Video Article

Chemical Amputation and Regeneration of the Pharynx in the Planarian *Schmidtea mediterranea*

Divya A. Shiroor[1], Tisha E. Bohr[1], Carolyn E. Adler[1]

[1]Department of Molecular Medicine, College of Veterinary Medicine, Cornell University

Correspondence to: Carolyn E. Adler at cea88@cornell.edu

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Abstract

Planarians are flatworms that are extremely efficient at regeneration. They owe this ability to a large number of stem cells that can rapidly respond to any type of injury. Common injury models in these animals remove large amounts of tissue, which damages multiple organs. To overcome this broad tissue damage, we describe here a method to selectively remove a single organ, the pharynx, in the planarian *Schmidtea mediterranea*. We achieve this by soaking animals in a solution containing the cytochrome oxidase inhibitor sodium azide. Brief exposure to sodium azide causes extrusion of the pharynx from the animal, which we call "chemical amputation." Chemical amputation removes the entire pharynx, and generates a small wound where the pharynx attaches to the intestine. After extensive rinsing, all amputated animals regenerate a fully functional pharynx in approximately one week. Stem cells in the rest of the body drive regeneration of the new pharynx. Here, we provide a detailed protocol for chemical amputation, and describe both histological and behavioral methods to assess successful amputation and regeneration.

Video Link

The video component of this article can be found at https://www.jove.com/video/57168/

Introduction

Regeneration is a phenomenon that occurs throughout the animal kingdom, with regenerative capacities ranging from full body regeneration in certain invertebrates to more restricted abilities in vertebrates[1]. Replacement of functional tissues is a complex process and often entails the simultaneous restoration of multiple cell types. For example, to regenerate the salamander limb, osteoblasts, chondrocytes, neurons, muscles, and epithelial cells need to be replaced[2]. These newly generated cell types also need to be organized properly to facilitate new limb function. Understanding these complex processes requires techniques that focus on regeneration of specific cell types and their integration into organs.

One of the strategies employed to simplify the study of regenerative responses is the targeted ablation of either certain cell types or larger collections of tissues. For example, in zebrafish, expression of nitroreductase in specific cell types leads to their destruction after application of metronidazole[3,4]. In *Drosophila* larvae, expressing pro-apoptotic genes under tissue-specific promoters can selectively ablate specific regions of the imaginal disc[5,6]. Both of these strategies cause rapid but controlled damage, and have been used to dissect the molecular and cellular mechanisms responsible for regeneration.

In this manuscript, we describe a method to selectively ablate an entire organ called the pharynx in the planarian *Schmidtea mediterranea*. Planarians are a classical model of regeneration, known for their prolific regenerative ability, where even minute fragments can regrow whole animals [7,8]. They have a large, heterogeneous population of stem cells consisting of both pluripotent cells and lineage-restricted progenitors[9,10,11]. These cells proliferate and differentiate to replace all missing tissues, including the pharynx, nervous, digestive and excretory systems, and muscle and epithelial cells[9,10,12]. While we know that these stem cells initiate regeneration, we do not fully comprehend the molecular mechanisms that drive them to replace all these different cell types. Defined wounding methods that elicit precise stem cell responses can help delineate this complex process.

The pharynx is a large, cylindrical tube required for feeding, and contains neurons, muscle, epithelial and secretory cells[13,29]. Normally hidden in a pouch on the ventral side of the animal, it extends through the animal's single body opening upon sensing the presence of food. To selectively amputate the pharynx, we soak planarians in a chemical called sodium azide, a commonly used anesthetic in *C. elegans*[14,15,16]. Its use in planarians was first reported by Adler *et al*., in 2014[12]. Within minutes of exposure to sodium azide, planarians extrude their pharynges, and with gentle agitation, the pharynx detaches from the animal. We refer to this complete and selective loss of the pharynx as "chemical amputation". One week after amputation, a fully functional pharynx is restored[12]. Because the pharynx is required for feeding, functional regeneration can be measured by monitoring feeding behavior. Below, we describe the protocol for chemical amputation, and for assessing the regeneration of the pharynx and restoration of feeding behavior.

