Video Article

Ablation of a Single Cell From Eight-cell Embryos of the Amphipod Crustacean *Parhyale hawaiensis*

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Abstract

The amphipod *Parhyale hawaiensis* is a small crustacean found in intertidal marine habitats worldwide. Over the past decade, *Parhyale* has emerged as a promising model organism for laboratory studies of development, providing a useful outgroup comparison to the well studied arthropod model organism *Drosophila melanogaster*. In contrast to the syncytial cleavages of *Drosophila*, the early cleavages of *Parhyale* are holoblastic. Fate mapping using tracer dyes injected into early blastomeres have shown that all three germ layers and the germ line are established by the eight-cell stage. At this stage, three blastomeres are fated to give rise to the ectoderm, three are fated to give rise to the mesoderm, and the remaining two blastomeres are the precursors of the endoderm and germ line respectively. However, blastomere ablation experiments have shown that *Parhyale* embryos also possess significant regulatory capabilities, such that the fates of blastomeres ablated at the eight-cell stage can be taken over by the descendants of some of the remaining blastomeres. Blastomere ablation has previously been described by one of two methods: injection and subsequent activation of phototoxic dyes or manual ablation. However, photoablation kills blastomeres but does not remove the dead cell body from the embryo. Complete physical removal of specific blastomeres may therefore be a preferred method of ablation for some applications. Here we present a protocol for manual removal of single blastomeres from the eight-cell stage of *Parhyale* embryos, illustrating the instruments and manual procedures necessary for complete removal of the cell body while keeping the remaining blastomeres alive and intact. This protocol can be applied to any *Parhyale* cell at the eight-cell stage, or to blastomeres of other early cleavage stages. In addition, in principle this protocol could be applicable to early cleavage stage embryos of other holoblastically cleaving marine invertebrates.

Video Link

The video component of this article can be found at https://www.jove.com/video/51073/

Introduction

The amphipod crustacean *Parhyale hawaiensis* has emerged over the last decade as a promising model organism with great potential for use in evolutionary developmental biology research[1]. Among the arthropods, most model systems are insects, and the most extensively studied of these is the fruit fly *Drosophila melanogaster*. *D. melanogaster* is a member of the insect order Diptera, and as such displays many embryological features that are derived with respect to those of basally branching insects[2]. Moreover, insects are nested within the subphylum Pancrustacea[3], meaning that insects have their closest relatives within the long-standing "natural" group called pancrustaceans, and that this group is paraphyletic. This suggests that in addition to basally branching insect models, studies of other crustaceans are required to gain a broader view of the evolutionary history of the developmental traits and molecular mechanisms that have been so well studied in *D. melanogaster*. However, very few crustaceans have been well established for experimental laboratory analysis of development. The amphipod *P. hawaiensis* is a highly tractable laboratory model system, amenable to a range of experimental techniques. Amphipods display many unique features within their parent superorder Peracarida (beach hoppers, scuds, and well shrimps), and are therefore thought to be relatively derived within this group of crustaceans. Nevertheless, the relative ease of embryological and functional genetic manipulation offered by *Parhyale* make this amphipod a valuable addition to the current inventory of model organisms.

As a laboratory animal, *P. hawaiensis* offers many advantages. Animals are tolerant to a wide range of temperatures and salinities, and survive well in large cultures of artificial seawater[1]. It is easy to distinguish between males and females based on clear morphological differences, most notably, the large, hooked, anterior trunk appendages that males use to grasp the females during mating. For embryological and developmental work, *P. hawaiensis* has several very appealing features. Embryogenesis lasts approximately 10 days and the time to sexual maturity is approximately six weeks at 28 °C (but note that *Parhyale* survives well at temperatures ranging from approximately 20-30 °C, and that detailed developmental staging information is available for embryos raised at 18 °C[4], 25 °C[4], and 26 °C[5,6]). Adults mate all year round in the laboratory, so embryos are available at any time of year. Females lay 2-20 (depending on the age of the female) fertilized eggs into a ventral brood pouch located between the first several pairs of legs (**Figures 1A** and **1B**), and it is possible to gather these embryos very early in development without killing the female or damaging the embryos (**Figure 1C**). The embryos survive in filtered artificial sea water through to hatching, can be fixed for subsequent gene expression or histological analysis[7], and a detailed staging table allows accurate identification of the progress through development[5]. Robust protocols have been used to perform gene expression analysis by *in situ* hybridization[8-15] or immunostaining[4,16,17],

