Chapter 5

Studying Ctenophora WBR Using *Mnemiopsis leidyi*

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Abstract

Ctenophores, also known as comb jellies, are a clade of fragile holopelagic, carnivorous marine invertebrates, that represent one of the most ancient extant groups of multicellular animals. Ctenophores show a remarkable ability to regenerate in the adult form, being capable of replacing all body parts (i.e., whole-body regeneration) after loss/amputation. With many favorable experimental features (optical clarity, stereotyped cell lineage, multiple cell types), a full genome sequence available and their early branching phylogenetic position, ctenophores are well placed to provide information about the evolution of regenerative ability throughout the Metazoa. Here, we provide a collection of detailed protocols for use of the lobate ctenophore *Mnemiopsis leidyi* to study whole-body regeneration, including specimen collection, husbandry, surgical manipulation, and imaging techniques.

Key words Ctenophore, *Mnemiopsis leidyi*, Wound healing, Whole-body regeneration, Husbandry, Surgeries, Live imaging, Time-lapse

1 Introduction

While regenerative capabilities are common across the animal kingdom, the ability to regenerate all the structures of the body (i.e., whole-body regeneration) is a rather unique feature only found in some species. Ctenophores (comb jellies) are one such animal with impressive whole-body regenerative capabilities; they are holopelagic, carnivorous marine invertebrates that represent one of the oldest extant metazoan lineages [1]. Ctenophores have a unique body plan characterized by a biradial symmetry (with no planes of mirror symmetry) and one primary body axis (the oral–aboral axis) delimited by a mouth (oral) and an apical sensory organ (aboral). The ctenophore body is composed of two epithelial layers: the ectoderm—including the epidermis, apical organ, pharynx, nerve net, ctene plates (or comb plates), and tentacle sheath—and the endoderm primarily composed of a system of endodermal canals that distribute nutrients to the periphery of the animal. The

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ectodermal and endodermal tissues are separated by a thick mesoglea mostly composed of extracellular matrix, but also containing several types of individual muscle and mesenchymal cells [2]. The characteristics of the mesoglea differ between ctenophore species. For example, in *Pleurobrachia* species (the sea gooseberry), the mesoglea is rather rigid, while in lobate ctenophores the mesoglea is highly pliable, presumably due to differences in hydration characteristics. Ctenophores' main mode of locomotion is via the coordinated beating of their comb plates. They possess eight longitudinally oriented rows of locomotory ctene plates, each plate composed of thousands of laterally arranged cilia which they coordinately beat to propel through the water column. Ctenophores have been accurately described morphologically for over a century, with the first volume of the *Flora and Fauna of the Statione de Napoli* being dedicated to Ctenophora by one of the world's first experimental embryologists, Carl Chun [3].

One of the best-studied species of ctenophores in the regenerative field is the lobate ctenophore *Mnemiopsis leidyi* [4–10]. Like the majority of ctenophores [11], *M. leidyi* is a self-fertile hermaphrodite, meaning that a single animal carries both female and male gonads. The eggs and sperm are released freely into a common sinus under each comb row and fertilization takes place upon release into the water column [12]. Like most ctenophores, *M. leidyi* produces embryos which are optically clear and, like all ctenophores, it has a very stereotyped, clade-specific cleavage program where rounds of division occur every 20 min at room temperature and the juvenile cydippid stage hatches from the fertilization envelope within 18–24 h after the first cleavage [13–15]. The cydippid is a feeding form characterized by a pair of long branching muscular tentacles that define the tentacular axis and bear specialized adhesive cells called colloblasts, used to capture prey [16, 17]. In lobate ctenophores like *Mnemiopsis*, the tentacles are progressively reduced and internalized during the transition to adulthood as the animal forms two large oral lobes that are extremely efficient at prey capture (Fig. 1). Under optimal conditions, the adult form can get sexually mature at ~4 weeks of age, though sexual reproduction at the juvenile morphological stage, termed "dissogeny," has been documented as early as 2 weeks [18, 19].

Our recent study shows that in *M. leidyi* cell proliferation is activated (after wound-healing) at the wound site and is indispensable for whole-body regeneration. EdU pulse and chase experiments after surgery together with the removal of the two main regions of active cell proliferation suggest a local source of cells in the replacement of missing structures. Time-lapse live imaging during *M. leidyi* wound healing shows evidence of cells forming actin-based protrusions while migrating to the wound site [10]. While lobate ctenophores show an outstanding capacity to