Protocol

1. Preparation

1. **Preparation of planaria water**[17]
    1. Maintain planarians in a 1X Montjuïc salt solution. To prepare planarian water, make individual stock solutions of 1 M $CaCl_2$, 1 M $MgSO_4$, 1 M $MgCl_2$, 1 M KCl and 5 M NaCl in ultrapure water. Filter-sterilize with a 0.2 µm bottle-top filterfor long-term storage.
    Note: Use only ultrapure deionized water (with a resistivity of 18.2 MΩ at 25 °C) to prepare Montjuïc salts.
    2. To prepare a 1 L stock of 5X salt solution, combine 5 mL of 1 M $CaCl_2$, 5 mL of 1 M $MgSO_4$, 0.5 mL of $MgCl_2$, 0.5 mL of KCl and 1.6 mL of 5 M NaCl solutions in 900 mL ultrapure water. To this solution, add 0.504 g of $NaHCO_3$ and stir to mix. Adjust the pH to 7.0 with hydrochloric acid.
    3. Dilute this 5X stock solution to a 1X working concentration in ultrapure water in a sterile container such as a large capacity carboy (see the **Table of Materials**). Use this 1X solution for maintaining asexual planarians.
    Note: As an alternative to Montjuïc salt solution, use locally-purchased spring water or ultrapure water containing a commercially available aquarium salt mix (see **Table of Materials**) at a concentration of0.5 g/L[18].
    4. To prevent bacterial infection in static culture[19], maintain planarians in water containing an antibiotic. Prepare a 50 mg/mL stock solution of gentamicin sulfate in ultrapure water and filter-sterilize. To containers where animals are maintained, add gentamicin sulfate to a final concentration of 50 µg/mL (1:1000 dilution).

2. **Preparation of liver paste**
    1. As planarians thrive on a diet of organic, grass-fed beef liver, purchase fresh liver and process within 24 h. Remove the membranous capsule encapsulating the liver by peeling it off gently. Cut the liver into ~1-cm cubes, and use a blade to scrape off and discard all hepatic veins and arteries.
    2. Macerate liver pieces using a food mill or a food processor and then pass it through a wire mesh food strainer. Combine into pastry bags, and dispense into syringes or 35-mm Petri dishes. Prior to feeding, centrifuge gently to remove air bubbles.
    3. Store aliquots of liver at -80 °C for up to a year and thaw prior to using. After thawing, re-freeze any leftover liver once, or store at 4 °C for up to 24 h.

3. **Maintenance of animals**
    1. Planarians will grow and shrink to various sizes depending on the frequency of feeding. Feed planarians every other week (once every 14 days). For long-term bulk cultures, use plastic containers of various sizes (see **Table of Materials**).
    2. To feed animals, use a metal spatula or plastic transfer pipet to place a pea-sized drop of liver in a box. Allow animals to eat for 1-2 h. Remove remaining food before cleaning the box.
    3. Clean worms twice a week if fed (once directly after feeding and once two days later), or once a week if not fed. To clean, drain water into a plastic beaker by carefully pouring out the water while retaining planarians in the box. Wipe box surface with a paper towel, then repeat on all sides until the box is clean.
    4. Replace water (to approximately ¾ of the box volume) and add gentamicin to a final concentration of 50 µg/mL. Store animals in a dark cabinet or in a 20 °C incubator.

4. **Selection of worms**
    1. Select worms that were fed 5-7 days prior.
    Note: It is significantly more difficult to amputate pharynges from worms that have been recently fed (1-2 days prior to amputation). Fed animals are also more sensitive to sodium azide.
    2. Select worms that are roughly 6 mm in length. To estimate the size of the worm, transfer it to a Petri dish using a plastic transfer pipette. Place either a flat 6-in ruler or 5 mm x 5 mm graph paper underneath the Petri dish and measure the worm length while it crawls.
    Note: Worms smaller than 6 mm in length are more difficult to amputate.

