***Oncopeltus* embryo fixation and in situ hybridization, Katie Reding**

**Embryo fixation**

1. Collect your aged embryos from the cotton and transfer about 100-200 uL of embryos to each tube.
2. Set about 300 mL of water to boil on the hot plate.
3. Add ~600 uL DEPC-treated ddH2O to each tube of embryos. Tap tube to try to submerge embryos, but some will likely remain floating.
4. Submerge tubes of embryos in boiling water bath for 3 minutes.
5. Carefully (but quickly) move tubes to ice and submerge. Leave for ~ 7 min or until cold.
6. Remove the water from tubes.
7. In fume hood, add 600 uL heptane and 600 uL *freshly thawed* (within a few days) 12% paraformaldehyde(PFA). Shake the tube manually until you see the embryos align between the two phases.
8. Shake at 250 rpm for 20 minutes.
9. Remove the paraformaldehyde (aqueous, lower phase) to hazardous waste. Remove heptane to hazardous waste.
10. Add fresh 600 uL heptane and 600 uL methanol (adding fresh heptane dramatically improves chorion cracking)
11. Shake tubes vigorously 15-30 s. Check how many embryos sink versus float (embryos still inside chorion tend to float). Continue shaking until nearly all embryos are sinking.
12. Remove MeOH/heptane.
13. Wash in 1 mL MeOH x 3. Try to remove eggshells as you remove washes.
14. Store in MeOH at -20C until use.

**Chorion removal**

I (KR) usually spend a day in between fixation and in situ removing embryos from their eggshells manually. You can do this on the first day of the in situ, but it is fairly time-consuming, so I prefer to do it ahead of time.

1. Rehydrate embryos from MeOH to PBST (remove 500 uL MeOH, add 500 uL PBST, let wash 5 min. Repeat 2-3 times)
2. Transfer embryos in PBST to dissection plate, and manually remove eggshells under the scope using very fine forceps.
	1. I find using a glass pipette is best during this step, because the eggshells (and the embryos inside) stick to the plastic tips. You may need to cut off the tip of the glass pipette, and will definitely need to cut off the end of a plastic 1 mL pipette tip.
	2. Make sure you are using a glass dissection plate with silicon filling a few of the wells. This helps keep all the embryos on one plane and prevents damage to your forceps.
	3. As you remove eggshells, try to remove them from the well to keep them from ending up in your tube. When all the embryos are in the well and my tube is empty, I also like to wash my tube a few times with water to completely clean it of eggshells, which will get in the way of the staining.
3. Move embryos back to tube and dehydrate into MeOH (remove 500 uL PBST, add 500 uL MeOH, let wash 3 min. Rinse a couple times in MeOH and store in MeOH). I like to mark the tube in some way that lets me know these embryos are free of eggshells.

**In situ hybridization**

**Day 1**

1. Warm up some hybridization buffer (1 mL per tube of embryos) in the **60°C** hyb oven
2. Rehydrate your embryos from 100% MeOH (remove 500 uL MeOH, add 500 uL PBST, let wash 5 min. Repeat this 2-3 times until solution is ~100% PBST)
3. Rinse 3X in PBST
4. Remove PBST and add 1 mL 4% PFA, let wash 1.5 h at RT
5. Remove PFA, rinse 3X with PBST
6. Remove PBST, add 1 mL pre-warmed hyb buffer
7. Let embryos incubate in **60°C** incubator for ~3-4 h.
8. Remove embryos from incubator and allow them to sit upright for at least 5 minutes as embryos settle to the bottom. Remove hybridization buffer and add at least 200 uL of probe (typically 1:500 of the 100 ng/uL works well) and place vertically in the **60°C** incubator.

