

Video Article

Inducing Complete Polyp Regeneration from the Aboral Physa of the Starlet Sea Anemone *Nematostella vectensis*

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Abstract

Cnidarians, and specifically *Hydra*, were the first animals shown to regenerate damaged or severed structures, and indeed such studies arguably launched modern biological inquiry through the work of Trembley more than 250 years ago. Presently the study of regeneration has seen a resurgence using both "classic" regenerative organisms, such as the *Hydra*, planaria and Urodeles, as well as a widening spectrum of species spanning the range of metazoa, from sponges through mammals. Besides its intrinsic interest as a biological phenomenon, understanding how regeneration works in a variety of species will inform us about whether regenerative processes share common features and/or species or context-specific cellular and molecular mechanisms. The starlet sea anemone, *Nematostella vectensis*, is an emerging model organism for regeneration. Like *Hydra*, *Nematostella* is a member of the ancient phylum, cnidaria, but within the class anthozoa, a sister clade to the hydrozoa that is evolutionarily more basal. Thus aspects of regeneration in *Nematostella* will be interesting to compare and contrast with those of *Hydra* and other cnidarians. In this article, we present a method to bisect, observe and classify regeneration of the aboral end of the *Nematostella* adult, which is called the physa. The physa naturally undergoes fission as a means of asexual reproduction, and either natural fission or manual amputation of the physa triggers re-growth and reformation of complex morphologies. Here we have codified these simple morphological changes in a *Nematostella* Regeneration Staging System (the NRSS). We use the NRSS to test the effects of chloroquine, an inhibitor of lysosomal function that blocks autophagy. The results show that the regeneration of polyp structures, particularly the mesenteries, is abnormal when autophagy is inhibited.

Video Link

The video component of this article can be found at <https://www.jove.com/video/54626/>

Introduction

The observation of regeneration in a single hydra was the seminal event in the advent of biology as an experimental science^{1,2}. Regeneration remains a phenomenon of extraordinarily broad appeal to biologist and lay person alike. The potential for developmental biologists, clinicians, biomedical scientists and tissue engineers to understand and overcome the limits on human regeneration makes *regeneration biology* more than intrinsically interesting.

Now, with the use of emerging technologies such as genome sequencing and gain and loss of function tools, the field is poised to tease apart regenerative mechanisms and to ultimately understand how various species can regenerate while others cannot. The degree of commonality in molecular, cellular and morphological responses remains to be elucidated, but so far it appears that the basic responses among animals that can regenerate is more similar than would have been imagined only a decade ago³.

Cnidarians in particular are facile at regenerating almost all of their body parts amid a broad spectrum of morphological diversity. From the solitary fresh water polyp, *Hydra* along with the tiny marine polyps that build immense coral reefs, to the complex colonial siphonophores, such as the Portuguese Man-O-War, regeneration is often a mode of reproduction, in addition to a mechanism for repairing or reforming damaged or lost body parts resulting from injury and predation. Whether the diverse species of Cnidaria use similar or different mechanisms for regeneration is a fundamentally interesting question⁴⁻⁶.

We, and others have been developing the anthozoan, *Nematostella vectensis* as a model for regeneration⁷⁻¹⁷. We recently developed a staging system for describing regeneration of an entire body from a morphologically uniform piece of tissue bisected from the aboral end of the polyp¹⁰. While *Nematostella* polyps can regenerate when bisected at any level, we chose to cut adults at an aboral position in the most morphologically simple region, the physa, in part because this is close to the normal plane of natural asexual fission¹⁸, and also because it permits observation and molecular analyses of how an entire body is reassembled from the simplest morphological components.

The *Nematostella* Regeneration Staging System (NRSS) provides a relatively simple set of morphological benchmarks that could be used to score the progress of any aspect of regeneration by an amputated physa, under normal culture conditions or experimentally perturbed situations

such as small molecule treatments, genetic manipulation, or environmental alteration. As anticipated, the NRSS is becoming adopted as a morphological scaffold on which the cellular and molecular events of regeneration can be referenced¹⁰.