functional knockdown by RNA interference[13,15] or morpholinos[12], and stable germ line transgenesis[18]. Using the transgenesis system, inducible expression[14] and enhancer trap[19] methods can also be used to investigate gene function in *P. hawaiensis*. While a publicly available genome sequence is not currently available, a transcriptome containing transcripts produced during oogenesis and embryogenesis has been *de novo* assembled and annotated[20], and deposited in a searchable database[21], facilitating gene discovery. In sum, *P. hawaiensis* is a highly tractable model organism suitable for multiple experimental and genetic approaches to understanding development.

Unlike the early syncytial cleavages of *D. melanogaster*, *P. hawaiensis* embryos cleave holoblastically following fertilization (**Figure 2A**). Lineage tracing analysis has shown that by third cleavage, each of the third cleavage blastomeres is specifically fated to give rise to one of the three germ layers or the germ line[6] (**Figure 2B**). These data, together with microarray data[22], cell lineage analyses[6,23], and blastomere isolation experiments[4] have suggested that developmental potentials are segregated to at least some third cleavage blastomeres by asymmetric inheritance of cell fate determinants. Accordingly, in blastomere ablation experiments in which the germ line precursor (termed "**g**" in the *Parhyale* cell lineage nomenclature[6]) was removed at the eight cell stage, embryos lacked germ cells at later developmental stages[4], as indicated by the absence of cells expressing the protein Vasa, which is a germ line marker in most metazoans[24]. In contrast, somatic blastomere ablation experiments showed that *P. hawaiensis* embryos also possess significant regulatory capabilities, such that the fates of mesoderm or ectoderm precursor blastomeres ablated at the eight-cell stage can be taken over by the descendants of some of the remaining blastomeres[25]. How regulative cell fate replacement can occur, and the extent of autonomous cell fate adoption by somatic blastomeres, remains unknown. Experimental embryological techniques such as blastomere ablation can be useful in understanding the relative autonomy and nonautonomy of cell fate decisions[26,27] and are therefore of interest in the study of *P. hawaiensis* embryogenesis.

In the experiments that demonstrated regulative replacement of ectodermal and mesodermal lineages, blastomere ablation was performed by injection[28] and subsequent excitation of phototoxic dyes[25]. While this technique is effective at killing the injected blastomere(s), it does not completely remove the dead cell body from the embryo. In addition, differences have been observed between cell lineage data gathered through to gastrulation stages of embryogenesis by injecting blastomeres with fluorescent lineage tracers[28,29], and data gathered by following unperturbed blastomeres through development of the same embryonic stages[23]. Complete physical removal of specific blastomeres may therefore be a preferred method of ablation for some applications.

We previously published the results of cell lineage analyses of embryos in which single cells were manually ablated[23]. However, the delicate operations required to remove single blastomeres from early cleavage stage embryos have not yet been fully described. Here we present a protocol for collection of *P. hawaiensis* embryos and manual ablation of a single blastomere from an eight-cell stage embryo. The goal of this method is to achieve complete removal of the cell body from the embryo, allowing observation of the cellular behaviors and cell fate competencies of the remaining cells during embryogenesis and post-embryonic development. Our protocol shows removal of the germ line precursor **g** (**Figure 2C**), but can be applied to any cell at the eight-cell stage, or to blastomeres of earlier cleavage stages. In principle, this protocol could be applied to remove single cells from early cleavage stage embryos of other holoblastically cleaving marine invertebrates.

