 1X 15 minutes at 60oC in inactivation buffer.
12. Rinse 1X, then wash at least 3X 10 minutes in PBST.
13. Leave overnight 4oC in PBST.

**Day 3 Detection of Second Color (~3.5 hr if inactivation already done. Add another hour or 1.5 hours for inactivation)**

1. Incubate 1.5-2 hours RT with 200-500ul of the appropriate second AP conjugated antibody in PBST (1/200 anti-fluorescein Fab or anti-dig Fab, 1/250 Goat anti-biotin).
2. Wash at least 4X10 minutes in PBST at RT.
3. Wash at least 1X 5 minutes in SB at RT.
4. Stain with INT/BCIP (7.5 ul of INT/BCIP stock solution in 1 ml of Staining Buffer).
	1. *You want nice dark orange signal and a light yellow background when you stop*
5. Rinse 3X with PBST immediately to stop the reaction.
6. Wash at least 3X 5 minutes with PBST.
7. Fix embryos in 4% PFA for 30 min.
8. Rinse 1X, then wash 3X 10 minutes with PBST.
9. Mount in 70% glycerol, 0.1M Tris pH 8.0.
	1. *Put two small coverslips on the ends of the slide. They will prevent the main coverslip from crushing the embryos. Remove as much PBST as possible and add about 50 ul of glycerol solution. Cut the end off a blue tip to suck up the embryos. Place them in the middle of the slide and cover with a large coverslip that sits on the small coverslips on the ends of the slide. To keep for more than a few days seal the sides of the large coverslip with nail polish. Store at 4oC.*
	2. *Note that if the pH is off, the NBT/BCIP turns blue overnight.*

**Hybridization Buffer (HB)**

|  | **5 ml** | **10ml** | **15ml** | **20ml** | **50ml** |
| --- | --- | --- | --- | --- | --- |
| **dH2O** | 1ml | 2ml | 3ml | 4ml | 10ml |
| **Formamide** | 2.5ml | 5ml | 7.5ml | 10ml | 25ml |
| **20X SSC** | 1.25ml | 2.5ml | 3.75ml | 5ml | 12.5ml |
| **10% Tween** | 50 ul | 100 ul | 150 ul | 200 ul | 500 ul |
| **Salmon Sperm DNA** | 50 ul | 100 ul | 150 ul | 200 ul | 500 ul |
| **Heparin** | 50 ul | 100 ul | 150 ul | 200 ul | 500 ul |
| **tRNA (20mg/ml)** | 25ul | 50ul | 75ul | 100ul | 250ul |

**Wash 1**

|  | **5 ml** | **10ml** | **15ml** | **20ml** | **50ml** |
| --- | --- | --- | --- | --- | --- |
| **dH2O** | 1.25ml | 2.5ml | 3.75ml | 5ml | 12.5ml |
| **Formamide** | 2.5ml | 5ml | 7.5ml | 10ml | 25ml |
| **20X SSC** | 1.25ml | 2.5ml | 3.75ml | 5ml | 12.5ml |
| **10% Tween** | 50 ul | 100 ul | 150 ul | 200 ul | 500 ul |

**Wash 2**

|  | **5 ml** | **10ml** | **15ml** | **20ml** | **50ml** |
| --- | --- | --- | --- | --- | --- |
| **dH2O** | 2ml | 4ml | 6ml | 8ml | 20ml |
| **Formamide** | 2.5ml | 5ml | 7.5ml | 10ml | 25ml |
| **20X SSC** | 0.5ml | 1ml | 1.5ml | 2ml | 5ml |
| **10% Tween** | 50 ul | 100 ul | 150 ul | 200 ul | 500 ul |

**PBST(phosphate buffered saline) PBTT (phosphate buffered saline + Triton)**

0.5% Tween-20 0.05% Tween 20

 0.1% Triton X-100

**Hyb Staining Buffer (10 ml)** **High Tween-Salt Wash for 50 ml**

8.2 ml H2O 5%Tween 20 24.75 ml H2O

1 ml Tris pH 9.5 650mM NaCl 2.5 ml Tween-20

200 ul 100mM NaCl 200mM KCl 6.5 ml 5 M NaCl

500 ul 1M MgCl2 125mM Tris pH 7.9 10 ml 1M KCl

100 ul 10% Tween 20 6.25 ml Tris pH 7.9

(Will be cloudy)

**4% PFA**

4% paraformaldehyde in PBST

**Inactivation buffer (1ml)** **Alternative**

480 ul formamide Glycine 100mM, pH 2.2

240 ul H2O

240 ul 20X SSC

10 ul 10% Tween-20

30 ul 10% SDS