Tribolium embryo collection and antibody staining

Embryogenesis in *Tribolium* takes around six days at 25°C. Neuronal axons can first be observed in 1-2 day old embryos, and mature ventral nerve cords are visible in 4-5 day old collections. (5-6 day old collections contain a high percentage of larvae, which are more difficult to stain and dissect.)

Setting up an egg collection

Set up an egg collection container with males and females in unbleached white flour with 5% (by weight) active dry yeast (it is helpful to pre-sift the yeast through a 250µm sieve to remove large fragments). Adults can be removed from the flour/yeast/embryos using an 850µm sieve, and embryos can be removed with a 300µm sieve. For large-scale collections, we add 5-10 ml of adults to 500g flour + 25g yeast in a two-quart plastic container with a mesh insert in the lid (cut a large hole in the lid and tape the mesh over it). Smaller collections can be done in a one-pint mason jar with ventilated lid or a *Drosophila* culture bottle with a standard plug.

- To collect all stages of embryonic development, allow adults to lay eggs continuously for six days at 25°C, then collect and fix embryos immediately. Embryos will range in age from 0-6 days (and the collection will also include some larvae).
- To collect all stages of neural development, allow adults to lay eggs for four days, then remove the adults and allow the eggs to develop in the flour for one additional day at 25°C. At the time of fixation, the embryos will be 1-5 days old.
- To examine mature nerve cords only, allow adults to lay eggs for 24 hours, then remove the adults and allow the eggs to develop in the flour for an additional 4 days at 25°C. At the time of fixation, all of the embryos will be 4-5 days old.

Embryo collection and fixation

1. If adults are still present, remove them first with an 850µm sieve.
2. Separate the eggs from the flour/yeast using a 300µm sieve.
3. Transfer the eggs to a nitex mesh basket.
4. Dechorionate embryos in 50% bleach for 2-5 min (squirt some 50% bleach into a small weigh dish, then dunk the collection basket into the bleach).
5. Wash dechorionated embryos well with H_2O.
6. Prepare fixative in glass scintillation vials (20 ml per vial):

H_2O	9 ml
37% formaldehyde	1 ml
Heptane	10 ml

7. Transfer embryos into the vial of fixative using a wet paintbrush.
8. Screw the cap onto the scintillation vial and fix for 30-45 min with vigorous agitation (taping the vials horizontally onto an orbital shaker stage works well).
9. Remove fixative (aqueous bottom phase) and heptane (organic upper phase) with a glass pasteur pipet, and discard. Leave the embryos behind!
10. Wash embryos 2-3 times with 100% methanol.
11. Fixed embryos can be stored indefinitely in 100% methanol at -20°C, or sonicated immediately.

Embryo sonication

Removal of extraembryonic membranes (especially the vitelline membrane) allows antibodies to access embryonic tissues and is essential for staining internal embryonic structures like the ventral nerve cord. Unlike in *Drosophila*, simply shaking fixed embryos in methanol does not efficiently remove the vitelline membrane from *Tribolium* embryos. Instead, embryos can be devitellinized by hand with fine forceps or needles (this is laborious, but produces a high percentage of intact embryos) or through low-intensity sonication. Sonication leads to fragmentation of a large proportion of the embryos, but will typically produce enough intact germbands or large germband fragments to allow examination of axon guidance phenotypes.

1. Return embryos (stored in 100% methanol) to a glass scintillation vial. If you have a large collection of embryos, you can split them up among multiple vials. At most, the embryos should cover the bottom of the vial in a single layer. If you have more embryos than this, use more than one vial.
2. Add enough methanol to bring the total volume to approximately 10 ml.
3. Add 10 ml heptane. Embryos should float at the heptane/methanol interface. If they fall to the bottom of the vial in large blobs, you have added too many embryos; split them into another vial.
4. Sonicate briefly to remove the vitelline membrane from the embryos (Note: we use a Branson S250 probe sonicator with a 1/8” microtip, and immerse the tip into the bottom (methanol) layer of the vial).
    - With the sonicator output at 5-10%, the duty cycle dialed to “constant”, and the timer dialed to “hold”, turn on the sonicator for five second bursts.
    - For the first round: sonicate for 5 sec, rest for a few seconds, then sonicate for another 5 sec. Remove the sonicator tip from the scintillation vial, screw on the cap, and shake the vial. Let the embryos settle, and remove any embryos or fragments that have fallen to the bottom of the vial. Transfer these to a new scintillation vial. Uncracked embryos and dissociated membranes should remain at the interphase. Add more methanol to replace what you removed with the embryos.
    - For subsequent rounds, sonicate once for 5 sec, then cap, shake and remove cracked embryos and fragments as above. Combine the cracked embryos and fragments from all rounds in the same new scintillation vial.
    - Repeat for 4-5 rounds total, or until nearly all of the embryos have cracked and fallen to the bottom. Discard remaining uncracked embryos (they will not stain well) and dissociated membranes.
5. Remove the methanol and residual heptane from the cracked embryos and fragments collected in step 4.
6. Wash cracked embryos and fragments 2-3 times with 100% methanol. Be aware that dissociated germbands and germband fragments will settle more slowly than intact embryos, so give them extra time to settle before removing the methanol after each wash.
7. Sonicated embryos can be stored indefinitely in 100% methanol at -20°C, or rehydrated immediately for antibody staining.

