Protocol

Live Imaging of Tardigrade Embryonic Development by Differential Interference Contrast Microscopy

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The tardigrade *Hypsibius exemplaris* was chosen as a model system in part because embryos and animals are optically clear at all stages, facilitating the viewing and filming of internal processes. Multiplane video recordings under differential interference contrast (DIC) microscopy have allowed early embryonic cell lineages to be reconstructed through seven rounds of division and have revealed invariant patterns of asymmetric cell divisions, nuclear migrations, and cell migrations. Here, we present a protocol for filming embryonic development of *H. exemplaris* by DIC microscopy.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Fed cultures of tardigrades (*Hypsibius exemplaris*)

See Protocol: ***Laboratory Culture of*** **Hypsibius exemplaris** *(McNuff 2018).*

Spring water

Use commercial bottled spring water found in grocery stores. Do not use tap water; the chlorine and/or chloramine common in tap water is harmful to many microscopic animals.

Valap sealant <R>

Equipment

Aspirator tube assembly for microcapillary tubes (Sigma-Aldrich A5177)
Compound microscope equipped for DIC optics and videorecording (see Step 9)
Glass beads (31-µm diameter; Whitehouse Scientific MS0031)
Glass microcapillary tube (e.g., Sigma-Aldrich P0674)

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From the Emerging Model Organisms collection.

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Glass Petri dishes (3.5-cm diameter or larger)

Plastic Petri dishes can be used, but in Step 3, the exuviae tend to adhere to the plastic.

Glass slides and coverslips (18 × 18 mm, with a thickness of 1 or 1.5)
Heat block (at 68°C) capable of holding test tubes
Kimwipes
Paintbrush
Paper clip or binder clip (see Step 1)
Pipette puller or flame
Stereomicroscope
Syringe needles (25G × 5/8″; e.g., BD PrecisionGlide 305122)
Test tubes

METHOD

Collect Embryos

H. exemplaris *mothers lay embryos into their own cuticles, and the adults molt soon thereafter, leaving embryos behind inside the exuvium (shed cuticle). The optically clear exuviae holding nearly synchronous embryos facilitate videorecording multiple embryos simultaneously.*

1. Prepare an aspirator pipette by pulling a glass microcapillary tube over a flame or in a needle puller, breaking the tapered end off to leave an opening that is ~0.5 mm wide, and inserting the blunt end of the tube into an aspirator tube assembly. Close the end of aspirator assembly's tubing furthest from the pipette by folding it over and clipping it with a paper clip or binder clip. Pinch the tubing between a finger and a thumb to control aspiration.

   This aspirator pipette can be used to pick up and deposit individual animals or exuviae. Aspiration is controlled by pinching the tubing between a finger and thumb. Aspirator pipets were traditionally controlled by mouth pipetting, but this method is disallowed by many modern safety protocols.

2. Examine fed cultures for embryos at the stages of interest under a stereomicroscope.

   - To collect 1- to 4-cell stage embryos, identify tardigrades that are sharing their cuticles with embryos.

   The mothers generally exit the cuticle within a few hours of laying.

   - To collect later-stage embryos, identify exuviae with only embryos inside.

   In these cases, the age of the embryos will not be known.

3. Fill a glass Petri dish halfway with spring water. Use the aspirator pipette to transfer the exuviae identified in Step 2 (either with embryos and mothers or with embryos alone) to the glass Petri dish with spring water. Minimize the transfer of algae. Remove algal cells from the Petri dish using the aspirator pipette if necessary.

   If exuviae can be collected cleanly at this stage, they can be transferred directly to a slide (see Step 7), foregoing this step.

4. (Optional) Separate the embryos from the exuviae.

   - For very early-stage embryos, manually push mothers out of their cuticles with a syringe needle. Be careful not to squish the embryos.
   - For early-stage embryos, wait until the mothers exit their cuticles.
   - For later stages, cover the Petri dish and leave the exuviae for the desired amount of time.

   Although embryos can be separated from exuviae before mounting onto slides, this can make it more difficult to mount and image multiple embryos at once, as there is no longer an optically clear exuvium holding the embryos in a defined area. We have not noticed a difference in image quality when filming embryos with or without exuviae.