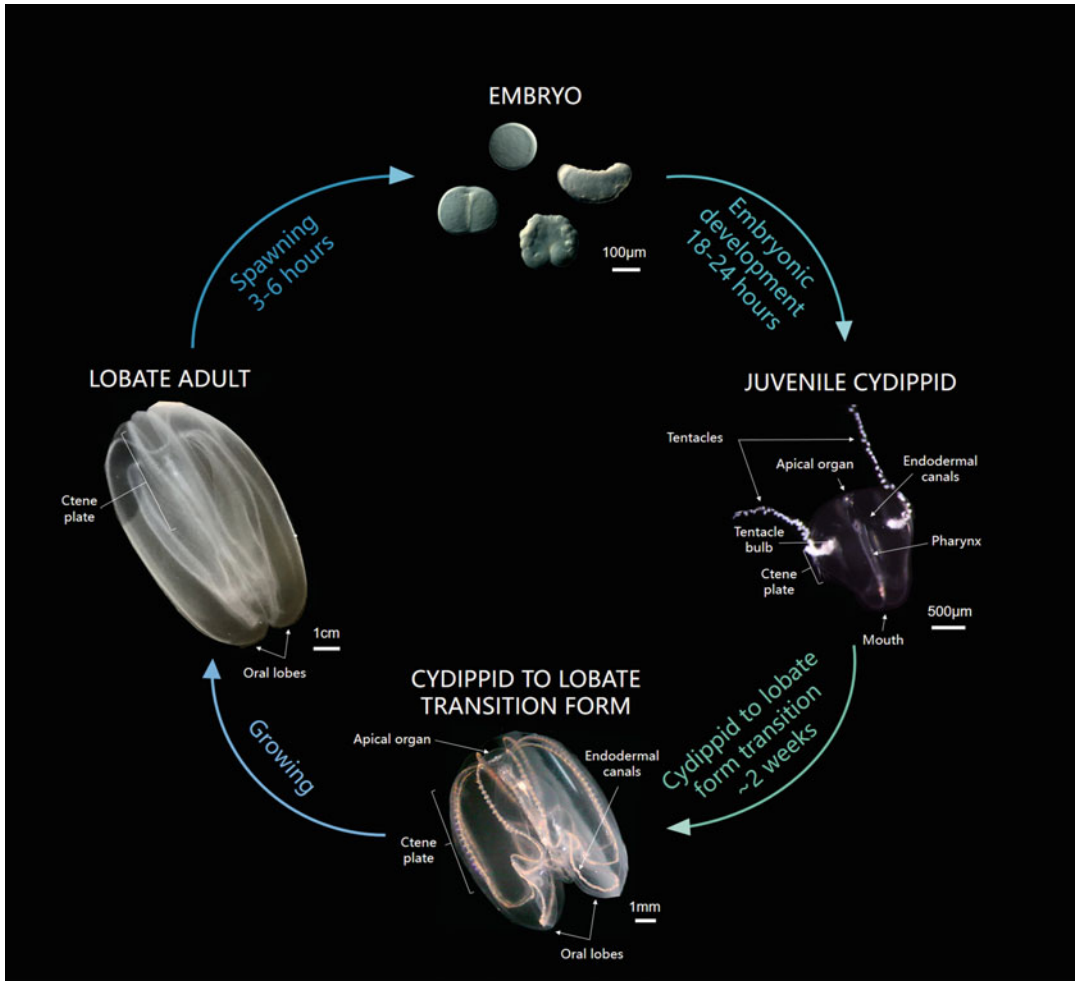

Fig. 1 The life cycle of *Mnemiopsis leidyi*. The adult body plan is referred to as "lobate," describing their prey-capture tissues (oral lobes) that extend from the oral end. Adults produce both eggs and sperm. Embryos are ~150 µm in diameter and develop from single cell to hatching over ~24 h. The hatched, free-swimming feeding juvenile body plan is referred to as "cydippid" and characterized by a relatively shortened body and long prey-capture tentacles. The cydippids will start to transition into the lobate body plan as their tentacles retract, body lengthens and lobes form. Adult lobates will continue to grow until they reach a maximum size of around 6–18 cm

regenerate all body parts, another group of ctenophores, the Beroids, have lost the ability to regenerate [9]. Hence, the comparison of cellular and molecular responses after amputation between lobate ctenophores and Beroids provides an ideal system to elucidate the core cellular and molecular responses required for the process (and loss) of adult regenerative potential.

The many favorable experimental features provided by ctenophores (optical clarity, stereotyped cell lineage, multiple cell types, sequenced genome available [20], comparative and functional

genomics [21–27], rapid regeneration (48–72 h) and their early branching phylogenetic position (potentially the earliest extant animal clade [1, 20, 28, 29]) make them a new powerful research organism for the study of regeneration at a cellular and evolutionary level. Here we provide a detailed protocol to use ctenophores to study the process of whole-body regeneration from the collection of specimens and husbandry in the laboratory to the deployment of basic techniques for the study and monitoring of regeneration at a cellular level.

2 Materials

2.1 Equipment

1. Ctenophore dipper: 1 L plastic beaker fixed at the tip of a 2-meter pole (Fig. 2A).
2. 20 L plastic bucket.
3. Ctenophore tank system: 200 L tank made of plexiglass (PMMA) walls consisting of an inner tank module enclosed between two outer compartments with drains both at the bottom and the top of the tank (Fig. 2B, C).
4. 6″ diameter glass bowls (Fig. 3).
5. 2 L glass beaker (Fig. 3).
6. 1 mL transfer plastic pipettes.
7. Rotifer culture system: 2 L glass beaker, a 5 × 5 cm piece of rotifer floss (e.g., Reed Mariculture, Inc), air pump (e.g., Tetra Whisper Aquarium Air Pump) connected by plastic tubing to a 1 mL serological pipette.
8. A coarse filter: 30-μm nylon mesh screening (e.g., Nitex) affixed to a section of pipe or plastic container with the bottom removed.
9. *Artemia* hatching system: 1.5 L plastic cone with a stopcock (valve) at the bottom placed inside a support, aeration system made from a 1 mL plastic pipette attached at one end to a plastic tube connected to a standard aquarium air pump from the other end.
10. Operating dish: 35-mm plastic petri dish, coated with a 2-mm thick silicon (SYLGARD-184) layer.
11. Microburner: 16-gauge syringe needle inserted into latex tubing attached to a propane source (Fig. 4A).
12. Pulled glass needles from Pyrex capillaries (Fig. 4A).
13. Pair of fine forceps (e.g., World Precision Instruments, Cat#500341).
14. Siliconized slide: microscope glass slide treated with a synthetic hydrophobic surface-applied product (e.g., Rain-X, Inc.).

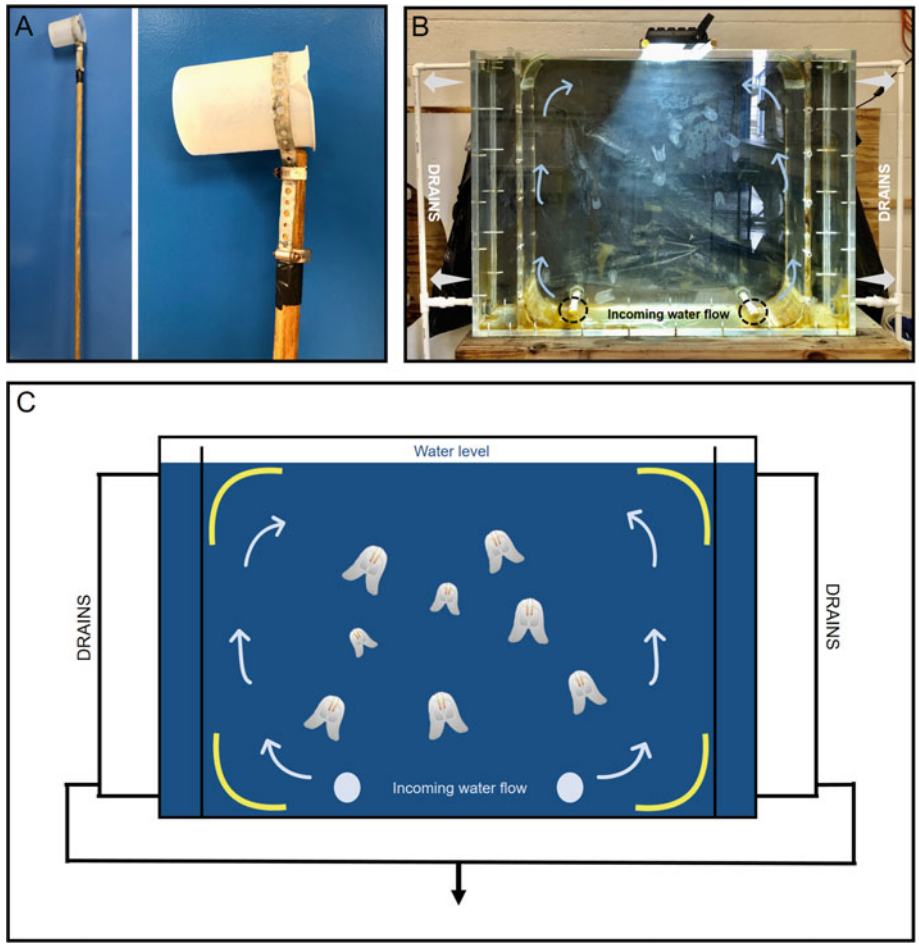

Fig. 2 Ctenophore collection and culturing materials. **(A)** A "ctenophore dipper" constructed to collect ctenophores from the field. Ctenophores located close to the surface of the water are gently scooped into the beaker portion of the dipper. (**B**) The pseudo-kreisel tank system used to contain adult ctenophores. (**C**) Diagram of a pseudo-kreisel tank system for culturing adult *M. leidyi* at the lab. Two water inlets located in the tank bottom generate a continuous flow that pushes water up the sides of the tank. Drains on the top and bottom of each side displace the input of water, while two partially perforated plexiglass sheets contain the animals in the central space. The flow of water keeps the ctenophores toward the tank's center, while constant water flow through the system prevents fouling

15. 35-mm plastic petri dishes.
16. Dissection microscope.
17. Microscope attached to a time-lapse camera—We use a Zeiss M2 Axio Imager coupled with a Rolera EM-C2 camera.