5. **Preparation of sodium azide**
    1. Prepare a 100 mM solution by dissolving sodium azide powder in planaria water.
    CAUTION: Sodium azide is toxic and should be handled carefully. Do not discard sodium azide down the drain. Following use, collect it separately for disposal as hazardous waste.

2. Pharynx Amputation

1. Place worms in a 35-mm diameter Petri dish. Carefully remove all planarian water from the dish by using a plastic transfer pipet.
Note: A maximum of 20 6-mm worms can be comfortably accommodated in a dish of this size.
2. Place the dish under the microscope and adjust magnification so that multiple worms can be viewed simultaneously (*e.g.* 10X magnification). Replace planarian water with 100 mM sodium azide solution.
Note: For a 35-mm Petri dish, approximately 5 mL of sodium azide is sufficient. The solution should completely submerge planarians. Adjust solution volume for larger dishes as necessary.
3. Monitor the animals under the microscope and refrain from moving the dish for the first 3-4 min.
4. Observe the extrusion of the pharynx through the microscope; the pharynx will protrude from the pharyngeal pouch and extend fully (approximately 1 mm in length) as shown in **Figure 1B**.
Note: It is important to wait until the pharynx has finished extending completely before proceeding further. Once the pharynx emerges from the animal, full extension takes roughly 1 min.

5. Use a plastic transfer pipet to squirt animals around the dish. Suck the animals into the pipet and forcibly release them into the dish a couple of times. If the pharynx is fully extended, this vigorous pipetting will cause it to detach.
6. Alternatively, grasp the pharynx either vertically along its length, or horizontally along its circumference using a pair of fine forceps (see **Table of Materials**) as shown in **Figure 1C**. With the pharynx pinched in the forceps, lift the animal upwards towards the meniscus. As the animal is raised, surface tension will cause the pharynx to detach from the animal.
   Note: This method can be used to remove the pharynges of smaller animals (1-3 mm in length) where forceful swirling is not adequate. Avoid poking or otherwise injuring the body of the animal.
7. Using a transfer pipet, move amputated animals to a new dish containing fresh planaria water.
8. Once transferred, rinse amputated animals thoroughly in planaria water, completely exchanging the water three times. Transfer to a new dish containing planaria water with 50µg/mL of gentamicin.
   Note: Complete buffer exchange during these three washes is essential to ensure removal of sodium azide.
9. The next day, replace water in the dish with fresh planaria water containing 50 µg/mL of gentamicin. Repeat every other day thereafter.

3. Assessment of Pharynx Removal After Amputation

1. Examine animals under a microscope (with 10-20X magnification) to determine whether a dark spot has appeared after successful removal of the pharynx, as shown in **Figure 2A**.
2. Alternatively, fix and bleach animals according to the protocol described by Pearson *et al.*[20]. Soak animals overnight in 4',6-diamidino-2-phenylindole (DAPI) or fluorescently-conjugated streptavidin (1 mg/mL diluted 1:500 in 1X Phosphate Buffered Saline containing 0.3% Triton-X). Mount animals on slides in 80% glycerol/20% 1X Phosphate Buffered Saline and image under a fluorescent stereomicroscope (**Figure 2B**).
   Note: All images in this manuscript were captured on a fluorescent stereomicroscope with a 1.0X objective.

4. Assessment of Pharynx Regeneration by Measuring Feeding Behavior

1. **Preparation of liver**
   1. Transfer required quantity of liver paste into a microcentrifuge or conical tube. Spin briefly to remove air bubbles. Estimate the volume of liver, and then add planaria water to 1/5 of its volume, and 2% of the total volume in red food coloring. For example, to 1000 µL of liver paste, add 200 µL of planaria water and 24 µL of food coloring.
   2. Using a plastic pestle, pipette tip or metal spatula, mix thoroughly until food coloring is evenly distributed in the liver paste. Spin again briefly. Liver paste can be stored at 4 °C for 24 h or frozen in aliquots at -80 °C for long-term storage.
   3. If testing up to 25 animals, prepare 25 µL of liver paste per dish (approximately 1 µL of liver paste per animal).