Finally our method of cutting produces a gaping hole several orders of magnitude greater than the pin point puncture used in a recent study¹⁷, yet both wounds heal in around 6 hours. Documenting the visually arresting and distinct phases of wound closure should suggest experimental approaches to explain the apparent independence of the size of a wound and the time it takes to close. Thus, a deeper visual understanding of the aboral amputation process, provided by this protocol, will aid further investigations into this model regeneration system and broaden the application of this staging system using *Nematostella vectensis*.

Protocol

1. Conditioning of Animals for Temperature, Nutrition and Light/Dark Cycle

1. Obtain *Nematostella vectensis* adults from one of the numerous *Nematostella* labs worldwide, or a non-profit supplier (**Table 1**)
2. Maintain *Nematostella* at constant temperature (typically between 18 and 21 °C) in the dark, in "1/3x" Artificial Sea Water (ASW) at a salinity of 12 parts per thousand (ppt). Maintain cultures in simple soda-lime glass culture dishes, typically 250 mL or 1.5 L capacity¹¹.
Note: These simple culture conditions are commonly used among labs that study *Nematostella*, but culture care can also be automated¹⁹.
3. Feed *Nematostella* freshly hatched *Artemia* nauplii 2 - 4x per week. Hatch *Artemia* cysts in full strength (36 ppt) or 1/3x ASW at 30 °C, in a shallow rectangular glass dish²⁰ or in any of a number of small scale, commercial or homemade brine shrimp hatcheries. If an incubator is not available, shrimp will hatch at RT but do so more slowly.
Note: This often requires more than 24 h for completion.
4. Replace anemone culture water at least once a week. For best adult health, thoroughly clean (without soap) the culture bowls once a week of accumulated mucous secretions, which coat the bowl and can trap uneaten food and waste, and entangle the animals.

2. Selection and Relaxation of Nutritionally Conditioned Animals

1. Select size-matched polyps of approximately the same length (3 - 5 cm, when naturally relaxed) and place them in a bowl separated from the colony for three days prior to amputation.
Note: The number of animals selected for cutting will be determined by the experiment being conducted, of course, but in general we recommend at least five animals per sample point with six replications. Thus, in a typical experiment a minimum of 30 animals would be preselected. In general, it is wise to select more than the minimum number (30) since amputations that are irregular (see below) can later affect scoring.
2. Remove the dish of selected animals from the incubator into room light at least one hour prior to amputation.
Note: Exposure to room light and vibrations of handling will likely cause the animals to contract, so they need to be adapted or "relaxed" by incubation on the lab bench. Animals will become refractory to touch and light exposure and at that point can be moved by gentle pipetting.
3. Optional: Anesthetize the animals by adding 7.5% MgCl₂ (in 1/3x ASW). Gently add the MgCl₂ solution to the bowl with a standard plastic 5 mL pipette.
Note: Although animals will eventually become habituated to the light and to physical manipulation, it may be advantageous to anesthetize animals to maintain or "fix" the relaxed state after they have become elongated^{16,21,22}.
4. Use a wide bore (>0.5 cm) plastic pipette to transfer (in 1/3x ASW) five animals from the pool to be amputated, into the bottom of a sterile glass cutting dish of 100 mm diameter containing 12 ppt ASW. Place the dish on to the stage of a stereomicroscope with variable magnification between 10 - 40X.
Note: If the animals have not been anesthetized and relaxed for cutting, they may still respond to touch and stereoscope illumination and thus may need a few minutes to become relaxed again.

3. Amputation

1. Using a sterile scalpel, amputate the aboral physa from each polyp, with the goal to obtain a section of the physa that is approximately as long as it is wide and containing no mesentery.
Note: The ideal cut site is just aboral to the termination of the mesentery. At the plane of cutting there is a transition from mesentery proper to thin lines corresponding to each mesenterial insertion (see **Figure 1**, arrows). Absence of mesentery is critical because it produces mucous that may facilitate 'plugging' the hole^{17,30}.
 1. Place the scalpel blade in contact with the animal at the desired site of amputation. Do this either unassisted (freehand), or by gently grasping the animal's body with a #5 forceps (Dumont style or similar).
 2. Cut through the tissue by leveraging the curved blade of the scalpel in a 'rocking' motion across the body.
Note: The tissue should sever cleanly as the scalpel is rocked and liberate the desired section of physa from the donor. However, if a small piece of tissue still connects the body and the physa, cut it with the scalpel. Do not attempt to separate the connected pieces by pulling, as this may damage the physa.
2. Remove each *amputated 'donor' polyp* from the dish and return it to a separate bowl labeled 'pooled amputees'; date the bowl and return it to stock culture.
Note: Amputated polyps will heal the aboral wound within a day and then can be fed normally. They will regenerate a normal looking physa within two weeks at which point the physa can be amputated again if desired.
3. Rinse the excised physa that remain in the cutting dish in 12 ppt ASW, then transfer each physa to a separate sterile well in a multi-well cell culture plate that already has 10 mL of 12 ppt ASW in each well.
Note: This example uses a six well plate, with each well holding 10 mL of seawater and five excised physa. In general seawater should cover the physa sufficiently to avoid exposure to air due to movement in handling and potential evaporation. The plate or wells should have a lid.

