Protocol

Isolation of *Amphimedon* Developmental Material

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INTRODUCTION

Fertilization occurs internally in *Amphimedon* and embryos are brooded in multiple chambers throughout the adult. Each chamber contains a mixture of developmental stages, from egg to late ring stages (i.e., prehatch late embryos). At the end of embryogenesis, swimming parenchymella larvae emerge from the adult. After several hours in the water column, the larvae settle and metamorphose into juvenile sponges. This protocol details how to obtain *Amphimedon* larvae and post-larvae/juveniles as well as embryos. Once isolated, these biological stages can be used for a variety of molecular and cellular analyses.

RELATED INFORMATION

For an introduction to *Amphimedon* as a model organism, see **The Demosponge *Amphimedon queenslandica*: Reconstructing the Ancestral Metazoan Genome and Deciphering the Origin of Animal Multicellularity** (Degnan et al. 2008). A protocol for **Whole-Mount In Situ Hybridization in *Amphimedon*** (Larroux et al. 2008) is also available.

MATERIALS

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

Reagents

Agarose, 2% in FSW (prepared as a thin layer in a deep Petri dish)
Seawater, 0.22-µm filtered (FSW)

Equipment

Beakers
Coverslips, plastic or glass (round 12-mm)
Field collection equipment (hammer, chisel, snorkeling gear, collection bucket/bag, 0.5- to 1-L screw-cap jars, large black tub [e.g., 32-L Nally bins], ties)
Forceps, fine (e.g., Dumont #5)
Light source (cold)

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Microscope, dissecting
Needle, teasing (curved)
Needle holder (equipped with screw chuck)
Pasteur pipettes

Use a Bunsen burner to reduce the diameter of the Pasteur pipette until it approximates the size of an embryo.

Petri dishes, plastic (including deep Petri dishes 90 × 25 mm; e.g., Labserv LBS61014)
Pins, stainless steel
Pipette, mouth (optional; see Step 16)
Plates, cell culture (six- and 24-well)
Scalpel and blades
Sharpening stone
Sucker cup

Drill two holes in the lid of a 250-mL plastic screw-top jar. Insert two lengths of 5-mm plastic tubing through the holes.

Thermometer

METHOD

Stages of the A. queenslandica *life cycle are shown in* Figure 1.

Larvae and Juveniles

1. Snorkel at low tide to locate adult sponges. Chisel away the rock around the sponge to collect adults on substrate.

   Avoid squeezing or touching adult tissue or exposing it to air. Although Amphimedon *is reproductive year-round, developmental materials are more readily available in the summer. After a strong tropical storm, brood chambers can be empty for a few days.*

2. Place sponges in a black tub filled with seawater. Transfer adult sponges gently and rapidly to minimize contact with air. Leave the tub in full sunlight, uncovered, and protected from the wind.

3. Monitor the temperature of the seawater. Do not allow it to increase more than 3°C-4°C above the ambient water temperature.

   This treatment induces larval release. The white larvae, clearly visible against the black background, will initially swim upward and tend to cluster in the corners of the tub.

   See Troubleshooting.

4. Check for larvae every 30 min (up to a maximum of 3-4 h).

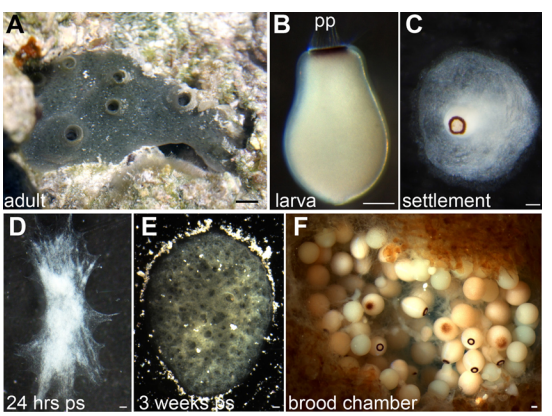

FIGURE 1. Stages of the *A. queenslandica* life cycle. (*A*) Adult animal; (*B*) swimming larva ([*pp*] posterior pole); (*C*) larva undergoing settlement with anterior part flattened on substrate; (*D*) post-larva 24 h post-settlement (*ps*); (*E*) 3-wk-old juvenile; (*F*) sliced brood chamber showing developing embryos at different stages. Scale bar, (*A*) 1 cm; (*B-F*) 100 µm. (Modified from Adamska et al. 2007).

5. Use a sucker cup to collect larvae:
    i. Place one tube into your mouth and the other into the seawater.
    ii. Use short bursts of suction to collect larvae into the jar.
    iii. Transfer the seawater with the larvae to screw-cap jars.
    iv. Store jars in a cool, shaded location.
6. Gradually lower the temperature in the black tub to acclimatize the adult sponges back to ambient water temperature.
7. Return adult sponges to where they were found. Secure to rocks with ties if necessary.
8. Use a Pasteur pipette to transfer larvae (with as little seawater as possible) into a beaker containing fresh FSW.

    These can then be maintained in the laboratory for days with regular changes of FSW (e.g., every 6-10 h).
9. Transfer larvae into a beaker containing fresh FSW.