Protocol

Comments that may be helpful in executing certain steps are indicated in *italics*.

1. Day 1: Preparation of Materials

1. Prepare the following materials (see Table of Materials and Equipment):
   - 15 cm and 22 cm Pasteur pipettes
   - Diamond scribe
   - filtered artificial seawater (salinity between 0.0018-0.0020) containing 1 mg/ml Amphotericin B (1:100 of a 100 mg/ml stock solution), 100 units/ml penicillin and 100 mg/ml streptomycin (1:50 of a stock solution containing 5,000 units/ml of penicillin and 5,000 mg/ml of streptomycin)
   - filtered artificial sea water (salinity between 0.0018-0.0020) without additives
   - a large plastic container for housing couples (at least 15 cm x 15 cm x 5 cm)
   - small (3 cm or 5 cm) Petri dishes lined with Sylgard
   - small (3 cm or 5 cm) Petri dishes
   - a watchglass or glass multi-well plate
   - forceps (we use blunt forceps derived from old Dumont #5 forceps that were accidentally damaged in other laboratory procedures, which we have repurposed by using a forceps sharpening stone to ensure that the ends of the forceps are smooth, that both tines are the same length, and that they no longer have sharp points. Extremely fine forceps, such as new Dumont #5 forceps, are undesirable as they may injure embryos and/or females.)
   - a Pasteur pipette with the end widened (see step 1.2 below)
   - an embryo retrieval tool consisting of a thin, curved tungsten wire embedded into the thin end of a Pasteur pipette (could be replaced by an alternative implement; see 1.3 below)
   - a mouth pipette with a small opening (see step 1.4 below)
   - a glass needle made from a pulled glass capillary (see step 1.5 below)

   No materials toxic to humans are used in this protocol. However, researchers should ensure that they wear gloves or other protective attire as required by the risk management guidelines of their institution.
2. To make the widened Pasteur pipette, first score around a 15 cm Pasteur pipette at the point where the pipette begins to narrow with a diamond scribe, wrap the scored region of the pipette in a Kimwipe or paper towel to protect the fingers, and then carefully break off the tip at the score line. Hold the broken end of the pipette over a Bunsen burner flame for several seconds to smooth the edges. The opening should be slightly narrower than the maximum width of the pipette.

3. To make the tungsten wire dissecting tool, insert a tungsten wire into the end of a 15 cm glass Pasteur pipette, and hold briefly over a Bunsen burner flame to melt the glass, fixing the wire in place. Use forceps to gently curve the end of the wire into a hooked shape. See **Figure 3** for an example of an appropriate shape for this tool.

4. To make a small opening for the mouth pipette, pull the thin end of a 22 cm Pasteur pipette into two parts over a flame, and break off the tip of the small end. Insert into the end of the mouth pipette holder.

5. Make the glass needle that will be used to puncture the blastomeres of interest during the ablation procedure using a needle puller. A suitably shaped needle is shown in **Figure 4**. This needle was pulled on a Sutter P97 needle puller using a program with parameters 2x(heat = 566, pull = 100, velocity = 20, time = 250) + 1X(heat = 566, pull = 100, velocity = 100, time = 250). The specific program and instrument used to make the needle can vary, but the needle must have a fine point, yet also be sturdy enough not to break when pushed against the chorion of the embryo.

2. Day 1: Gathering *Parhyale* Mating Couples

1. Use the widened Pasteur pipette (step 1.2 above) to collect mating *P. hawaiensis* couples from the large culture container, and transfer them to a separate container containing clean artificial seawater. It is not necessary that this seawater be filtered. It is best to collect mating pairs in the later afternoon and leave the couples at room temperature (20-23 °C) overnight, so that the next morning, most embryos will have been recently fertilized and deposited, and thus at early cleavage stages suitable for ablation. Gather at least 20 mating pairs to ensure that some females will be carrying early cleavage stage embryos by the following morning.