2. **Feeding animals**
   1. Seven days after chemical amputation, transfer animals to a new Petri dish. If testing 10-15 animals, use a 35 mm dish; more than 15 animals should be tested in a 60-mm Petri dish. Keep animals in the dark, undisturbed, for approximately 1 h prior to feeding.
   2. Using scissors, increase the width of a P200 pipette tip by trimming roughly ½ cm off of the narrow end. Pipet 25 µL of red liver paste into the dish.
      Note: Trimming the end makes pipetting the viscous liver paste easier. To prevent the liver from floating on the surface, touch the tip to the bottom of the dish while dispensing.
   3. Allow animals to feed for 30 min.
   4. Score the number of animals that have eaten by placing the animals on a white background or examining them under a microscope with 10-20X magnification.
   5. After feeding, remove liver from the dish and clean.

Representative Results

Exposure to sodium azide disrupts the normal motility of planarians, causing animals to stretch and writhe. These movements force the pharynx to emerge from the ventral side of the animal, and after approximately 6 min in sodium azide solution, the white tip of the pharynx can be seen **(Figure 1B-left panel)**. A few minutes later, animals actively contract and fully extend the pharynx by forcefully pushing it out of the body. **(Figure 1B-middle panel)**. Approximately 11 min after azide exposure, the animals and the pharynx relax. At this stage, the characteristic bell shape of the pharynx is clearly visible **(Figure 1B-right panel)**. For easy amputation, it is important to wait for the pharynx to relax.

Because the pharynx is a large, unpigmented mass of tissue, removing it results in the appearance of a dark spot on the dorsal side of amputated animals **(Figure 2A)**. This darkened region is a visual indicator of successful amputation in live animals. The spot is visible immediately after amputation and becomes more prominent the next day. As the pharynx regenerates, this region lightens. To monitor pharynx regeneration more precisely, animals can be stained with DAPI or fluorescently-conjugated streptavidin overnight **(Figure 2B)**. To quantitatively assess the extent of pharynx regeneration, its area can be measured using ImageJ software.

The pharynx is an essential organ for chemosensation and food ingestion. To determine when these functions are restored during regeneration, we used feeding assays to monitor animal behavior. By combining food coloring with liver paste, we were able to distinguish animals that ate from those that did not **(Figure 3A)**. We then quantified the number of red animals to assess the percentage of animals with a functional pharynx. Based on the results shown in **Figure 3B**, successful feeding behavior was restored 7 days after amputation, indicating that one week after pharynx removal, animals have regenerated a functional pharynx. To test whether sodium azide exposure affected feeding behavior, we soaked animals in sodium azide but washed it out immediately prior to pharynx ejection. One day later, we evaluated whether food-seeking behavior was altered by sodium azide exposure with the feeding assay. In contrast to chemically-amputated animals lacking a pharynx, all animals retaining an intact pharynx after sodium azide exposure ate (n=30 animals for each condition) the food. This result indicates that sodium azide exposure does not affect feeding behavior, as long as the pharynx remains intact.

