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Protocol

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INTRODUCTION

The great diversity of arthropod body plans, together with our detailed understanding of fruit fly development, makes arthropods a premier taxon for examining the evolutionary diversification of developmental patterns and hence the diversity of extant life. Crustaceans, in particular, show a remarkable range of morphologies and provide a useful outgroup to the insects. The amphipod crustacean *Parhyale hawaiensis* is becoming established as a model organism for developmental studies within the arthropods. This protocol describes the dissection and fixation of *P. hawaiensis* embryos. Embryonic tissue fixed in the following manner is suitable for in situ hybridization experiments to study mRNA expression or for immunocytochemistry to study protein localization.

RELATED INFORMATION

The fixed embryos prepared in this protocol can be used to study protein localization as described in **Antibody Staining of *Parhyale hawaiensis* Embryos** (Rehm et al. 2009a) or for in situ hybridization to probe mRNA expression, as in **In Situ Hybridization of Labeled RNA Probes to Fixed *Parhyale hawaiensis* Embryos** (Rehm et al. 2009b). Cell-lineage analysis can be performed following injection of *P. hawaiensis* embryos with fluorescent tracers, which is detailed in **Injection of *Parhyale hawaiensis* Blastomeres with Fluorescently Labeled Tracers** (Rehm et al. 2009c). An introduction to *P. hawaiensis* that discusses husbandry, the developmental stages of this crustacean, and its utility as a model organism for studies of arthropod development and evolution is presented in **The Crustacean *Parhyale hawaiensis*: A New Model for Arthropod Development** (Rehm et al. 2009d).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

Clove oil (Oshadhi)
<**!**>Formaldehyde (37%; Fisher)
<**!**>Methanol (100%, 90%, 70%, and 50%; all dilutions in PT)
<**!**>NaOH (1 M)

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P. hawaiensis (gravid females)
<R>PT
<R>Seawater (filter-sterilized)

Equipment

Alligator clamp leads (two) that can be plugged into the transformer
Autotransformer (variable type 3PN1010, 0-120 V, 10A; Staco Energy Products Co.)
Beaker (50 mL)
Bunsen burner
Centrifuge tubes (50 mL; BD Biosciences)
Forceps
Humidity chamber

This chamber is a small, lidded plastic box either lined with moistened paper towels or containing an inverted pipette tip holder and filled with 2 mm of water.

Incubator pre-set to 26°C
Medicine cups
Microcentrifuge tubes
Microscope (dissection)
Needle (26G1/2; BD Biosciences)
Pasteur pipettes (5.75 in.; VWR)
Petri dish (100 × 15 mm) coated with Sylgard 184

Prepare a dissection dish by coating a plastic Petri dish with 2-3 mm of Sylgard 184 following instructions for the Silicone Elastomer Kit (World Precision Instruments).

Pyrex spot plate (three or nine well)
Syringe (1-mL tuberculin slip tip; BD Biosciences)
Timer (optional; see Step 18)
Tissue culture dishes (35 × 10 mm; Falcon)
Transfer pipettes (Samco Scientific Corp.)

Clip the tip of a plastic transfer pipette for use in Step 13.

Tube rack for microcentrifuge tubes
Tungsten wire (0.005-in. diameter; Ted Pella)

METHOD

Making Dissection Tools

Pasteur Pipettes

Drawn and blunted Pasteur pipettes are used to extract embryos from brood pouches of gravid female P. hawaiensis. *Wear eye protection to protect against small pieces of flying glass when breaking drawn pipettes.*

1. Heat a glass Pasteur pipette, ~1 in. from the end, over a Bunsen burner flame until the glass softens. Remove it from the heat and pull gently on the end with forceps, drawing the glass to a finer point.
2. Break the drawn end carefully by flexing it with forceps. Discard the small piece.
3. Heat the newly formed end so that it rounds up.

   The objective is to obtain a small and rounded end that fits easily into the brood pouch and does no harm to the female.

Tungsten Needles

Fine tungsten needles are used for dissecting P. hawaiensis *embryos away from their surrounding membranes. They are honed to a fine point electrochemically. Wear protective eyewear and gloves while sharpening the needles. Care should be taken to avoid electric shock.*

4. Thread a short piece of 0.005-in. diameter tungsten wire through a 26G1/2 needle. Crook the back end of the wire so that it stays attached to the needle. Attach the needle to a 1-mL syringe.
5. Set up a 50-mL beaker with 1 M NaOH.
6. Attach an alligator clamp from one lead to the beaker lip, ensuring that the lead makes contact with the NaOH solution. Attach the clamp from the other lead to the base of the needle.