2.2 Reagents

1. 1× UV-FSW: UV treated 1.0-μm filtered full strength seawater (e.g., 35 g/L).
2. 1× 0.2-μm UV-FSW: UV treated 0.2-μm filtered full strength seawater.

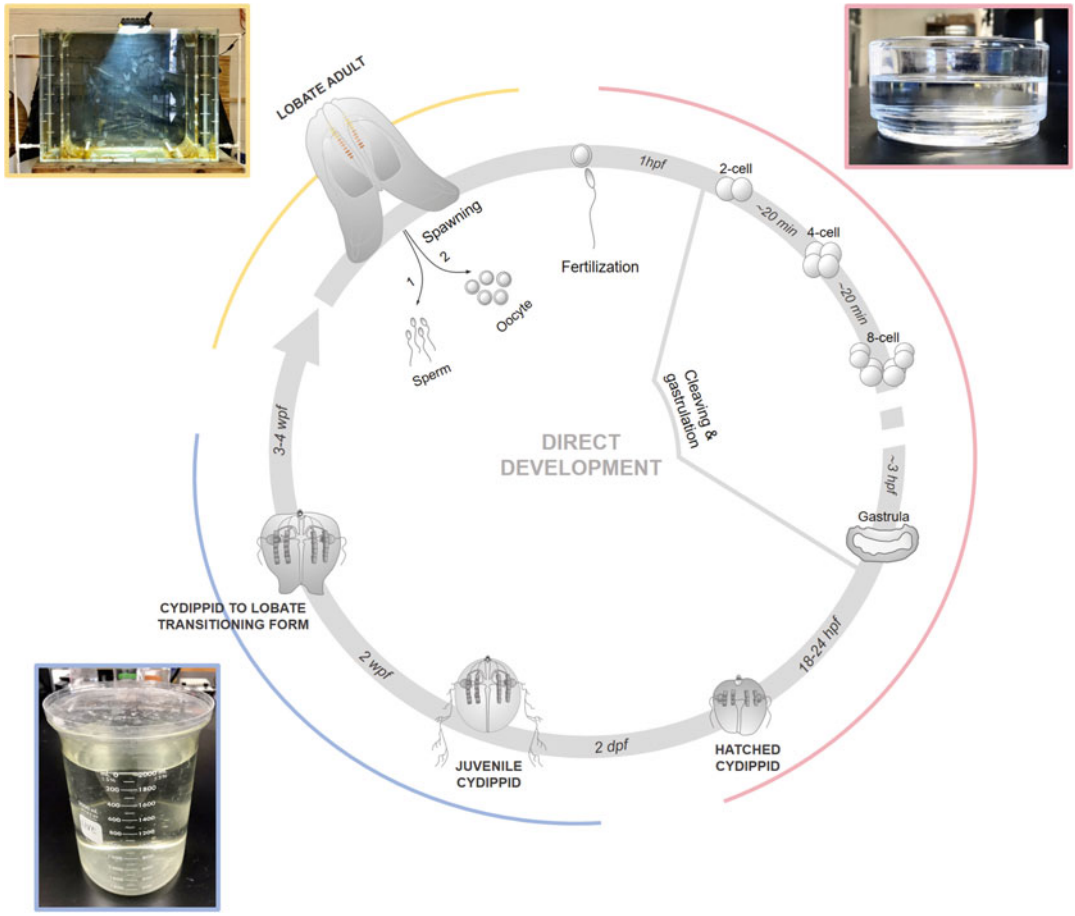

Fig. 3 Husbandry and culture of *Mnemiopsis leidyi*. Culturing conditions for each stage of the *M. leidyi* life cycle. Spawning is induced in wild-caught or captive adults by manipulating their light exposure. Embryos are collected and placed in a 6″ diameter glass finger bowl in 1× UV-FSW. Hatching occurs 18–24 h post-fertilization (hpf). Hatched cydippids (*M. leidyi* juveniles) are grown in 2 L glass beakers to provide space for hunting behavior as their tentacles tend to get tangled with other individuals in smaller containers. Cydippids grow into adults in the next 3–4 weeks postfertilization as they grow in size, retract their tentacles, and develop oral lobes for prey capture

3. 20 ppt FSW: 500 mL 1× UV-FSW, 500 mL deionized water (*see* **Note 1**).
4. Rotifers: one million *Brachionus plicatilis* (L-type) starting bag.
5. Concentrated microalgal based rotifer feed (e.g., RGcomplete, Reed Mariculture, USA). Store at 4 °C.
6. *Artemia* nauplii larvae. We use *Artemia franciscana* from Great Salt Lake Origin (www.brineshrimpdirect.com).
7. Mysid shrimps (*Americamysis bahia*, U.S. Mysids, Saint Augustine, FL USA).