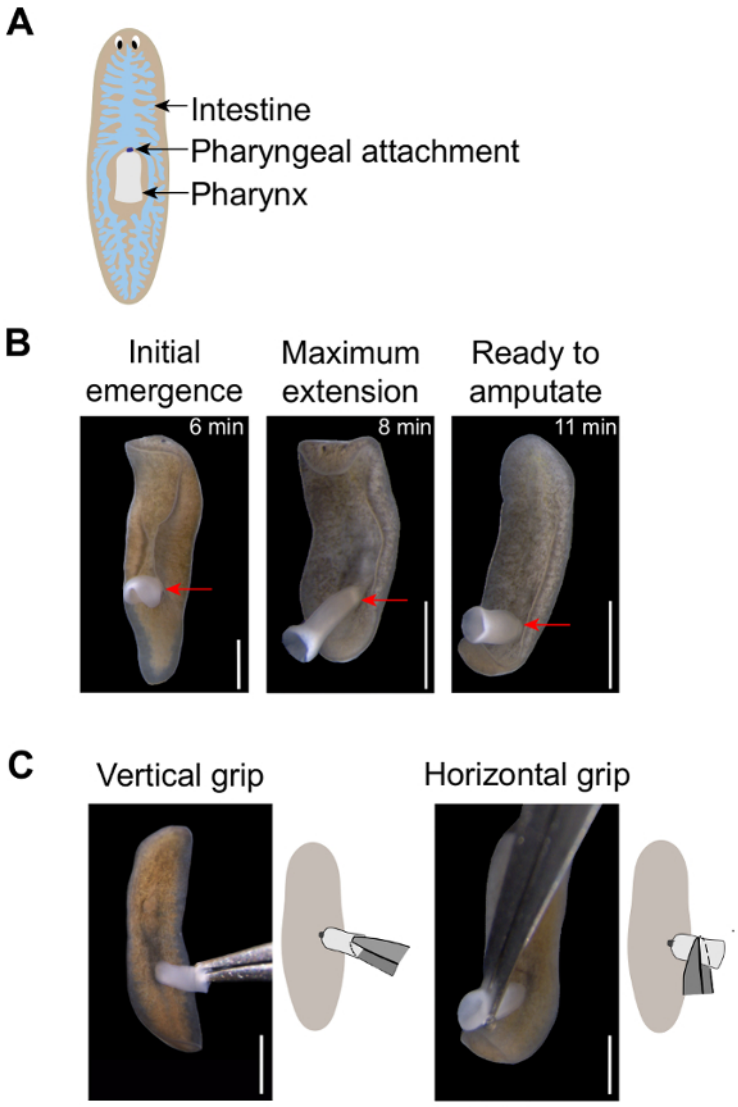

Figure 1. Pharynx ejection and amputation.(A) Schematic of planarian anatomy. **(B)** Images of live animals showing pharynx ejection upon soaking in 100 mM sodium azide. Red arrows highlight pharynx. Scale bars = 750 µm. **(C)** Image of live animal (left) and schematic (right) of amputation using forceps. Panels show two different options for gripping the pharynx. Ventral side of the animal faces up. Scale bars = 500 µm.
Please click here to view a larger version of this figure.