   Take care to avoid touching the two leads together because this may cause a shock and damage the transformer.
7. Make sure that the variable autotransformer is turned off. Plug both leads into the transformer. Adjust the output knob to no more than 5% of maximum voltage output (~6 V).
8. Turn on the transformer while holding the needle with its attached lead by the plastic barrel of the syringe.
9. Dip the tip of the needle into the NaOH solution, which completes an electrical circuit.

   Small bubbles at the needle's point of contact with the solution will indicate that the tungsten is dissolving. Typically, <1 min of steady up-and-down motion produces a sufficiently sharp needle tip.
10. If unsure of the proper voltage setting, attach the leads and dip the needle into the NaOH solution with the transformer turned off. While holding the plastic syringe, turn on the transformer with the output voltage knob positioned at zero. Slowly increase the transformer's voltage until bubbles begin to form.

Extracting *P. hawaiensis* Embryos from Gravid Females

Gravid females are anesthetized, and the embryos are scooped out of the brood pouch.

11. Add 10 µL of clove oil to 50 mL of filtered seawater in a centrifuge tube. Shake vigorously for 5 sec.
12. Collect gravid *P. hawaiensis* in a medicine cup. Remove as much seawater as possible before replacing it with the clove oil/seawater mixture.

    The animals should be fully asleep within 10-15 min.

    Typically, up to 20 gravid females can be anesthetized in 10 mL of the clove oil/seawater mixture.

    Do not leave the animals in the clove oil/seawater mixture for longer than 1 h.
13. Transfer the anesthetized amphipods in a drop of clove oil/seawater mixture to a plastic Petri dish that has been coated with Sylgard. Females may be moved using forceps or a plastic transfer pipette whose tip has been clipped to allow the animals to pass freely.
14. Under a dissection microscope, expose the brood pouch by using forceps to gently hold the animal on its back. Gently plunge the forceps tips into the Sylgard surface of the dish, straddling the gravid female, to ensure that it rests on its back and the brood pouch remains exposed. Extract the embryos by sweeping the blunted end of a drawn Pasteur pipette through the brood pouch in a posterior-to-anterior direction.

    One-cell embryos recently deposited into the brood pouch are particularly soft and difficult to remove without damage. The one-cell stage lasts for ~4 h at 26°C. It is best to wait 1 h following deposition before extracting one-cell embryos from the brood pouch.
15. Wash the embryos with three or four changes of sterile seawater in medicine cups. Store the embryos in 35- × 10-mm tissue culture dishes in sterile seawater until ready for use. Keep in small humidity chambers to avoid evaporation. Periodically change the seawater to prevent fungal infections.

    At 26°C, embryos will develop in accord with the established staging system (Browne et al. 2005). Embryos incubated at 18°C will develop significantly more slowly, but otherwise normally.
16. Revive the females by transferring them to a medicine cup of seawater. Ensure that they are fully awake before returning them to tanks with other amphipods.

Dissection and Fixation of *P. hawaiensis* Embryos

Embryos are hand-dissected away from membranes in a seawater/formaldehyde fix.

17. Prepare fixative of 9 parts seawater to 1 part 37% formaldehyde. Place a few embryos (start with two or three and increase with experience) in one well of a three- or nine-well Pyrex spot plate containing the fixative.

    Glass pipettes can be used to move embryos from one well to another, but designate different pipettes for the different solutions so that fixative is not inadvertently introduced into a dish of living embryos.

    See Troubleshooting.

18. Poke a hole in the eggshell with a dissecting needle, choosing a region of the egg with little embryonic tissue. Fixative will begin to enter the egg, so start a timer if fix duration is critical (see Step 22). If necessary, stabilize the embryo with either a pair of forceps or a second dissecting needle while poking the hole.

    Very young embryos (0-18 h) are quite yolky and contain only a single membrane. It is difficult to maintain the embryo's shape when dissecting away this membrane. Begin by poking a shallow hole. It may help to keep the embryo in fix for 5 min before making the hole. Start the timer, as usual, only after *making the hole.*

    Older embryos (1-2 d) are relatively easy to dissect because they are surrounded by only a single membrane and the embryo has condensed to one side of the egg. Target the side of the egg with little embryonic tissue for the initial hole and subsequent dissection. This allows the membrane to be removed easily without damaging many embryonic cells.