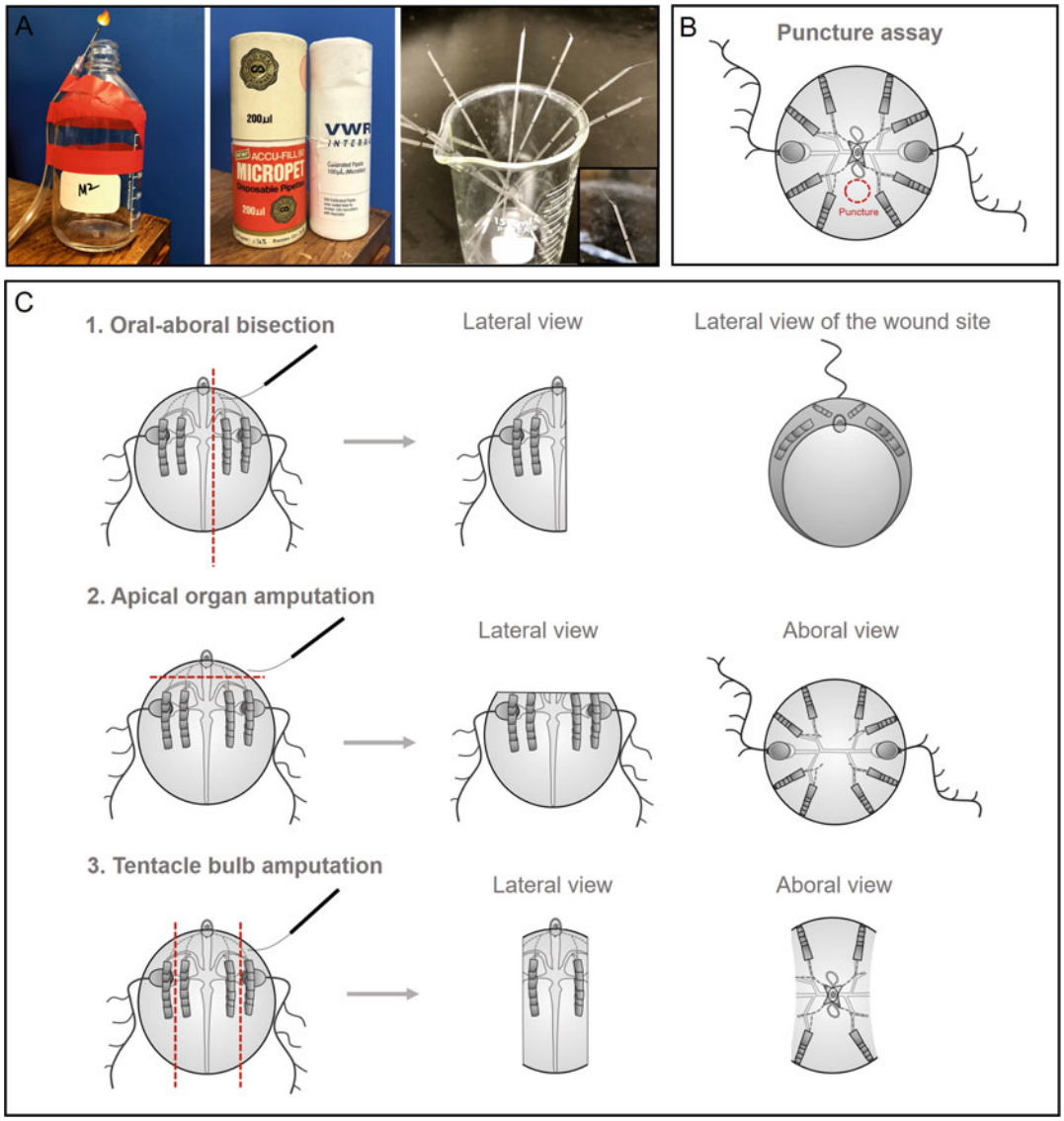

Fig. 4 Methods for ctenophore tissue regeneration assays. (**A**) Surgery instruments. Glass needles are hand-pulled using a microburner. (**B**) Puncture assay for *M. leidyi* cydippids. Tissue is punctured in a space that is clear of organs (tentacle bulb, comb rows, etc.) to assay wound healing of the epithelia. (**C**) Designs for multiple types of amputations. Oral–aboral bisection is performed by cutting tissue parallel to the esophageal canal and slightly to the side of the apical organ. This is so one half of the animal maintains an intact apical organ, as it is more likely to regenerate with this feature. Apical organ amputation includes cutting the space between the top of the comb rows and the base of the apical organ. Careful attention should be taken to ensure that the canals connected to the comb rows are not damaged. Tentacle bulb amputation requires cutting tissue between two adjacent comb rows, cutting out one or both tentacle bulbs on either side of the body and leaving behind 4 of the 8 comb rows. Make sure the tentacle bulbs are completely removed, including the dense cluster of cells at the base of the bulb

8. 100× hydroxyurea (HU) stock solution: 500 mM HU in distilled water. 1 mL aliquots can be kept at −20 °C for several months. Keep at 4 °C once thawed.
9. 1× HU: 100× HU stock solution in 1× UV-FSW.
10. 25 °C low-melt agarose (e.g., Sea-Plaque).
11. 1.2% (w/v) low-melt agarose in 1× UV-FSW.
12. 1× PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 in 800 mL of H_2O. Adjust the pH to 7.4 with HCl, add H_2O to 1 L. Autoclave and store at room temperature (*see* **Note 2**).
13. Fixative solution: 100 mM HEPES-HCl, pH 6.9, 0.05 M EGTA, 5 mM $MgSO_4$, 200 mM NaCl, 1× PBS, 3.7% (w/v) formaldehyde (e.g., Sigma-Aldrich, #F8775), 0.2% (v/v) glutaraldehyde (e.g., EMS #16216), 0.2% (v/v) Triton X-100 (e.g., Sigma-Aldrich,#X100-500ML) in 1× 0.2-μm UV-FSW.
14. PBS-0.02% Triton X-100: 0.02% (v/v) Triton X-100 in 1× PBS.
15. Optically transparent jammed microgel: 7.5% (w/w) azobisisobutyronitrile and *N*′-methylene bisacrylamide microgel dried powder dispersed in 1× 0.2-μm UV-FSW. Kept at 4 °C. *See* [10] for details on microgel elaboration.
16. Mounting clay for microscope slides.
17. Vaseline.

3 Methods

3.1 Sources and Collection

We collect *M. leidyi* on the northeast coast of Florida, around the Saint Augustine area where the University of Florida's Whitney Lab for Marine Bioscience is located. The confluence of the Matanzas intercoastal river with the Atlantic Ocean creates a system of estuarine saline waters which favors the appearance of *M. leidyi* specimens all year long. *M. leidyi* can also be found in coastal waters along the Atlantic coast of North and South America and it has become an invasive species in European waters through ballast water introduction, most notably in the Black Sea, eastern Mediterranean, and Caspian Sea [30]. Collecting ctenophores is an art, but once an experienced collector has seen several in the wild it is relatively easy to find them.

1. Choose optimal environmental conditions for ctenophore collection (*see* **Notes 3** and **4**).
2. Navigate to a calm marine area (*see* **Note 5**).
3. Orient yourself with the sun at your back.

4. Spot ctenophores by the light reflection generated by the movement of the comb plates creating a colorful rainbow-like iridescence running down the comb rows or from food in the gut.
5. Collect the animal using the ctenophore dipper (Fig. 2A, *see* **Note 6**).
6. Transfer the specimen into a plastic bucket filled with seawater (*see* **Note 6**).
7. Repeat **steps 4–6** until sufficient specimens are collected (*see* **Note 7**).
8. Transfer the ctenophores to the laboratory.

3.2 Laboratory Setup for Mnemiopsis leidyi *Culture*

Different culturing systems have been optimized for each *M. leidyi* life cycle stage. Adult specimens prefer to move vertically on an hourly regime, so keeping them in a large volume, with a tall height aspect to cross sectional area is optimal. If adult animals are allowed to interact with the bottom surface of their container, they will erode their epidermal surface and die. For example, *M. leidyi* can be kept in 20 L buckets for short periods of time as long as the water is changed once or twice daily, but after several days animals will start to deteriorate. The ideal situation is to keep them in a tank installed in open sea water system room at ambient temperatures that allows continual sea water circulation keeping adults off tank surfaces. Hatchlings and juvenile cydippid stages can be grown and cultured in the laboratory (room temperature, 20–22 °C) in a variety of different types of glassware, but low-density cultures (~1 embryo/5 mL) are preferred. Generally, we dilute embryonic cultures to 2× larger volumes every 2–3 days to give them more space to set their tentacles and feed (*see* Subheading 3.4).

We keep both *M. leidyi* and the atentaculate *Beroe ovata* in pseudo-kreisel tanks made of clear 2 cm thick plexiglass sheets connected with stainless steel screws and sealed with silicone, that generate a circular flow which keeps animals suspended in the water column. In the pseudo-kreisel depicted (Fig. 2C), the inner tank module where the animals are kept is 90 cm in diameter and has rounded corners with small perforations allowing water to exchange into two outer compartments (10 cm each) that have drains both at the top (that also serve as water level overflows) and the bottom of the tank. Upwelling inlets at the bottom of the inner tank introduce fresh sea water and direct it to the edges. By controlling the volume of water entering and exiting the tank, the overall position of the ctenophores can be controlled. For example, if the flow rate is too high, all of the animals are concentrated in the center of the tank and if it is too low the animals may sink to the bottom. The optimal flow rate allows animals to swim freely but prevents them from approaching the bottom because abrasion of

ctenophore epidermis on the bottom of the tank causes wounds that lead to death. Incoming seawater at Whitney is naturally sand filtered and devoid of zooplankton or phytoplankton.