3. Day 2: Gathering *Parhyale* Embryos

- The next morning, infuse filtered artificial seawater (FASW) with CO_2. Some of the females will have swum free from their males during the night; collect these separated females and anesthetize them in FASW / CO_2. The remaining unpaired males can be removed from the mating pair container and returned to the large culture. Remaining mating pairs can be saved for embryo collection later in the day or the next day.
- Transfer a single anaesthetized female carrying embryos to a watch glass in FASW / CO_2. All separated females will likely have deposited eggs, which will be visible by eye as pale pink or purple spheroids through the transparent coxal plates of the females (**Figure 1A**). To determine if the embryos are at early cleavage stages and thus appropriate for blastomere ablation, the researcher will have to remove the embryos from the brood pouch and examine them under a microscope.
- Viewing the procedure under a dissecting microscope, grasp the coxal plates along one side of the body with forceps. Embryos will be visible in the ventral brood pouch between these coxal plates (**Figure 1B**).
- Insert the thin, curved wire tool (**Figure 3**; step 1.3 above) into the posterior end of the brood pouch, and gently lift the wire towards the anterior end of the brood pouch to separate the brood pouch coverings (these are modified ventral appendages called oostegites[30]), opening the pouch and loosening the embryos. Embryos that are within the first hour or so of being laid are all enclosed within a very thin, transparent membrane that will easily be disrupted by the wire tool; this membrane disappears by approximately 2-3 hr after egg laying, and embryos are not bound to each other or to the female in any way from this point onwards throughout embryogenesis. If researchers find it inconvenient or difficult to maneuver the curved wire tool to remove embryos from the brood pouch, an alternative implement may be used, as long as the movements are gentle and no harsh pressure is applied to the embryos or the brood pouch. Other tools appropriate for removing embryos from the brood pouch include blunted forceps or a glass capillary with a sealed and smoothed end.
- Use an unaltered 15 cm Pasteur pipette to flush water through the opened brood pouch, pushing out the embryos, which should settle to the bottom of the watch glass. Transfer the female to clean FASW (without CO_2) to allow recovery before reintroducing her to the main culture. Multiple females can be placed into the same recovery dish, but allow females to recover fully before returning them to the main culture, as they may be eaten by other animals if they are still partially anesthetized.
- Use an unaltered 15 cm Pasteur pipette to transfer the embryos to a Petri dish with clean FASW, and view under the microscope to determine their embryonic stage. Separate embryos that are at the third cleavage (stage 4 [5]), which are suitable for this procedure. It should also be possible to apply this protocol to any cleavage stage prior to germ disc formation (stages 7-8 [5]).

4. Day 2: Ablating Single Cells

1. Fill Sylgard-lined Petri dish with a shallow layer of FASW. Use an unaltered 15 cm Pasteur pipette to transfer a single embryo to the plate.
2. Using blunt forceps, gently roll the embryo to identify the cell to be ablated (**Figures 2A** and **2B**). The germ line precursor **g** can be identified as the smallest micromere, which sits on top of the smallest macromere **Mav**. Additionally, **g** does not directly contact the cell **en**, which is the micromere directly across from it, as the mesoderm precursor micromeres **ml** and **mr** share a cell border in the middle of the embryo. **mr** is the blastomere to the right of **g**, and **ml** is the micromere to the left of **g**. The sister macromeres of **mr**, **ml,** and **en** are the ectoderm precursors **Er**, **El**, and **Ep** respectively.
3. With forceps in the left hand (or in the right hand if the researcher is left-handed), orient the embryo with the cell of interest to the right (or to the left if the researcher is left-handed), and gently grasp the embryo between the forceps to stabilize it.
4. Using a pulled glass needle (step 1.5 above; **Figure 4**), gently puncture the cell of interest. Do not insert the needle too far, so as not to push the needle into the cells neighboring or below the cell of interest. The needle only has to puncture the chorion and underlying cell membrane, and does not need to extend deeply into the cell to be ablated.
5. Exchange the needle used to puncture the embryo for the glass end of a mouth pipette with a fine pulled Pasteur pipette tip (step 1.4 above). Hold the tip of the pipette close to the hole created in the cell of interest, and apply very gentle suction.
6. Very gently squeeze the embryo with the forceps. As the content of the blastomere is pushed out of the chorion through the hole created in step 4.4, suck it into the mouth pipette to allow an unobstructed view of the embryo. If the hole is large enough, the nucleus of the punctured cell may be seen to emerge as well. This is a good check to make sure the entire cell contents have been removed and cannot be regenerated.
7. Carefully observe the embryo as the blastomere of interest is removed. The border of the punctured cell will recede towards the hole in the chorion, making the intersections of the underlying cells visible. Continue applying pressure until all of the blastomere of interest has been removed. Use forceps to roll the embryo from side to side and visually confirm that all of the blastomere of interest is gone.