    This ensures that the seawater carried over from the initial larval collection is diluted, minimizing the risks of contamination and infection.
10. Transfer ~40 larvae into a deep plastic Petri dish with 100 mL of FSW. Allow the larvae to settle undisturbed in the dark.

    It is important not to overcrowd the dishes because this increases the rate of fusion between settling larvae. Settlement and metamorphosis in FSW occur 4-72 h after release.

    See Troubleshooting.
11. Within 6 h after settlement, use a curved teasing needle to gently detach newly settled larvae. Transfer to small round coverslips placed in wells or dishes submerged in FSW. Use 24-well plates if subsequently conducting whole-mount in situ hybridization (see **Whole-Mount In Situ Hybridization in *Amphimedon*** [Larroux et al. 2008]).

    Post-larvae reattach rapidly to the coverslips and proceed with development.
12. Raise post-larvae/juveniles to the age of interest. Change the FSW regularly, and scan the dishes for protist outbreaks.

    Osculae and choanocyte tracts become apparent 3 d after metamorphosis.

    See Troubleshooting.

Embryos

Keep the sponge, slices, and embryos submerged in FSW throughout the procedure.

13. Snorkel at low tide to locate adult sponges. Chisel away the rock around the sponge to collect adults on substrate. Carefully "peel" an adult sponge away from its substrate. Gently remove debris from the sponge.

    Avoid squeezing or touching adult tissue or exposing it to air. Brood chambers are often located at the base of the sponge, and squeezing can damage embryos.

    Although Amphimedon *is reproductive year-round, developmental materials are more readily available in the summer. After a strong tropical storm, brood chambers can be empty for a few days.*
14. Use a scalpel to slice the sponge into 3-mm sections. Inspect each section for the presence of brood chambers (Fig. 2).

    Glass spicules embedded in the adult can readily dull scalpel blades. Replace these regularly because cutting with dull blades crushes the embryos.

    See Troubleshooting.
15. Transfer a slice with chambers to a deep Petri dish containing a thin base of 2% agarose in FSW. Use pins to secure the slice to the agarose.
16. Under a dissecting microscope, using a cold light source, carefully use fine forceps and/or sharpened pins to release embryos from the white maternal tissue. Use a mouth or Pasteur pipette to gently blow on and/or extract embryos.

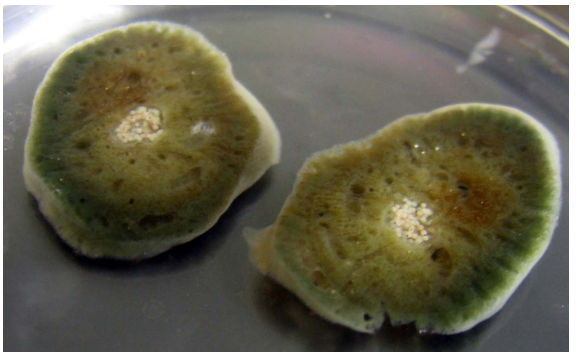

FIGURE 2. Detecting a brood chamber (white embryos) by making thin slices through an adult.

17. Transfer isolated embryos to a separate dish of FSW. Ensure that there is space around each embryo to prevent them from fusing together. If embryos detach from the chamber in a clump, fix the clumps within 1 h to avoid fusion.

TROUBLESHOOTING

Problem: Embryos, larvae, or post-larvae stick to the inside of Pasteur pipettes or plastic pipette tips.
Solution: Precoat tips with seawater or FSW every time a fresh pipette or tip is used. To dislodge an embryo from the pipette/tip, draw liquid and air bubbles up past the embryo or fill the pipette/tip with liquid and tap the outside.

Problem: Weather conditions are not appropriate to significantly raise the temperature of the seawater in the tub.
[Step 3]
Solution: Use an aquarium heater (100 W) to heat the water.

Problem: Larval settlement rate is low.
[Step 10]
Solution: Settlement occurs at a higher rate on certain types of plastic. Consider the following:

1. Use new deep Petri dishes or test various plastic dishes for settlement rate.
2. If desired, add small shards of coral rubble collected from the natural habitat of *Amphimedon* to induce settlement. These shards are typically a decaying calcium carbonate substratum consisting of crustose coralline algae, the boring sponge *Cliona* sp., and undescribed biofilm biota.
3. Alternatively, "age" Petri dishes in running seawater for 1-4 d. These dishes develop a natural biofilm that also can induce settlement.
4. Note that whereas induction increases settlement rates, it can also lower the chances of post-larval/juvenile survival because of the higher risk of bacterial and protozoan infections.

Problem: Settling larvae or settled post-larvae arrest development and appear to degenerate.
[Step 12]
Solution: Avoid contaminating the FSW with organisms introduced from seawater. Transfer larvae intended for settlement to fresh FSW several times with a Pasteur pipette to dilute seawater carried over from the initial collection. Check cultures regularly under a dissecting microscope for the presence of contaminants. If possible, avoid using coral rubble or biofilm to induce metamorphosis.

Problem: Brood chambers only contain late embryonic stages.
[Step 14]
Solution: This can occur when sponges are kept in an open system aquarium for more than 1 wk. Collect new sponges from the field or keep sponges in a closed system.

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