Water quality is important for the successful culturing of ctenophore embryos and adults. We routinely rear embryos and early hatched cydippids in glass bowls (Fig. 3) filled with 1× UV-FSW until they reach ~1.5–2 mm diameter (1 week old). They are then transferred to 2 L glass beakers in order to give them more vertical space for swimming and feeding and are kept in this container until they transition into the lobate state (Fig. 3) (*see* Subheading 3.4).

3.3 *Culturing Live Feed for* Mnemiopsis leidyi *Husbandry*

Juvenile and adult ctenophores feed on zooplankton present in the water column. Adults typically feed on copepods and other pelagic organisms (including larval fish). In captivity, the best first food source for *M. leidyi* is rotifers (e.g., *Brachionus plicatilis)* because of their small size and the ability of *M. leidyi* cydippids to catch them with their tentacles. We feed adults with *Artemia* and/or mysid shrimp daily. Note that feeding *Mnemiopsis* on *Artemia* is not sufficient to maintain reproductive ability, so mysids must be fed at least 1–2 times per week and ideally daily. Other labs use fish eggs and larvae rather than mysids. This nutritional requirement is currently a bottleneck in the rearing of reproductive colonies of *Mnemiopsis* in laboratory culture (*see* **Notes 8–10**).

A small-scale rotifer culture is sufficient to cover the feeding regimes of growing cydippids. We follow the instructions provided by the supplier (Reed Mariculture, Inc) except that we keep a smaller culture volume (*see* **Note 11**).

1. Fill two separate rotifer culture systems with 1 L of 20 ppt FSW.
2. Place the closed rotifer starting bag in the culture system.
3. Wait 30 min for the animals to acclimate.
4. Open the bag releasing the rotifers into each beaker.
5. Top up each beaker with 20 ppt FSW.
6. Shake the microalgal concentrate rotifer feed.
7. Pipette 2 mL of microalgal concentrate into each beaker.
8. Wait 6 h for the rotifers to feed.
9. Repeat **steps 6** and **7** to feed the rotifers again.
10. Wait 24 h for the culture to expand, feeding twice per day (morning and afternoon).
11. To harvest fresh rotifers, filter ~30–40% (600 mL) of the culture through the coarse 30-μm nylon mesh.
12. Backwash the mesh into a small glass beaker using a squirt bottle filled with 10 mL 1× UV-FSW.

13. Use these isolated rotifers to feed the juvenile ctenophores (Subheading 3.4). Stir rotifers with a plastic transfer pipette to make concentration uniform before feeding.
14. Repeat **steps 6–13** to culture the rotifers for a week.
15. Pour the cultures into clean 2 L glass beakers avoiding the transfer of waste deposited at the bottom.
16. Clean the glass beaker wiping the green residue on the sides and bottom with deionized water, avoiding any kind of soap.
17. Repeat **steps 14–16** for three more weeks.
18. For deeper cleaning, once a month, filter the whole culture through the 30-micron nylon mesh.
19. Backwash the mesh into two clean 2 L glass beaker filled with 20 ppt FSW.
20. Feed the clean culture following **steps 6** and **7**. It is convenient to perform the cleaning before feedings.
21. Follow **steps 11** and **12** to isolate a backup culture of rotifers.
22. Pipette 5 mL of microalgal concentrate into the backup culture.
23. The backup culture may be kept uncovered at 4 °C for a week.
24. Repeat **steps 6–23** to maintain the rotifer culture.

We use just-hatched *Artemia* to complement the diet of growing cydippids once they reach a certain size (Subheading 3.4) as well as to feed adult ctenophores.

1. Set up the *Artemia* hatching system by placing the plastic cone inside a support.
2. Fill the hatchery cone with 1 L of 1/3× FSW.
3. Add 6.5 g/L of *Artemia* cysts to the hatchery cone.
4. Start the aeration system.
5. Incubate for 20 h.
6. Stop the aeration, turn on the light source and wait for 5 min for the unhatched cysts to sink and accumulate at the bottom of the cone (*see* **Note 12**).
7. Open the water flow using the valve at the end of the cone to discard the unhatched cysts.
8. Collect hatched *Artemia* nauplii larvae from the bottom of the cone.
9. Use these isolated *Artemia* to feed growing cydippids and adult ctenophores (*see* Subheading 3.4).

We use mysid shrimps to feed adult ctenophores (*see* Subheading 3.4). We obtain the mysids directly from a shrimp farm and feed adult specimens with ~2 mysid shrimps per ctenophore twice a week (*see* **Note 13**).

3.4 Spawning and Husbandry of *Mnemiopsis leidyi*

M. leidyi has a natural circadian rhythm and spawns according to the light-dark cycle. Our protocol for *M. leidyi* spawning at the Whitney Lab in St. Augustine, FL. has been modified from Pang and Martindale, 2008 [31]. Under normal summer conditions in Cape Cod, MA (Woods Hole), *M. leidyi* spawning is triggered by the onset of darkness and it normally occurs ~8 h after sunset. In northeast Florida, spawning occurs after 3–4 h of darkness. In order to get *M. leidyi* to spawn at any time of the day, we keep animals under constant light conditions and then place them in the dark to induce spawning. It takes 2–3 days of constant light exposure to erase the endogenous circadian rhythm of wild caught animals so they reliably spawn 3–4 h after putting them in the dark. When spawning freshly caught specimens, wild caught adult ctenophores are kept in a 20 L bucket filled with 1× UV-FSW in the laboratory under constant light for 48–72 h (more detailed protocol in [32]). Here we describe the protocol to spawn adult ctenophores cultured in captivity at the lab. All steps are performed at room temperature (20–22 °C).

1. Take adult lobate stage animals from the tank and transfer them into a plastic bucket filled with fresh 1× UV-FSW using a plastic or glass beaker (*see* **Note 14**).
2. Transfer the specimens into individual 6″ glass culture bowls filled with fresh 1× UV-FSW. We place 2 ctenophores per bowl when specimens are ~5 cm long, up to three animals if they are smaller or only one for larger animals (*see* **Notes 15** and **16**).
3. Repeat **step 2** to fill 4 more bowls with adult ctenophores (*see* **Note 17**).
4. Place the bowls into complete darkness (*see* **Note 18**).
5. Wait 3 h for the animals to start spawning.
6. Check for cloudy meridional canals which indicate the presence of sperm (*see* **Notes 19** and **20**).
7. Wait 20–30 min for spawning to complete.
8. Transfer up to 200 fertilized embryos with a transfer pipette into a 6″ bowl filled with fresh 1× UV-FSW.
9. Cover the glass bowl with a plastic lid to avoid evaporation of water.
10. Place the bowl at room temperature in the lab.
11. Return the adult specimens to the pseudo-kreisel tank (*see* **Notes 21** and **22**).
12. Repeat **steps 8–10** to process all the fertilized embryos.
13. Wait for 24 h for the embryos to hatch (*see* **Note 23**).