Mount Embryos on Slides

We use microscopic glass beads as precisely sized spacers to support the coverslip near the height of the embryos, and we seal the coverslip with Valap, which is gas-permeable, to prevent evaporation. We keep Valap warmed at 68°C in a test tube containing a paintbrush at all times.

5. Place a small drop of spring water (about 25 µL) on the center of a glass slide.
6. Dip a clean micropipette tip into a vial of glass beads, tap the tip against the sides of the vial to remove excess beads, and then transfer the beads onto the slide by dipping the micropipette tip coated with beads into the droplet of spring water on the slide.
7. Use an aspirator pipette to transfer an embryo-containing exuvium (or multiple exuviae) to the center of the droplet, and allow the embryo(s) to settle.

   The tip of the aspirator pipette can be used to further move the exuvium to the center.
8. Carefully place a coverslip on top, and remove excess water by lightly touching the edge of the coverslip with a Kimwipe. Seal the coverslip with Valap by painting each side of the coverslip with a single stroke, adding extra Valap to fill holes if necessary.

Perform Imaging

9. Place slide on compound microscope under DIC optics with Köhler illumination (with the embryo(s) and edges of the field diaphragm simultaneously in focus). Use a 60×–100× DIC objective, and ensure that condenser setting matches the DIC setting of lens. Rotate the polarizer until the image appears as desired. Film at room temperature for the desired length of time.

   Embryos will develop through hatching at 4 to 4.5 d.

   See Troubleshooting.

TROUBLESHOOTING

Problem (Step 9): Embryos cannot be found.

Solution: The embryos may be stuck under the Valap; alternatively, the field of view on the compound microscope may be too small to easily find the embryos. To avoid these issues, place the embryo(s) in the center of the area where the coverslip will be placed in Step 7, and after Step 8, find the embryo(s) under a stereomicroscope and use a marker to draw a circle around them on the slide (not on the coverslip, as objective oil may dissolve the ink). On the compound microscope, find the embryos under low power before switching to the 60×–100× lens.

Problem (Step 9): Embryos do not develop.

Solution: The embryos may die if they are overheated or if the slide dries out. Embryos can become overheated if the slide is left on a hot surface while the Valap is applied in Step 8, if the microscope is too warm in Step 9, or if the light overheats the slide in Step 9. To minimize heat resulting from lighting the embryos during filming, shutter the light source and close down the microscope's field diaphragm to only just larger than necessary to light the embryo(s) being filmed. To prevent slides from drying out, check that the Valap seal is complete after Step 8 by examining the slide under a stereomicroscope, and then fill in any holes left in the seal.

Problem (Step 9): Embryos move as the objective lens is lowered and during filming.

Solution: Consider the following.

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- The prep may be too thick. Use slightly smaller glass beads, place less water on the slide in Steps 5 and 7, and wick away excess water.
- The embryos may be mounted too close to the edge of the coverslip. If the objective lens comes into contact with the Valap that lines the edges of the coverslip while imaging, it may generate an uneven downward force on the coverslip that causes the embryos to be displaced. Prevent this by centering the embryos in the droplet of water in Step 7, and take care to minimize the thickness of Valap added in Step 8.
- The slide may slowly dry out during filming. Check under a dissecting microscope that the Valap seal is complete after Step 8, as described above.

RELATED INFORMATION

The suitability of tardigrades for imaging is one of the reasons they were chosen as a model system for studying development and evolution. For additional background on tardigrades as model organisms, see Gabriel et al. (2007) and Introduction: The Emergence of the Tardigrade *Hypsibius exemplaris* as a Model System (Goldstein 2018).

RECIPE

Valap Sealant

Add equal weight of petroleum jelly (e.g., Vaseline), lanolin, and paraffin together in a 1-L beaker. (The preparation of a few hundred milliliters of this mixture should be a lifetime supply.) Heat the mixture at a low setting on a hot plate. Stir occasionally until thoroughly blended. Take extreme care not to overheat this mixture. Warm it just enough to make the components liquefy. The final product should be golden yellow, the color of corn or canola oil. If it is dark brown, it was heated too much. In that case, start again. Aliquot the final mixture into several small screw-capped jars (~50-mL capacity). Store at room temperature. Keep a small amount in a 25-mL beaker, and warm at a low setting on a hot plate before use.

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