Embryo Immunostaining: This protocol is based closely on an earlier immunostaining protocol developed for *Hybsibius dujardini* (Gabriel and Goldstein 2007).

Each wash step is followed by centrifugation.

All centrifugation steps are at 18,500 rcf for 3 min.

Note that steps 1-16 are identical to steps 1-16 of the *in situ* hybridization protocol, except 0.5X PBT is used in this protocol rather than 0.5X PBtween.

Day 1. (~7.5 hrs)

A. *Collecting* Hypsibius dujardini *Embryos (~1 hrs/200 embryos)*

1.) Collect embryo-filled exuviae in a 60mm dish (either Pyrex or polystyrene) filled with Poland Springs (or another brand of spring water suitable for rearing tardigades). In order to expedite the collection of embryos, preference should be given to exuviae with the most embryos. We normally find exuvia with between 3 and 12 embryos encased.

2.) With a 25-gauge needle (BD 305125) attached to a syringe, slice through the maternal exuvia near its middle, while being careful not to damage the embryos inside. Most embryos will simply spill out. Gently push down on the exuvia to force any remaining embryos out (See video 1).

3.) Transfer embryos to an autoclaved 1.5 ml low retention tube (Fisherbrand 02-681-320) filled with 1 ml of 0.5X PBT.

B. *Primary Permeabilization (~1.5 hrs)*

4.) Centrifuge the 1.5 ml tube containing the embryos at 18,500 rcf for 3 minutes.

5.) Remove supernatant using a 25-gauge needle (BD 305125) attached to a syringe, leaving embryos suspended in approximately 20 ul of 0.5X PBT. Keep the needle tip close to the surface of the water in the tube in order to avoid sucking up embryos. We have found that by using a needle and syringe, very few embryos are lost during wash steps.

6.) Add 20 ul of Chymotrypsin/Chitinase solution and let stand for 1 hr at RT.

7.) Wash 3X-5 min. in 0.5X PBT, centrifuging after each wash and following the procedure from step 5 to remove supernatant, leaving ~50 ul of 0.5X PBT in the tube after removal of supernatant.

C. ***Methanol Dehydration (~1 hr)***

8.) Centrifuge and remove supernatant, leaving the embryos undisturbed in approximately 20 ul of 0.5X PBT in the bottom of the tube.

9.) Add 1 ml of -20° C 100% MeOH. Manually rock the tube gently a few times to ensure that the embryos are not clumped. Set the tube on its side at 4°C for at least 20 minutes.

10.) Wash 5 min. each in 90%, 70%, and 50% MeOH in 0.5X PBT at RT, centrifuging and removing the supernatant as in step 5. Manually rock the tube gently a few times between washes.

D. ***Paraformaldehyde Fixation (~1 hr)***

11.) Remove supernatant leaving ~20 ul of 50% MeOH. Fix at RT for 10 min. in 1 ml of 4% Paraformaldyhyde [750 ul 0.5X PBT, 250 ul 16% PFA (Electron Microscopy Sciences 15700)].

12.) Continue Paraformaldyhyde fixation on ice for 15 min.

13.) Wash 5X-5 min. in 0.5X PBT, centrifuging after each wash.

E. ***Secondary Permeabilization (1 hr/ 30 embryos)***

14.) After the final wash, centrifuge the embryos at 18,500 rcf for 3 min. Pull the embryos out of the 1.5 ml tube using an autoclaved 9" glass Pasteur pipette (Fisherbrand 13-678-20C). So as to not lose embryos in the pipette, be careful to not suck embryos up past the pipette's narrower tip. Expel the embryos into a 60 mm dish filled halfway with 0.5X PBT.

15.) With the sharp edge of a 25-gauge needle (BD 305125) attached to a syringe, gently scratch at the outer most surface of the embryo. When the eggshell has been penetrated, a space between the embryo and eggshell will become apparent as buffer flows in (See video 2). At this point, stop so as to prevent damage. Usually, the embryo will slowly fall out of the eggshell, but as long as the eggshell has been penetrated, the embryo will be permeable to antibodies.