14. Transfer about two freshly isolated rotifers (*see* Subheading 3.3) per embryo in each bowl within 24 h after fertilization to ensure food availability immediately after hatching.
15. Wait for 24 h for cydippids to grow.
16. Transfer 1-day old cydippids using a plastic transfer pipette into a clean glass bowl filled with fresh 1× UV-FSW (*see* **Notes 24** and **25**).
17. Feed about two freshly rotifers isolated per cydippid in each bowl.
18. Repeat **steps 15–17** for three more days.
19. Transfer 4-day old cydippids (about ~1.5–2 mm in size) into a 2 L glass vertical beaker (*see* **Note 26**).
20. Complement feeding with just-hatched *Artemia* nauplii larvae (1 *Artemia* per cydippid, *see* Subheading 3.3). Alternate rotifer and *Artemia* feedings every other day (*see* **Note 27**). This is a convenient size to perform regeneration experiments (*see* **Note 28**) (*see* Subheading 3.5).
21. Wait 12–24 h for animals to feed.
22. Clean the cultures by transferring the animals into a clean 2 L glass beaker filled with fresh 1× UV-FSW.
23. Repeat **steps 19–22** for two more weeks (*see* **Note 29**).
24. Transfer the lobate stage ctenophores into the ctenophore adult tank using a beaker.
25. Feed lobate ctenophores with a diet combining *Artemia*, mysid shrimps, and wild zooplankton and/or fish larvae if possible (*see* Subheading 3.3).

3.5 Animal Surgeries to Study Wound Healing and Whole-Body Regeneration

Although lobate stage adults have a high capacity to regenerate, we utilize *M. leidyi* cydippid stages due to their smaller size, speed of complete regeneration, and ease of visualization [5].

1. Use the microburner to hand-pull several Pyrex glass needles utilized for surgical operations (*see* **Notes 30–32**).
2. Collect cydippids of 1.5–3 mm diameter (~6–14 days postfertilization) and place them in a separate small glass dish (*see* **Note 33**).

Follow the steps described below according to the type of operation. All operations are performed at room temperature (20–22 °C).

Puncture assay (Fig. 4B):

3. Place one cydippid in a small drop of water on a siliconized microscope slide.

4. Using a pair of sharp forceps, puncture the animal by pinching the epithelium layer.
5. Check for the presence of an epithelial gap with the edges of the wound forming a small gap (diameter ~200–400 μm), exposing the mesoglea (*see* **Note 34**) [10].
6. Immediately after puncture, mount animals for live imaging (*see* Subheading 3.7 for details).

Whole-body regeneration studies (WBR):

7. Transfer cydippids using a plastic or glass pipette larger than the diameter of the specimen to an operating dish filled with just enough 1× 0.2-μm UV-FSW to cover the specimens (*see* **Note 35**).

We use three types of operations to recover all the structures/cell types of the cydippid's body.

8. To perform **oral–aboral bisections**, cut the cydippid longitudinally through the esophageal plane generating two "half animals" (*see* **Note 36**). The operations are performed such that one half retains an intact apical organ while the remaining half lacks the apical organ. Only the halves retaining the apical organ are kept for studies of WBR as these halves regenerate to whole animals in a high percentage of the cases (Fig. 4C) [5, 10]. Oral–aboral cuts that lack the apical organ either regenerate complete animals, or remain as "half" animals [5, 33] (*see* **Note 37**).
9. To perform **apical organ amputations,** remove the whole apical organ structure (including the entire statolith system, dome cilia and ciliated grooves) by cutting perpendicular to the oral–aboral axis above the level of the tentacle bulbs (Fig. 4C).
10. To perform **tentacle bulb amputations**, dissect the epidermis surrounding the tentacle bulb so the entire sheath and endodermal tentacular canal are removed (Fig. 4C) [10].
11. Transfer operated cydippids into a 35-mm plastic petri dish filled with 1× 0.2-μm UV-FSW using a pipette larger than the diameter of the specimen.
12. Clean operated cydippids once a day every day during regeneration by transferring them into a new small plastic petri dish filled with fresh 1× 0.2-μm UV-FSW (*see* **Notes 38** and **39**).

3.6 Cell Proliferation Inhibitor Treatment with Hydroxyurea (HU)

The role of cell proliferation in replacement of missing cell types was first proposed by TH Morgan more than 100 years ago [34]. Cell proliferation inhibitor experiments are a straightforward way to evaluate the requirement of cell proliferation in regeneration. We expose amputated cydippids to hydroxyurea (HU) treatments, a drug that inhibits cell proliferation by inhibiting the ribonucleotide reductase enzyme and thereby arresting cells in S-phase [35].

1. Prepare a 500 mM stock solution of HU by dissolving HU powder in distilled water.
2. Dilute the HU stock 1:100 to a working concentration of 5 mM in 1× UV-FSW (*see* **Note 40**). Once you have thawed an aliquot of stock solution for use, keep it at 4 °C.
3. Add 3 mL of 5 mM HU into a 35 mm plastic petri dish and transfer the amputated cydippids (not more than 50 per dish) (*see* **Note 41**).
4. Incubate HU treatments at room temperature for the desired time (*see* **Note 42**).
5. Replace HU working solution with fresh HU every 12 h (*see* **Note 43**).
6. You may now process the animals with downstream treatments like fixation (*see* Subheading 3.7) or wash out the HU to continue the experiment with living animals (*see* **Notes 44** and **45**).

3.7 Fixation of Mnemiopsis leidyi *Cydippids*

The gelatinous body of *M. leidyi* cydippids is mostly composed of mesoglea with varying osmotic proprieties, which makes standard fixation protocols challenging; standard fixative preparations in direct contact with the cydippid's body generate osmotic changes that cause the structural mesoglea to collapse and tissue to disintegrate. To preserve both the cellular and gross anatomic integrity, we use a fixation protocol based on embedding of specimens in a low melting point agarose [32].

1. Melt the 1.2% low melt agarose by warming the solution to 30 °C in a water bath (*see* **Notes 46** and **47**).
2. Place the specimens with the minimum volume (~10 μL FSW) in a 35-mm plastic petri dish.
3. Carefully add 100 μL of liquid agarose (approximately 10:1 agar:water drop volume) to the specimens (*see* **Note 48**).
4. Mix well but gently until the mixtures is homogenous and the specimens are fully embedded into the agar.
5. Let the embedded cydippids in agar cool for ~2 min (*see* **Note 49**).
6. Carefully place a coverslip over the agar drop and press down to form an agar lamina of homogenous thickness with the cydippids embedded inside. This step will facilitate mounting for microscopy.
7. Let the agar cool completely by incubating the petri dish on ice for ~5 min.
8. Once the agar is solidified, carefully remove the coverslip with forceps to allow better penetration of the fixative solution.

9. Add 3 mL of ice-cold fixative solution to the plastic petri dish (*see* **Note 50**).
10. Incubate for 1.5–2 h on a rocking platform at room temperature.
11. Discard the fixative solution and wash twice with PBS-0.02% Triton X-100 for 10 min on a rocking platform at room temperature.
12. Using a razor blade, carefully cut the agar lamina into cubes such that each cube contains an intact cydippid.
13. Transfer the agar cubes into a 2 mL tube using a plastic transfer pipette with a widened opening.
14. Specimens may now be labeled as desired. Fixed cydippids are kept in agar for all downstream preparations.

3.8 Live Imaging During Wound Healing and Regeneration

The remarkable optical clarity and small size of *M. leidyi* cydippids make them an ideal system for live-imaging experiments. Here we describe a combination of differential interference contrast (DIC) live-imaging and time-lapse techniques to monitor wound healing. *M. leidyi* wound healing involves cell migration and formation of actin-based cellular protrusions, resulting in a scar-less wound epithelium [10]. Under normal conditions wound healing is completed in around 30 min to 1 h after injury, depending on the size of the cut.