19. Allow the fixative to enter the embryo for 1 min. This will assist in the subsequent dissection.

    Do not fix too long before completing dissections, however, because the membranes will begin to attach irreversibly to the embryo.

20. Remove the outer membrane by carefully reentering the initial hole with two dissecting needles and tearing a larger opening.

    This often results in loose flaps of membrane that can then be peeled away using forceps or dissecting needles. Alternatively, the outer membrane pops off the embryo as a more or less intact capsule.

21. If the inner membrane has not been removed with the outer, repeat Step 20 to remove the inner membrane.

    At various stages, the inner membrane routinely remains snugly wrapped around the embryo/yolk mass after removal of the outer eggshell. The inner membrane is visible as a slight sheen over the opaque, whitish embryo. This inner membrane is often quite challenging to remove. If left more than a few minutes in fix, the inner membrane tends to fuse to the embryo and yolk, making it difficult or impossible to remove.

    i. After 2.5-3 d, the embryo has developed an inner membrane that frequently does not come off with the outer membrane. Poke the initial hole along the dorsal midline of the embryo, which is readily identified by the small number of large cells that will give rise to the dorsal organ. Most of the embryo lies on the other, ventral side of the egg. Do not attempt to dissect too many embryos at once because the inner membrane can quickly become permanently fixed to the embryo. Quick and efficient removal of the outer membrane enables the inner membrane to be peeled away before becoming overfixed.

    ii. After 4 d, the embryo has developed appendages, making it difficult to remove the membranes without dismembering the animal. Poke a hole along the dorsal midline, just behind the embryo's head. It may help to wait 2 min before continuing to dissect the membranes. Often, both membranes will come off together.

    iii. By day 7, cuticle deposition begins to cause significant levels of background, especially where limbs emerge from the developing body. Beyond day 9, as the embryonic cuticle attains a yellowish coloration, detection of gene expression by in situ hybridization becomes impossible.

22. Remove the dissected embryos from the fixative after sufficient fixation time has elapsed. Collect the fixed and dissected embryos in a medicine cup filled with 20 mL of PT. Store them in this solution until all dissections are completed.

    If the dissected embryos are to be used for in situ hybridization, they can be fixed for 15 min or up to a few hours. However, embryos that are to be used for antibody staining must not be overfixed. Embryos dissected for antibody staining are typically exposed to fixative for no longer than 15-20 min, although these conditions may require optimization depending on the antibodies to be used. Underfixation typically causes high background, whether the embryos are used for in situ hybridization or antibody staining.

23. Rinse the embryos by gathering them from the medicine cup and transferring them into a microcentrifuge tube. Fill the tube with ~1 mL of PT. Swirl the contents by spinning the tube as it rests

in a tube rack. Allow the embryos to settle, and reduce the PT down to ~25 µL. Repeat two to three times.

Dehydration of *P. hawaiensis* Embryos

P. hawaiensis *embryos can be dehydrated and subsequently stored in 100% methanol at -20°C. Dehydrated embryos work fine for in situ hybridizations and many antibody-staining reactions. However, some antibodies do not stain tissue that has been dehydrated. In these cases, it is important to begin antibody reactions directly after dissection and fixation.*

24. Dehydrate the embryos as follows:
    i. Transfer the embryos to a microcentrifuge tube containing 1 mL of 50% methanol.
    ii. Swirl the tubes to mix, and allow them to sit for 5 min.
    iii. Carefully remove the methanol solution and replace it with 1 mL of 70% methanol.
    iv. Swirl and wait 5 min.
    v. Remove and replace the solution with 1 mL of 90% methanol. Swirl and wait 5 min.
    vi. Complete the dehydration with three quick washes of 100% methanol.
25. Store the dehydrated embryos in 100% methanol at -20°C.

TROUBLESHOOTING

Problem: Dissected embryos stick readily and permanently to the inner surfaces of glass pipettes. **[Step 17]**
Solution: Consider the following:

1. Pass yolk from the first group of dissected embryos into pipettes that will be used to move the embryos, coating them with a nonstick layer of yolk proteins.
2. Alternatively, rinse pipettes with PT before using them to move embryos.
3. It also helps to avoid drawing embryos past the neck of the pipette; embryos tend to stick more frequently to the wider part of the pipette.

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