Antibody staining

Begin with sonicated embryos in 100% MeOH (stored at -20°C or freshly sonicated). Note, as above, that dissociated germbands and germband fragments will settle more slowly than intact embryos, so give them extra time to settle before removing the buffer after each wash/rinse.

1. Transfer 50-100ul embryos to a 1.5ml microcentrifuge tube.
2. Rehydrate embryos by rinsing 3x with 1ml PBT, then washing 2x 5min (on nutator) with 1ml PBT.
3. Block embryos in PBT+5% NGS. Incubate on nutator at room temp for at least 30min.
4. Add 1° antibody, diluted in PBT+5% NGS. 500ul per tube (1ml per tube can be used if staining a larger volume of embryos).
5. Incubate overnight on nutator in cold room (4°C).
6. Next morning: rinse 3x with 1ml PBT, then wash 2x 5min with 1ml PBT.
7. Add 2° antibody, diluted in PBT+5% NGS. 500ul per tube.
8. Incubate at least 1 hr on nutator at room temp.
9. Rinse 3x with 1ml PBT, then wash 2x 5min with 1ml PBT.
10. Rinse with 1ml PBS.
11. Resuspend in 500ul 70% glycerol/PBS.

Store stained embryos in a cardboard freezer box at room temp or 4°C. Protect from light! Some antibody/fluorophore combinations will remain discernible for a year or more; others will fade in a few months. Best practice is to dissect/score/image fluorescently stained embryos as soon as possible after staining.

PBT (1xPBS with 0.1% Triton X-100) recipe (store at room temp):

100 ml 10X PBS
5 ml 20% Triton X-100
H_2O to 1 L

PBT + 5% NGS recipe (store at 4°C):

95 ml PBT
5 ml Normal Goat Serum (NGS)

Antibody working dilutions:

<u>1° antibodies</u>

Rabbit anti-TcFasII	1:1000
Goat anti-HRP FITC	1:100

<u>Fluorescent 2° antibodies</u>

Goat anti-rabbit Cy3	1:1000

Equipment and reagents:

Brass test sieves from Fisher:

850µm sieve	Fisher cat # 04-881M
300µm sieve	Fisher cat # 04-881T
250µm	Fisher cat # 04-881U
receiving pan	Fisher cat # 04-886B

Nitex mesh (120µm pore size) Genesee Scientific cat # 57-102

- To prepare egg collection baskets: cut off a 50 ml conical tube approx. 1” from the cap, briefly heat the rim of the tube to melt the plastic, press the melted rim onto the mesh until cool, then trim off the excess mesh. Make sure that the mesh is securely attached to the rim all the way around, so that no embryos are lost during washing.

Unbleached white flour	from the supermarket; we use Gold Medal unbleached all purpose flour
Active dry yeast	VWR cat # IC10140005
Glass scintillation vials with caps	VWR cat # 66022-128
37% Formaldehyde	Fisher cat # F79-500
Heptane	VWR cat # EM-HX0080-6
Methanol	Fisher cat # A412-4
10x PBS	Fisher cat # BP3994
Triton X-100	VWR cat # EM-9410
Normal goat serum (NGS)	Invitrogen cat # 16210064
Glycerol, molecular biology grade	Fisher cat # BP2291

Goat anti-HRP FITC	Jackson Immunoresearch cat # 123-095-021
Goat anti-rabbit Cy3	Jackson Immunoresearch cat # 111-165-003