1. Place one punctured cydippid on a siliconized slide in the minimum volume (~10 μL drop of FSW).
2. Immobilize the animal by slowly applying 100 μL of optically transparent jammed microgel around the drop of water.
3. Carefully mix using the pipette tip until the specimen is surrounded by homogenous microgel (*see* **Note 51**).
4. Using a pair of fine forceps, orient the animal so that the epithelial wound faces upward (*see* **Note 52**).
5. Prepare a coverslip with clay feet on each of the four corners and place it over the preparation.
6. Press down the coverslip gently, being careful not to overcompress the animal.
7. For longer live-imaging times (>2 h), seal the edges of the coverslip with Vaseline to prevent evaporation (*see* **Note 53**).
8. Mount the slide on the microscope for image acquisition.
9. Locate the specimen using the bright-field mode of your microscope in ocular position and a low magnification objective (e.g., 5× or 10×).
10. Bring the region of interest (wound gap) into focus.

11. Change to a higher 20× magnification objective for wound-healing time-lapse experiments (*see* **Note 54**).
12. We recommend acquiring a z-stack at each time point. The z-stack should go into the mesoglea in order to visualize migration of cells from the mesoglea to the wound edges (*see* **Note 55**).
13. We found wound healing to be sufficiently resolved with a time-lapse setting of 30 s to 1-min intervals over 2 h.

4 Notes

1. Ratios of FSW: deionized water might need to be adapted depending on the starting salinity of the seawater.
2. PBS can be made directly as a 1× solution or diluted 1/10 from a 10× PBS stock solution.
3. Optimal conditions for ctenophore collection consist of a clear, sunny day (to avoid cloud reflections in the water) with no wind and slow water currents at ebb tide.
4. The best time of the day for collecting is when the sun is high in the sky and at your back. Polarized sunglasses can improve visibility.
5. Ctenophores swim to the surface of the water column during sunny hours, however, even the slightest wave action will make them sink to calmer water.
6. Ctenophores are extremely fragile animals and care must be taken to avoid touching specimens with nets or bare hands which could damage their outer epidermis. They should always be kept submerged and transferred gently between containers (e.g., plastic or glass beakers).
7. Ctenophores are open ocean animals but some species, like *M. leidyi,* are relatively common along the coast and can be found in a wide variety of different salinities (e.g., salt marshes). Ctenophores tend to be found in groups. If you see one *M. leidyi* it is likely you will see others.
8. In the wild, ctenophores feed on a diverse diet consisting in all types of zooplankton, fish eggs and larvae, so a diet of *Artemia* alone is not sufficient for maintaining healthy *M. leidyi* for long periods of time. Hatchlings can be efficiently grown with a diet of rotifers, however, a diverse diet including *Artemia* brine shrimp accelerates their growth rate. Wait until cydippids are larger than the brine shrimp (>1.5 mm diameter) before attempting to feed *Artemia*. Once cydippids transition into the lobate state, their diet should be diversified with foods such as mysid shrimp, fish larvae, and ideally wild plankton.

9. If adult *M. leidyi* are starved, they will begin to resorb their oral lobes and slowly shrink in size.
10. Overfeeding should also be avoided. Overfed *M. leidyi* will regurgitate ingested food and the undigested food will foul the water.
11. Rotifers:
    (a) We use *Brachionus plicatilis* (L-type) rotifers (Reed Mariculture, Campbell, CA, USA).
    (b) We recommend growing two asynchronous rotifer cultures at the same time and alternate harvesting between them. Excessive repeated harvesting (>50%) in one single culture could lead to the crash of the culture.
    (c) In order to maintain the productivity, it is important to not let the rotifers run out of food. Maintain a detectable light green tint in the water between feedings (https://reedmariculture.com/support_rotifers.php).
    (d) It is recommended to feed rotifers every day. If this is not possible, add the volume of food required for the days they will not be fed (ideally not more than 4). The accumulation of debris at the bottom could be a sign of overfeeding.
    (e) The stability of the rotifer culture is based on finding the right balance between harvesting and feeding. In a healthy culture all or the majority of the rotifers will be females and will reproduce clonally. An increase in the proportion of males (smaller individuals) in the culture is indicative of an unbalanced and stressed culture.
    (f) In case the rotifer culture cannot be maintained/harvested for longer than 4 days, there is a way to put rotifers in "hibernation mode" by setting up a backup culture. Harvest ~40% of your culture and transfer it into a 1 L container filled with 20 ppt FSW. Add a bit of extra algae concentrate to darken the culture. Let the culture uncovered or cover loosely to allow oxygen to enter and keep it at 4 °C to slower the metabolism of rotifers. After 7 days 50% of your rotifers should be alive.
12. Brine shrimp show positive phototaxis so hatched swimming *Artemia* larvae will concentrate at the light point high in the water column after few minutes.
13. Feeding the adult ctenophores with ~2 mysid shrimps per ctenophore the day before spawning increases the success and rate of gamete production.

14. Use plastic or glass beakers to dip out individual animals when transferring animals between tanks and containers in order to avoid damage to their gelatinous body.
15. *M. leidyi* are self-fertile hermaphrodites—a single animal carries both female and male gonads—and viable embryos can be obtained from the spawning of a single individual but they produce more viable offspring in the presence of conspecifics [36].
16. It is important to keep specimens in clean water while spawning in order to avoid debris and mucus which could interfere with fertilization and development.
17. One single healthy and well-fed specimen can give up to 500 embryos. We recommend using between 5 and 10 adults for spawning taking into account that from the starting number of embryos, around 2/3 end up developing into the cydippid stage.
18. You can place the bowls inside an incubator or cover them with a cardboard box.
19. Before spawning, the meridional canals below the comb rows may turn cloudy indicating the presence of sperm. Sperm is released before the eggs. After sperm are released the water of the bowl gets cloudy. Then the eggs are released 10–15 min after and are fertilized upon release.
20. Ctenophores can sometimes release a few eggs before the bulk of spawning starts. To ensure a batch of synchronously developing embryos, animals can be checked after 1.5–2 h of dark treatment and transferred into a bowl of fresh 1× UV-FSW if gametes are detected early.
21. Avoid keeping adult specimens in the small glass culture bowls after spawning otherwise both adult and embryo quality will deteriorate rapidly as the adult's waste and mucus accumulate. As soon as the eggs are released, remove adult specimens from the bowl and transfer fertilized embryos into another glass bowl filled with fresh 1× UV-FSW.
22. *M. leidyi* adults can be spawned multiple times a week if fed well and given sufficient time to recover between spawning cycles. We recommend waiting 1–2 days to spawn the animals again and feed them with shrimps within this time period.
23. Embryo development should be relatively synchronous, with the first cleavage occurring 1 h after fertilization and subsequent cleavages every 20 min thereafter. Gastrulation occurs at 3–4 h and hatching at 18–24 h [31].
24. Embryos are very sensitive and exposure to any type of chemical could drastically affect their development. Make sure that all culturing glassware and solutions are dedicated to this purpose

and kept free of detergents or any other harmful chemicals. Culturing materials should be cleaned exclusively with tap water followed by a final rinse with distilled water.