4. Repeat steps 3.1 - 3.3 to collect at least 5 physa in each well reserved for each experimental treatment.
5. Incubate the physa at a temperature that will provide the best rate of regeneration for the planned experimental interrogations. Place the plate containing the physa into a temperature regulated incubator, at a fixed temperature determined by the desired rate of regeneration.
Note: The physa will regenerate missing tissues and form a full polyp when incubated at temperatures between 15 and 27 °C. The rate of regeneration is temperature dependent except for the first two stages. The average day for reaching Stage 4 for all temperatures is 7 d after cutting and this also coincides with regeneration at 21 °C. At 27 °C, Stage 4 is reached about 3 days earlier and at 15 °C, Stage 4 is delayed by about 3 d compared to regeneration at 21 °C (also see Reference 10).

4. Assessing Regeneration with the *Nematostella* Regeneration Staging System (NRSS)

1. Score the physa using a stereo-compound microscope with variable magnification (10 - 80X). Score the freshly cut *Nematostella* physa as Stage 0 and continue scoring at the same time each day post amputation (dpa) using the NRSS¹⁰.
Note: For key staging criteria and details refer Reference 10.
 1. Score physa as Stage 0 (Open Wound) if a freshly cut physa appears as a cup- shaped mass resembling a flaccid balloon, with an open wound site is likely visible.
Note: The wound edges might also stick together from the outset, but the tissue will still be collapsed and lack rigidity. The edges of the open wound may be observed undergoing radial contraction as the wound heals.
 2. Score physa as Stage 1 (Wound Closed) if the amputation wound appears closed.
Note: Wound location will correspond to the future oral pole. The outer surface around the future oral pole may begin to display distinct arches corresponding to the underlying radially symmetric endodermal mesenterial insertions.
 3. Score physa as Stage 2 (Radial Arches) if the surface of the oral pole appears inflated, revealing eight raised arches arranged in a radially symmetric pattern and separated by grooves. Observe small, hemispherical blebs at the apex of the arches. They will be about as tall as wide, likely transient, and initially comprised by a single ectodermal cell layer.
Note: In some cases double-layered blebs may stabilize. Note: At this or later stages a mucous layer may appear to encapsulate the physa (**Figure 2**) in a membranous 'sheath'. This encapsulating material should be removed to facilitate scoring.
 4. Score physa as Stage 3 (Tentacle) if the buds of the tentacles containing endodermal and ectodermal tissue layers are stably formed at the oral end of at least some radial arches.
Note: The tentacles are longer than they are wide and are minimally motile. The physa will show increased, but variable inflation so that mesentery rudiments may become visible extending from the mesenterial insertion into the body cavity (coelenteron).
 5. Score physa as Stage 4 (Linear Mesenteries) if the physa contains eight distinct, visible mesenteries that extend into the coelenteron from insertions in the body wall, with oral-aboral lengths that are more than twice their radial width measured from where they appear to connect to the pharynx at its aboral end (enterostome).
Note: Four or fewer mesenteries have "pleated" internal free edges. The pharynx is visible. More than eight tentacles are visible, motile and sometimes they contract into the body.
 6. Score physa as Stage 5 (Predominantly Pleated Mesenteries) if the physa has more than four mesenteries with pleating, and the pleating is more full and sinuous than at Stage 4. The animal has an almost "normal" adult appearance, but there are no visible gonadal cells.