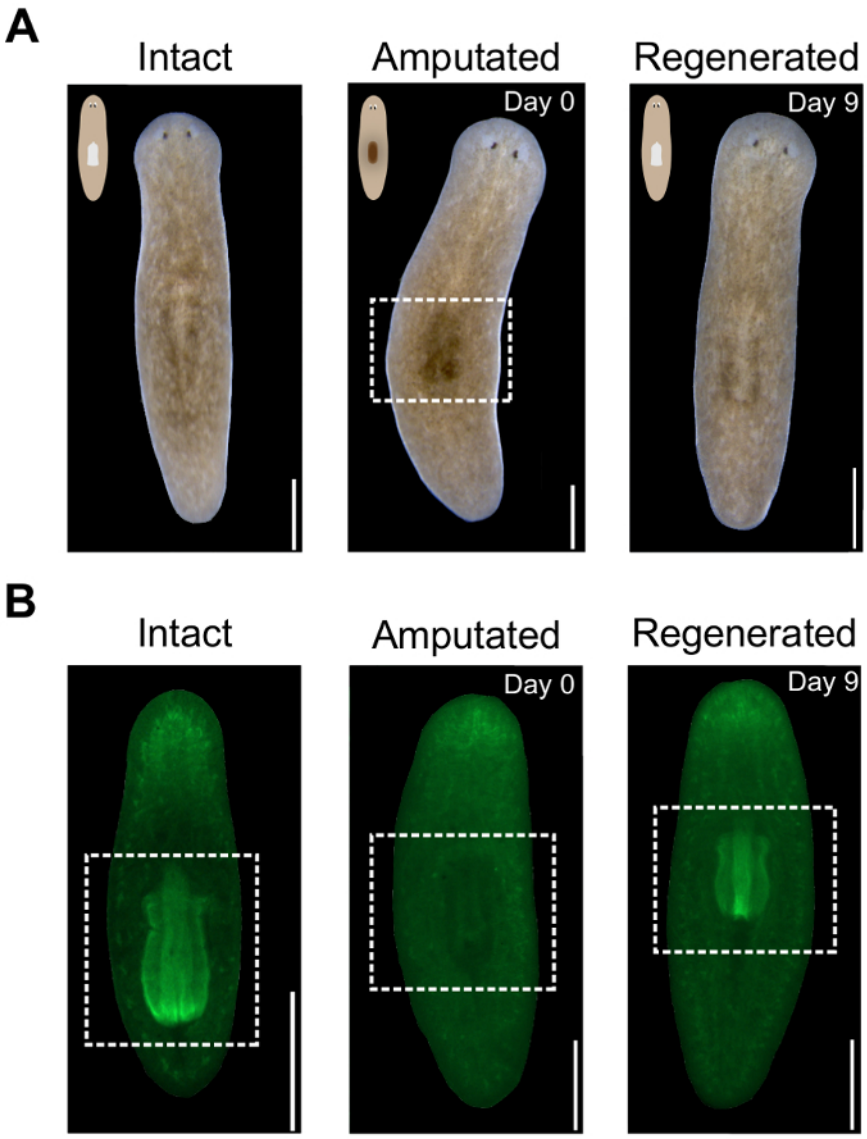

Figure 2. Assessment of pharynx amputation and regeneration. (A) Representative images of live animals at specified times before and after amputation. Middle panel shows dark spot in pharyngeal region after amputation, highlighted by dashed white box. Dorsal side faces up. Scale bars = 500 µm. **(B)** Representative images of animals stained with Alexa488-streptavidin at specified times before and after amputation. Middle panel shows absence of pharynx after amputation. Pharyngeal region highlighted by white box. Ventral side faces up. Scale bar = 500 µm. Please click here to view a larger version of this figure.