25. Clean water is essential to ensure the survival of just-hatched cydippids. Cleaning should be done once a day during the first 3 days and every other day thereafter. Use a plastic transfer pipette two times the diameter of the specimens for transferring cydippids into the clean glass bowl. To fit cydippids into the pipette as they grow, the plastic pipette opening can be widened by cutting with a razor blade.
26. Transfer growing cydippids into a larger volume and taller container (e.g., 2 L glass beaker) ~4 days after hatching in order to give them more space for swimming vertically and feeding in the water column. Cydippids feed by setting their tentacles and if they are too crowded their tentacles get tangled.
27. Use newly hatched *Artemia* (18–20 h after starting the culture) for feedings. *Artemia* rapidly loose nutritional value after hatching due to their use of internal stores for swimming. They can be fed with live algae and grown to larger sizes if desired.
28. We find that 1.5–3 mm diameter animals are a good size for performing regeneration experiments, since at this stage cydippids have completely developed all the organs of their body. Moreover, cydippids at this size have high fitness and the ability to withstand starvation which increases the success of regeneration.
29. Properly fed cydippids transition to the lobate state 3–4 weeks after hatching. To culture into adulthood, animals at this stage should be transferred to the ctenophore adult tank and feeding must be complemented with foods such as wild zooplankton, mysid shrimps, and/or fish larvae.
30. Pyrex glass is stiffer than borosilicate/aluminosilicate needle stock used for microinjection.
31. We recommend pulling several operating needles before you start. Because every needle will be a little bit different than the others, you will find the size and length that works best for you. Plus, if you break a needle during a surgery, you will have "backups" so that you can finish your experiment. Once you learn what shape you like, you can continue to pull additional needles that work best for you.
32. We like needles that are flexible enough so that you can put the tip on the bottom of the dish on the opposite side of the region you want to remove, and then move the needle down, sort of like a paper cutter. You can drive the needle down into the bottom of the dish to complete the cut.

33. Stop feeding the day before the operations and starve animals during regeneration in order to avoid food particles that can interfere with live imaging and cause nonspecific staining.
34. For live-imaging of wound healing, we puncture animals to generate small epithelial gaps approximately 200–400 μm in diameter which have been consistently proven to heal rapidly when exposed to the live-imaging setup conditions and can be visualize with high magnification objectives.
35. Keeping a low level of water in the surgical dish can help reduce the mobility of animals when cutting. If more immobilization is required, the dish with cydippids can be incubated on ice ~10 min before cutting.
36. We find it effective to use two glass needles when cutting, one for immobilizing and orienting the animal and the other for performing the cut.
37. When juvenile or adult ctenophores are bisected along the oral–aboral axis, an incomplete regeneration event will occur in a substantial percentage of the cases [4, 5]. Wound healing proceeds normally, but instead of replacing the missing parts, a stable "half-animal" forms. These animals possess half the typical complement of body structures, that is, one tentacle, four comb rows and a half apical organ. They are considered stable since they can feed, grow, and reproduce. We found that bisected cydippids containing an intact apical organ regenerate into whole animals in nearly 100% of cases in contrast to bisected cydippids with a half or missing apical organ which are more likely to forego whole-body regeneration and remain instead as "half animals" [10].
38. Accumulation of debris in the water can inhibit regeneration. Clean regenerating cydippids daily by transferring them into a new small plastic petri dish filled with 1× 0.2-μm UV-FSW.
39. We do not feed during regeneration as food in the gut could interfere with sample imaging but amputated animals could be fed when used for other experimental purposes (i.e., scoring ability to regenerate after amputation).
40. We set 5 mM HU as the working concentration for our cell proliferation inhibitor experiments since at this concentration control nonamputated cydippids remain in good condition, swimming normally, with no cell death over a 72 h time course incubation. We found concentrations higher than 5 mM to cause the degeneration and eventually death of most of the animals during the first 24 h of incubation [10].
41. Start incubating cydippids with 5 mM HU ~30 min before performing the surgeries to be sure that cell proliferation is completely blocked from the very beginning of the regenerative response.

42. HU is light-sensitive so dishes need to be protected from light (wrapped with aluminum foil or covered with a cardboard box).
43. HU loses efficacy over time so 5 mM HU working solution must be replaced every 24 h or less when doing a continuous incubation. Make 5 mM HU working concentration fresh each time you replace the solution.
44. Always confirm that HU treatments were effective by checking for cell proliferation in treated animals (e.g., with EdU labeling).
45. The inhibition of the ribonucleotide reductase enzyme by HU is reversible [37]. Wash out the HU working solution and replace it with 1× 0.2-μm UV-FSW to allow proliferation to resume.
46. It is important to homogenize the liquid agar very well (e.g., by vortexing) when first preparing the 1.2% agarose solution and again just before use when melting previously prepared 1.2% agarose. Incomplete homogenization can lead to irregular agar concentrations within the solution, impairing the embedding process.
47. The temperature of the liquid agar is crucial for the success of embedding. Too hot liquid agar disintegrates the cydippids while too cool agar sets before it can be thoroughly mixed with the sample. We recommend melting the agar at higher temperature (water bath above 30 °C or submerge in boiling water in a glass beaker) to ensure complete melting, and then letting it cool down for ~5 min until reaching a temperature slightly above the working temperature of 25 °C (~30–40 °C).
48. Do not pour the liquid agar directly on top of the drop containing live cydippids since the high temperature can cause instant disintegration. Instead, gently pipet the liquid agar from the sides surrounding the drop containing the sample and allow it to merge with the seawater. It is also important to homogenize the agar with the cydippids in seawater by carefully pipetting up and down 2 or 3 times.
49. Let the agar cool down for ~2 min before placing the coverslip on top. This helps in creating a small resistance from the agar when placing the coverslip on top which favors the formation of a homogeneous agar lamina with intact cydippids inside. If the agar is too liquid, cydippids get squished when placing the coverslip on top.
50. Use fresh fixative solution; it can be kept at 4 °C for up to 3 days.
51. For time lapse imaging immobilizing the specimen is of critical importance. For some experiments, increasing the viscosity

with methyl cellulose can suffice. For longer periods synthetic hydrogels [10] may be of use. We find 7.5% hydrogel as described the optimal concentration for our wound-healing time-lapse experiments, in terms of keeping a good balance between osmolarity of the medium and immobilization of the specimen. However, we recommend trying different hydrogel concentrations depending on the mobility of the animal and the length of the experiment. Lower hydrogel concentrations are more osmotically compatible with the animal but they also are less effective for immobilization while higher hydrogel concentrations allow for better immobilization but tend to dehydrate the specimens. Optimize this tradeoff for specific experiments.

52. It is convenient when orienting and immobilizing the animals to make the epithelial puncture on the aboral side of the cydippid's body since cydippids mounted with the aboral pole facing upward tend to stay more still compared to laterally mounted cydippids.
53. A coverslip with clay feet placed over the microgel drop is sufficient for maintaining humidity during short live-imaging experiments. For longer time-lapse experiments (>2 h) seal the edges of the coverslip with Vaseline to prevent evaporation. Under ideal circumstances, we have been able to image regeneration in *M. leidyi* for 12–16+ h. Specimens can also be mounted into a glass depression slide with a larger volume of microgel in order to maintain greater humidity; however, animals are less immobilized in this system and the sample is thicker which may restrict the magnification to lower-powered objectives.
54. For wounds of approximately 200–400 μm in diameter, we recommend using a 20× objective which provides sufficient magnification for detailed visualization of individual cell crawling and cytoskeletal extensions with a large enough field of view to include the complete epithelial gap.
55. Specimens are likely to move out of focus during the acquisition so setting up a range of stacks along the *z* axis will allow you to select the better planes upon playback.

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