8. Using an unaltered 15 cm Pasteur pipette, transfer the embryo to clean FASW+ (FASW + 1 mg/ml Amphotericin B, 100 units/ml penicillin and 100 mg/ml streptomycin).
9. Raise blastomere-ablated embryos in individual wells of a clean 48-well plate in FASW+, changing 50% of antibiotic-supplemented FASW + daily. In our hands embryos survive and develop well, even following ablation, at a range of temperatures from 18-28 °C. Researchers should raise their embryos at a temperature for which they have a clean (but not necessarily sterile) surface on which to raise the embryos with minimal disturbance. To be able to compare the timing of developmental events in blastomere-ablated embryos with controls, we refer the reader to the detailed developmental staging information that is available for embryos raised at 18 °C[4], 25 °C[4], and 26 °C[5,6].

Representative Results

Following successful ablation of single third cleavage *P. hawaiensis* micromeres as described in this protocol, the remaining micromeres gradually shift their positions slightly so as to partially occupy the space formerly occupied by the ablated blastomere. For example, when **g** is removed, the neighboring blastomeres **mr** and **ml** shift slightly and come to share the lateral cell borders that were formerly in direct contact with **g** (compare **Figure 2A** and **2C**). Following successful ablation, the remaining blastomeres may display a slight delay (of less than one hour) before entering fourth cleavage, but they should then resume the same cleavage patterns and timing that they would have displayed in unmanipulated embryos at least through to gastrulation stages[23]. Following ablation of any of the four micromeres, descendants of the remaining blastomeres should also show normal cleavage timing and cellular behaviors, including formation of a characteristic cellular arrangement termed the "rosette" and the initiation of gastrulation movements[23]. The proportion of embryos that survive the ablation procedure and complete embryogenesis through to hatching may initially be on the order of around 10% for a researcher new to the technique, but with experience should average approximately 50%, and can be as high as 75% with practice and vigilant care of embryos following blastomere ablation. **Table 1** shows examples of numbers of embryos ablated and survival rates for a series of successive ablation experiments performed by the same researcher, showing that survival rates are generally at least 80% for the first few days following ablation, and that survival rates through to hatching rise with increasing experience of the researcher.

Incomplete blastomere removal would be evidenced by seeing remnants of the blastomere that should have been ablated, which could include membrane components, cytoplasm, or yolk granules, still situated within the chorion of the embryo. A delay in fourth cleavage of more than two hours, or irregular cleavage patterns or cellular behaviors of descendants of the remaining blastomeres, could also indicate unsuccessful ablation. These phenomena can be the result of damage caused to other blastomeres during the ablation procedure. If blastomeres other than the one targeted for ablation display morphological abnormalities or disintegrate, then again damage has likely been caused to other blastomeres during the ablation procedure.