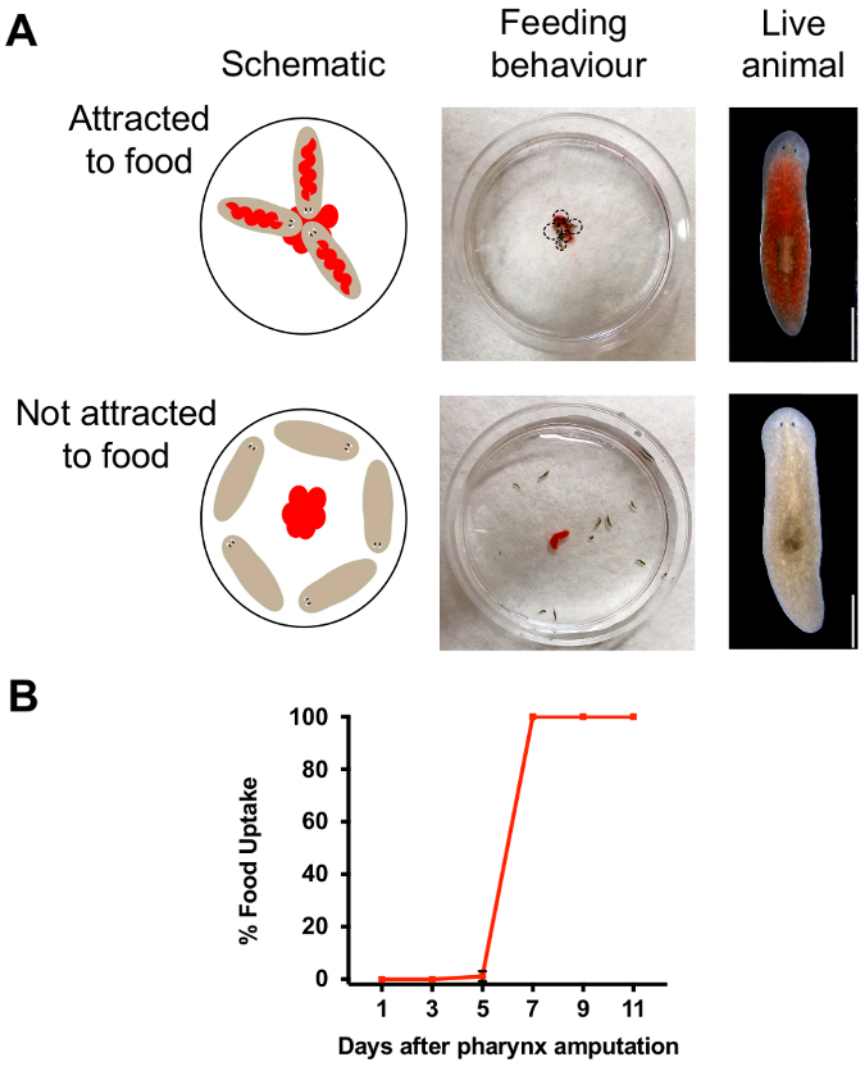

Figure 3. Evaluation of pharynx regeneration. **(A)** Images and schematic of feeding assay, conducted by placing 25 µl of liver in the center of a 60 mm Petri dish. Schematic (top, left) and image (middle) show feeding behavior in animals with intact pharynges. Top right, image of animal that has eaten. Schematic (bottom left) and image (middle) of feeding behavior in animals 1 day after amputation. Bottom right, animal that has not eaten. Scale bar = 750 µm. **(B)** Results of feeding assay. Percentage of animals that have ingested food (quantified by red coloration) at specific times after amputation. For each time point, n=10 animals, repeated in triplicate. Error bar = SD. Please click here to view a larger version of this figure.

Discussion

This protocol describes a method of selective ablation of the pharynx using sodium azide. Other targeted ablation studies in planarians have used modified surgery to remove photoreceptors[21] or pharmacological treatment to ablate dopaminergic neurons[22]. One significant advantage of chemical amputation over existing methods is that it does not require surgery. The rigid structure of the pharynx compared to the rest of the planarian body facilitates its complete removal from the animal, which is much softer-bodied. Also, the pharynx detaches from the animal at a small junction (the esophagus)[30] joining the pharynx and the intestine, generating a more reproducible wound than one induced by a larger surgical tool. Based on its physical properties and small anatomical linkage to the rest of the animal, pharynx removal therefore generates more homogeneous wounds than other surgical amputations.

Length of exposure to sodium azide is also critical for the success of this technique. Although brief exposure to sodium azide does not adversely affect planarians, prolonged exposure is toxic and will eventually kill animals. Sodium azide is known to inhibit energy-dependent processes through inhibition of cytochrome oxidase[23,24]. In addition, it suppresses transcription and translation, likely by accumulation of key components of these reactions in stress granules[25]. In planarians, sodium azide exposure suppresses mitosis for 24 h, after which cells resume proliferation[12]. Modifications such as limiting the time of azide exposure, removing azide with thorough rinsing, and accounting for the transient mitotic suppression in experimental design can help overcome these limitations.

The unique anatomical position of the pharynx and its characteristic radial symmetry allow for the easy distinction of newly regenerated pharyngeal tissue from pre-existing stem cells. Pharynx regeneration can be monitored at the cellular and anatomical levels using tissue stains like streptavidin and DAPI, immunohistochemistry, or by *in situ* hybridization[26]. The feeding assay described here assays functional regeneration

of the pharynx. Chemotaxis towards food requires the pharynx[27,28] and animals without a fully functional pharynx cannot ingest food. This simple, quantitative assessment of organ function therefore complements structural regeneration. Additionally, chemical amputation can be performed on populations of animals, and can generate numerous animals with identical injuries. The procedure is therefore convenient for large-scale studies. These advantages, coupled with the fact that regeneration of a mature pharynx requires stem cells, makes pharynx amputation an ideal model to study organ regeneration. Used in combination with broader amputations, pharynx removal can be used to test hypotheses about how wound size and anatomical position impact the stem cell response to injury, which is a key question in the regeneration field[21].

Disclosures

The authors have nothing to disclose.

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