Embryo *In-situ* Hybridization: This protocol is based closely on an immunostaining protocol for *Hypsibius dujardini* (Gabriel and Goldstein 2007) and available *in-situ* protocols for both insects (Tomoysu et al. 2009) and *Caenorhabditis elegans* (Seydoux and Fire 1995).

Each wash step is followed by centrifugation.

All centrifugation steps are at 18,500 rcf.

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

A. *In-situ* probe synthesis: Genes of interest were amplified from *H. dujardini* embryonic cDNA and cloned into the pCR 4-TOPO vector (Invitrogen K4575-01), following the manufacturers protocol. M13 forward and reverse vector primers were used to amplify the insert. Amplified fragments included a flanking T3 site at one end and a T7 promoter site at the other end from the vector. T7 or T3 RNA polymerase and DIG RNA labeling mix (Roche 11277073910) were used to produce either a sense or antisense DIG labeled probe.

Day 1 (~8 hrs)

B. *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 PBtw.

C. *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 PBtw, centrifuging after each wash and following the procedure from step 5 to remove supernatant, leaving ~50 ul of 0.5X PBtw in the tube after removal of supernatant.

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

8.) Centrifuge and remove supernatant, leaving the embryos undisturbed in approximately 20 ul of 0.5X PBtw 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 PBtw at RT, centrifuging and removing the supernatant as in step 5. Manually rock the tube gently a few times between washes.

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

11.) Remove supernatant leaving ~20 ul of 50% MeOH. Fix for at RT for 10 min. in 1 ml of 4% Paraformaldyhyde [750 ul 0.5X PBTw, 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 PBtw.

F. ***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 beveled tip. Expel the embryos into a 60 mm dish filled halfway with 0.5X PBtw.

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 *in-situ* probes and antibodies.

16.) Recollect embryos in a 1.5 ml tube in ~1ml of 0.5X PBtw.

G. *Prehybridization (~2 hrs)*

17.) Centrifuge, remove ~half the supernatant, replace it with Hybe buffer (resulting in 1:1 PBTw:Hybe) and rock gently on Nutator (Clay Adams Model # 421105) or other appropriate device for 20 min at RT.

18.) Centrifuge and replace with Hybe and rock gently on Nutator or other appropriate device for 20 min at RT.

19.) Replace with Hybe heated to 55°C and leave in water bath at 55°C for 1 hr.

H. *Hybridization*

19.) Incubate DIG-labeled riboprobe in 200 ul of Hybe buffer (final concentration ~ 0.5 ug/ml DIG-labeled riboprobe) for 5 min at 100°C. Different riboprobes may have different optimal concentrations. Reduce the riboprobe concentration if strong background staining is recovered.

20.) Incubate the riboprobe solution on ice for 2 min

21.) Centrifuge the 1.5 ml tube holding the embryos, remove most of the supernatant and add the Riboprobe solution.

22.) Hybridize overnight at 55°C in a waterbath. Different riboprobes may have different optimum hybridization temperatures

Day 2 (3 hrs)

I. *Posthybridization Washes*

23.) Wash in Hybe for 20 min at 55°C, centrifuge and remove supernatant.

24.) Wash in 4:1 Hybe:0.5X PBTw for 20 min at 55°C, centrifuge and remove supernatant.

25.) Wash in 3:2 Hybe:0.5X PBTw for 20 min at 55°C, centrifuge and remove supernatant.

26.) Wash in 2:3 Hybe:0.5X PBTw for 20 min at 55°C, centrifuge and remove supernatant.

27.) Wash in 1:4 Hybe:0.5X PBTw for 20 min at 55°C, centrifuge and remove supernatant.

28.) Wash 2 X 20 min in 0.5X PBTw at 55°C, centrifuge and remove supernatant

29.) Wash 20 min in 0.5X PBT, centrifuge and remove supernatant

30.) Incubate in 1 ml blocking solution for 1hr at RT.

J. *2° Antibody and probe detection*

29.) Add 0.5 ul of anti-DIG antibody (Roche) and leave overnight at 4°C.

Day 3

30.) Wash embryos 3X-20 min in PBT, centrifuge and remove supernatant.

31.) Wash embryos 3X-5 min in developing solution buffer, centrifuging after each wash before removing supernatant.

32.) Remove as much supernatent as possible and add 500 ul of color reaction substrate to each tube and monitor until signal develops. For the probe we were testing (1020 nt/*Distal-less*), signal developed rapidly, and so we monitored the embryos every 10 min.

34.) Wash 3X in PBTw to stop reaction, centrifuging after each wash before removing supernatant, and mount on microscope slides using Fluoromount-G (SouthernBiotech 0100-01) or another appropriate mounting medium.

Reagents

DEPC H_2O

DEPC (MP Biomedicals, Inc. 150902)	0.5 ml
ddH_2O	500 ml

Stir overnight in the dark and autoclave.

Final volume	**~500 ml**

10X PBS (store at room temp)

DEPC H_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**

20X SSC (pH 6.0)

ddH_2O	400 ml
NaCL	87.65 g
Sodium Citrate	44.1 g

Adjust pH to 7.0 with 10N NaOH, fill to 500 ml with ddH_2O, and autoclave.

Final volume	**500 ml**

0.5X PBTw

10X PBS	2.5 ml
10% Tween20	500 ul
DEPC H_2O	47 ml
Final volume	**50 ml**

0.5X PBT

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**

50X Denhardt's (store at -20°C)

Ficoll (Fisher Scientific BP525-5)	5 g
polyvinylpyrrolidone (Fisher Scientific BP431-100)	5 g
Bovine Serum Albumin Fraction V, protease-free (Roche 03117332001)	5 g
ddH_2O	to 500 ml
Final volume	**500 ml**

Hybe buffer (store at -20°C)

100% Formamide	25 ml
20X SSC (pH 6.0)	12.5 ml
100 mg/ml heparin	50 ul
10% Tween20	500 ul
10 mg/ml yeast RNA	500 ul
10% CHAPS	500 ul
50X Denhardt's	1 ml
DEPC H_2O	9.95 ml
Final volume	**50 ml**

Blocking buffer (store at 4°C)

Bovine Serum Albumin	1 g
0.5X PBT	50 ml
Final volume	**~50 ml**

Developing solution buffer (make fresh)

1 M Tris HCl (pH 9.5)	1 ml
5 M NaCl	200 ul
1 M $MgCl_2$	500 ul
10% Tween20	100 ul
ddH_2O	8.2 ml
Final volume	**10 ml**

Color reaction substrate

Developing solution buffer	1.5 ml
50 mg/ml BCIP	5.25 ul
50 mg/ml NBT	10.125 ul
Final volume	**~1.5 ml**

Works Cited

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

Seydoux G, Fire A. 1995. *Methods Cell Biol* **48**:323-37.

Tomoyasu Y, Arakane Y, Kramer KJ, Denell RE. 2009. *Curr Biol* **19**(24):2057-65.

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, right between the 2nd and 3rd embryo 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.