If cell fate markers are available to label the descendants of specific third cleavage blastomeres whose fates are not subject to regulative replacement, then additional confirmation of successful ablation can be obtained by labeling manipulated embryos with those markers following the ablation. Markers of some mesodermal and ectodermal territories have been described at mid to late stages of embryogenesis[8,14,15]. However, because both of these germ layers can undergo regulative replacement upon loss of one of their founder blastomeres[25], these markers may not unambiguously determine whether or not blastomere ablation was successful. In the case of the germ line precursor **g**, all of its descendants are marked by the expression of *vasa* transcript[12] and protein[4] throughout embryogenesis, and embryos in which **g** has been ablated lack germ cells at least through to early germ band stages[4]. Successful ablation of **g** can thus be confirmed by examining *vasa* expression following ablation in later stages of embryogenesis (**Figure 5**).
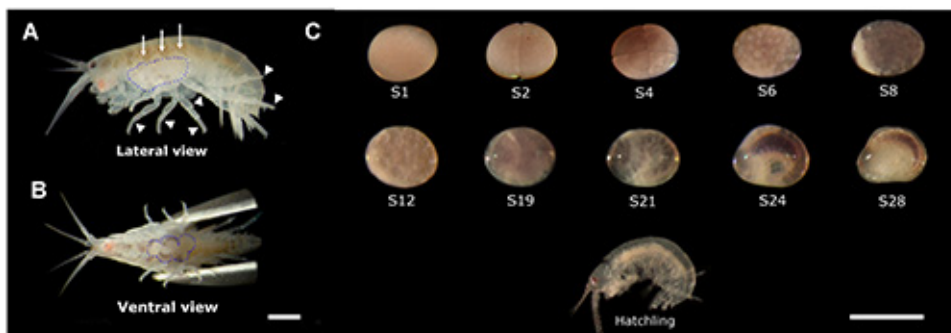

Figure 1. Adult *Parhyale hawaiensis* female and embryos. (**A**) Lateral view of an adult female *P. hawaiensis*, showing coxal plates (arrows), thoracic appendages (arrowheads) and embryos visible as pink or purple spheroids through the transparent coxal plates (blue dotted outline). Anterior is to the left. (**B**) Ventral view of an adult female *P. hawaiensis* showing embryos visible in the brood pouch (blue dotted outline). Anterior is to the left. (**C**) Embryos released from brood pouch develop normally in FASW. A variety of stages ranging from S1 through hatching are shown, staged according to Browne *et al.*[5] Scale bar = 1 mm in (**A**) and (**B**); 500 µm in (**C**). Please click here to view a larger version of this figure.
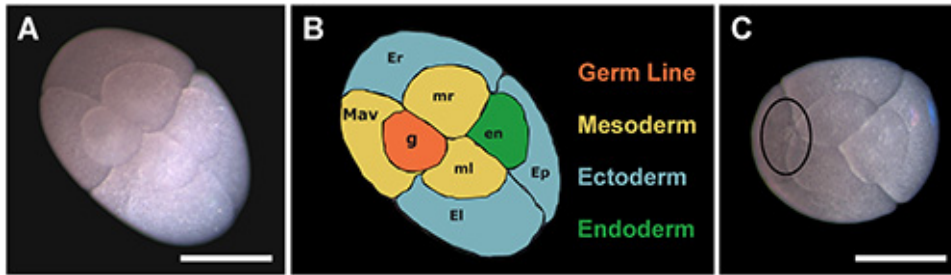
Figure 2. Eight cell stage embryos of *P. hawaiensis*. (**A**) Live eight-cell stage (stage S4[5]) unmanipulated embryo, comprising four micromeres (smaller cells) and four macromeres (larger cells). (**B**) Schematic of an eight-cell stage embryo showing cell fate designations of all blastomeres in a wild type embryo as revealed by lineage tracing based on fluorescent markers[6]. (**C**) Live S4 stage embryo that has had the **g** blastomere removed using the present protocol. The circled region shows the region formerly occupied by the **g** blastomere; the remaining micromeres have shifted position slightly as a result of the ablation of **g**. Scale bar = 250 μm in (**A**) and (**C**). Please click here to view a larger version of this figure.