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

**Day 2**

1. Put 2 wash volumes hyb buffer and 1 wash volume 2X SSC (saline-sodium citrate)/0.1% Tween-202 in **60°C** incubator to warm up (wash volume = 1 mL/tube of embryos)
2. Remove probe.
3. Wash in the following solutions, rocking:
	1. 2 x 30 minutes pre-warmed hyb buffer **at 60°C** from Step 1
	2. 1 x 30 minutes pre-warmed 2X SSC/0.1% Tween-20 **at 60°C** from Step 1
	3. 1 x 30 minutes 2X SSC/0.1% Tween-20 **at RT**
	4. 1 x 30 minutes 0.2X SSC/0.1% Tween-20 **at RT**3
4. Rinse 3X in PBST
5. To block, wash in the following solutions, rocking, **at RT**:
	1. 10% sheep serum in PBST for >2 hours
6. Add 800 uL of the antibody (anti-Dig-AP or anti-Fluorescein-AP) at 1:1600 dilution in 10% sheep serum in PBST. Incubate overnight at **4°C** OR at **RT** for at least 1 h, rocking. If incubating at RT, remove antibody and leave embryos in PBST rocking at **4°C** overnight.

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

**Day 3**

1. Wash in 1 mL the following solutions, rocking **at RT**:
	1. 5 x 20 minutes in PBST
	2. 3 x 5 minutes in AP staining buffer4
2. When embryos are in their last wash of staining buffer, move them to a 24-well plastic plate.
3. Remove buffer and add 500 uL of BCIP/NBT staining solution5. Place in a dark drawer or cover with aluminum foil. You can typically wait a few hours before checking them for the first time. Check every hour or so for staining. They may need to stain overnight.
4. To stop staining, rinse 3X in PBST followed by 3 x 10 minute washes in PBST **at RT**.
5. If you used NBT/BCIP:
	1. Wash with 1:1 PBST/MeOH for 5 min.
	2. Rinse 2X in MeOH
	3. Rinse 1X in EtOH
	4. Rinse 2X in MeOH
	5. Wash 1:1 PBST/MeOH for 5 min

If you used INT/BCIP:

* 1. Fix embryos in 4% PFA for 30 min
1. Wash with PBST 3 x 5 min; store in PBST at **4°C**.
2. Clean the 24-well plate:
	1. Rinse with tap water
	2. Add some 10% SDS to the wells, scrub with a bottle brush, rinse with tap water

**Mounting germbands on slides**

1. Manually dissect germbands away from yolk using very fine forceps in a glass dissecting dish lined with silicone. A very small brush may be useful for removing bits of yolk sticking to the embryo but usually isn’t necessary.
2. Move dissecting germbands to a well with fresh PBST to wash away all bits of yolk.
3. Using a 200 uL tip with the end cut off, move germbands to a slide.
4. Carefully wick away PBST with a kimwipe, being very careful not to wick up embryos as well.
5. Add a drop (~ 20-25 uL) 70% glycerol to embryos
6. Use forceps to extend germbands flat. This is most easily done by moving germband to the edge of the glycerol droplet where the layer of glycerol is thinnest.
7. Add cover slip to distribute glycerol toward embryos. Some embryos may be picked up and relocated in this wave of glycerol, but as long as you the droplet isn’t too big, this shouldn’t happen too frequently.
8. Use clear nail polish to seal cover slips, let dry overnight.

1 Hybridization buffer recipe

|  |  |  |  |
| --- | --- | --- | --- |
|  | **50 mL** | **25 mL** | **15 mL** |
| 20X SSC | 12.5 mL | 6.25 mL | 3.75 mL |
| Heparin (10 mg/mL) | 250 uL | 125 uL | 75 uL |
| Yeast tRNA (20 mg/mL) | 125 uL | 62.5 uL  | 37.5 uL |
| 10% Tween-20 | 500 uL | 250 uL | 15 uL |
| Water | 12 mL | 6 mL | 3.75 mL |
| Formamide | 25 mL | 12.5 mL | 7.5 mL |

2 2X SSC/0.1% Tween-20

 1.5 mL 20X SSC DEPC

15 uL Tween-20

To 15 mL with ddH2O

3 0.2X SSC/0.1% Tween-20

150 uL 20X SSC DEPC

15 uL Tween-20

To 15 mL with ddH2O

4AP staining buffer (pH 9.5)

300 uL 5M NaCl

750 uL 1M MgCl2

1.5 mL 1M Tris-HCl, pH 9.5

15 uL Tween-20

ddH2O to 15 mL

5 BCIP/NBT staining solution (use 500 uL/tube)

 4.5 uL NBT

 3.5 uL BCIP

 1 mL staining buffer