16.) Recollect embryos in a 1.5 ml tube of 0.5X PBT.

F. ***Blocking (~1 hr)***

17.) Centrifuge and remove supernatant, leaving ~20 ul of 0.5X PBT.

18.) Add 1 ml of blocking buffer and leave at RT for 1 hr.

G. ***Primary Antibody*** (The volumes used in steps18 and 19 are antibody specific.)

19.) Centrifuge and remove supernatant, leaving an appropriate volume for diluting the antibody. For experiments including either a cross-reactive mouse anti-histone monoclonal primary antibody (Chemicon, MAB052) or a cross-reactive mouse anti-RNA Polymerase II monoclonal antibody (Covance, MMS-1), we left a volume of 500 ul of blocking buffer.

20.) Add appropriate volume of primary antibody and incubate at 4°C overnight. [For both of the primary antibodies mentioned above, we added 1 ul of antibody to the 500 ul of blocking buffer in the 1.5 ml tube with embryos.] Rock the tubes gently a few times to ensure that the primary antibody and embryos are adequately mixed, and leave the tube on its side, so that embryos do not become clumped.

Day 2 (2 hrs)

H. ***Secondary Antibody (2 hrs)***

21.) Wash 3 X 5 min, then 2 X 20 min in 0.5X PBT, centrifuging and removing supernatant as above after each step.

22.) Add 1 ul of fluorescently-labeled secondary antibody to embryos in ~200 ul of 0.5X PBT and leave in the dark for 1 hour at RT. Make sure to rock gently a few times to ensure that the primary antibody and embryos are adequately mixed, and leave the tube on its side, so that embryos do not become clumped.

23.) Wash 3 X 5 min in 0.5X PBT and mount on microscope slides using Fluoromount-G (SouthernBiotech 0100-01) or another appropriate mounting medium.

Reagents

10X PBS (store at room temp)

ddH_2O	750 ml
NaH_2PO_4	2.56 g
$Na_2H\ PO_4$	11.94 g
NaCl	102.2 g

Adjust pH to 7.4 with HCl, fill to 1 L with ddH_2O, and autoclave.

Final volume	**1 L**

0.5X PBT (store at room temp)

10X PBS	2.5 ml
10% Triton X-100	500 ul
ddH_2O	47 ml
Final volume	**50 ml**

Chymotrypsin/Chitinase solution (store at 4°C in the dark)

Chitinase (Sigma-Aldrich C6137)	50 mg
Chymotrypsin (Sigma-Aldrich C4129)	15 mg
50 mM potassium phospate buffer (pH 6.0)	1 ml
Final volume	**~1 ml**

Blocking buffer (store at 4°C)

Bovine Serum Albumin Fraction V, protease-free (Roche 03117332001)	5 g
0.5X PBT	50 ml
Final volume	**~50 ml**

Work Cited

Gabriel WN, Goldstein B. 2007. *Dev Genes Evol* **217**(6):421-33.

Video 1 Caption: This recording shows the dissection of a maternal exuvia containing four embryos. At 3 seconds, the exuvia is cut open, using a slicing motion and the sharp edge of a 25-gauge needle (BD 305125) attached to a syringe. The middle region of the exuvia, between the 2nd and 3rd embryos, was targeted. At 4 seconds, the first embryo spills out of the exuvia. By gently pushing against the other embryos, they are also forced out of the opening in the exuvia.

Video 2 Caption: This recording shows an embryo being dissected out of its eggshell. Using the sharp end of a 25-gauge needle (BD 305125), the surface of the

eggshell was carefully targeted with a scratching motion. At 14 seconds, the eggshell is penetrated. At 15 seconds the eggshell becomes apparent as buffer rushes in and it expands away from the embryo. At this point the embryo begins to fall out of the eggshell. At 20 seconds the eggshell is completely pulled off the embryo.