Figure 3. Wire tool used for removing embryos from the brood pouch. The tool shown here was made by inserting a tungsten wire into the narrow end of a Pasteur pipette and gently melting the glass to seal the wire in place, then using forceps to introduce a gentle curve into the wire. Other tools appropriate for removing embryos from the brood pouch include blunted forceps or a glass capillary with a sealed and smoothed end. Scale bar = 1 cm.
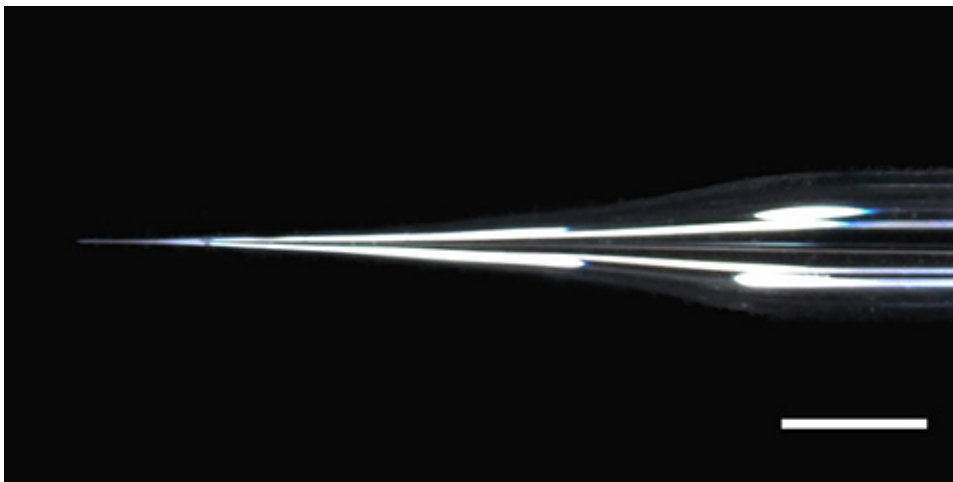

Figure 4. Glass needle used for puncturing blastomeres during the ablation procedure. The needle shown here was pulled on a Sutter P97 needle puller using a program with parameters 2 x (heat = 566, pull = 100, velocity = 20, time = 250) + 1 x (heat = 566, pull = 100, velocity = 100, time = 250). Other instruments or programs may be used to pull needles suitable for ablation, as long as they are of the same general shape as the one shown here. Scale bar = 1 cm.
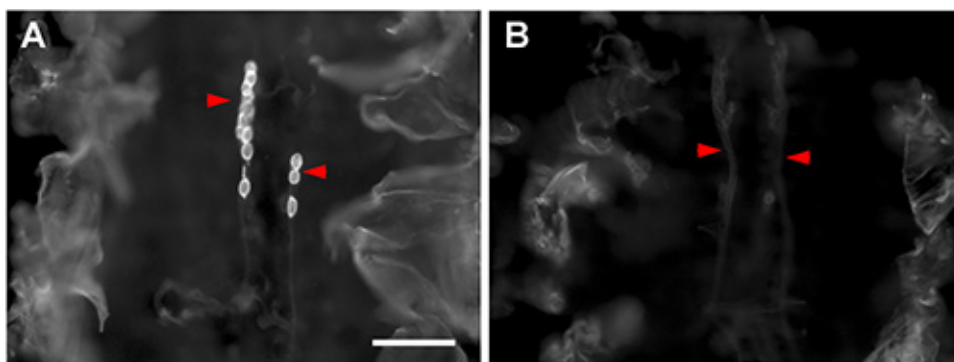
Figure 5. Assessing the results of blastomere ablation during embryogenesis. (A) Gonad region of a wild type stage S29 embryo shortly before hatching, showing germ cells labeled by anti-Vasa immunostaining (arrowheads). The anti-Vasa antibody used here is specific to *P. hawaiensis* Vasa (Linsler, Alwes and Extavour, unpublished data). **(B)** Gonad region of a stage S29 embryo that had the **g** blastomere removed at the eight-cell stage, showing absence of germ cells as revealed by absence of specific anti-Vasa signal in the gonad region (arrowheads). Scale bar in **(A)** = 100 µm and applies also to **(B)**. Please click here to view a larger version of this figure.

Ablation round	# Ablated	# (%) Survived to 50% development	# (%) Survived to 90-100% development
1	49	41 (83.7)	6 (12.2)
2	48	46 (95.8)	17 (35.4)
3	31	25 (80.6)	22 (71.0)
4	97	72 (74.2)	56 (57.7)
5	108	100 (92.6)	65 (60.2)
6	50	36 (72.0)	37 (74.0)
TOTAL	383	320 (83.6)	203 (53.0)

Table 1. Representative ablation survival statistics. Following micromere ablation, survival was scored both within the first 50% of development (5-6 days at 25 ºC) and then at 90-100% development (10-12 days at 25 ºC). Ablation rounds 1-6 are representative examples of experiments performed by a single researcher (A. R. Nast) in chronological order over the course of an eight-month period. Researchers should find that, as shown here, survival rates to 90-100% development increase with experience.

Discussion

We describe a protocol for manual ablation and complete physical removal of single blastomeres from early cleavage stages of the amphipod *P. hawaiensis*. We demonstrate use of this protocol by removing the single germ line precursor cell **g** from an eight-cell stage embryo, and show that ablation has been successful by confirming absence of **g**'s daughter cells in later embryogenesis. This protocol can be used to remove any of the micromeres from the embryo at early cleavage stages. To use this protocol to remove macromeres at this stage, or blastomeres at first or second cleavage stages, users can simply apply the slight modifications of creating of a slightly larger hole in the chorion at step 4.4, and applying pressure and suction for a longer time to remove the larger volume of cell contents at step 4.6. We have found that following use of this protocol, development of the remaining blastomeres after ablation is unaffected with respect to cleavage and cell movement patterns at least through to the time of gastrulation[23].

This protocol could be useful for continued investigation into the developmental potential of early cleavage blastomeres in this amphipod. Many questions remain to be answered in this area, including why some cells but not others can change fates to replace those of ablated blastomeres, and the precise timing and mechanisms of these regulative changes. The protocol can also be modified to accommodate ablation of more than one cell, by simply repeating steps 4.2-4.7 successively on the blastomeres of interest.

It is important to use a high-quality stereomicroscope with appropriate lighting in order to view and correctly identify the early cleavage blastomeres. We find that lateral incident white light from a fiber optic cold light source is the most useful for this purpose. Incident light directly above the specimen creates reflections that can obscure the cellular morphologies and boundaries, while transmitted light fails to penetrate the dense yolk of the embryos and blastomeres are therefore difficult to distinguish. It is most helpful for the watch glass or Petri dish containing embryos to be placed on a black surface rather than a white or transparent glass surface, as this provides best contrast for the pale embryos.

One limitation to this protocol is that the blastomere of interest must be correctly and unambiguously identified before beginning the procedure. Photoablation techniques would be more useful for researchers new to the system, since the embryos can be left to develop for a few cleavage cycles following injection of the fluorescent dye[28], and the identity of the injected blastomere confirmed post-injection, but before photoablation, by analyzing the patterns of the cellular descendants, which have been well described in previous cell lineage analyses[6,23]. Once researchers are familiar with the *P. hawaiensis* embryo and can identify all blastomeres with confidence, the manual ablation described here could be used for applications where complete physical removal of a blastomere, rather than killing the cell without removing the dead cell body from the embryo, is desirable.

This procedure could in principle be applied to remove single blastomeres from cleavage stage embryos of other holoblastically cleaving crustaceans, or other marine or freshwater invertebrates whose chorions or fertilization membranes cannot be easily removed. Modifications could include using incubation media with appropriate salinity characteristics, and modifying the shape of the needle to accommodate more delicate membranes (longer, thinner needles) or more robust embryonic coverings (shorter, rapidly tapering needles).

Disclosures

The authors have nothing to disclose.

Acknowledgements

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