Representative Results

The progression of morphological events during regeneration in severed physa is shown in **Figure 1A**, which includes representative views of physa at each NRSS stage. The typical physa cut site is indicated on the adult (arrowheads). The photographs in **Figure 1A** show progressive regeneration of oral and body structures from freshly cut physa through fully formed polyp. **Figure 1B, C** show the arrangement of internal septa, the mesenteries, at Stage 4 and Stage 5, respectively. Note that some mesenteries at Stage 4 will lack "pleating", but to qualify as a Stage 5 the majority must have developed pleats. **Figure 1D** shows a physa enveloped in a membrane of mucous which can be removed with forceps (**Figure 1E**). While usually not harmful to regeneration (unless it unreasonably traps the animal), the membrane can impede scoring the physa and doing experimental manipulations, such as microscopy, sample fixation or harvesting for molecular/biochemical analysis. This is best removed after Stage 1, or later if it reforms.

Figure 2 shows how the staging system can be used to score the results of an experiment to assess the effects of inhibiting autophagy. Physa were cut and treated with chloroquine at 10, 50 and 100 μ M, or physa were untreated (controls). Chloroquine inhibits lysosomal functions, which are required for autophagy. The NRSS criteria were used to score the physa over the course of regeneration and results were plotted in **Figure 2E**. Photographs of representative control (**Figure 2A**) and chloroquine treated physa (**Figure 2B - D**) were made when controls reached stage 5. Chloroquine treated animals did not progress beyond stage 4, and they typically exhibited incomplete mesentery regeneration (most lacked pleating), short tentacle size, and in some cases short body length.

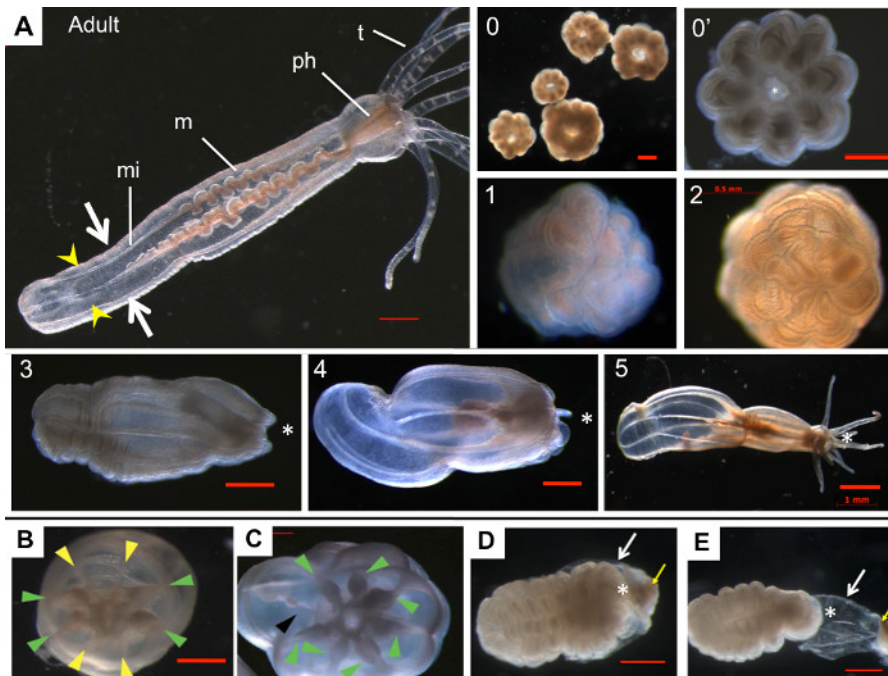


Figure 1. Features of Regeneration. (A) Examples of physa regenerating oral and body structures staged according to the NRSS. Panel labeled *Adult* shows features of a mature animal with tentacles (t) pharynx (ph), mesenteries (m) and mesentery insertions (mi). White arrows show where pleated region of mesenteries transition to the mesentery insertion, a ridge of endoderm extending to the aboral terminus. This region constitutes the physa. Yellow arrows show ideal physa bisection site. **Panel 0** shows five physa minutes after bisection, and **Panel 0'** is a magnified view of one of those physa, with open wound at center, defining Stage 0 of the NRSS. **Panel 1** shows a physa with the wound now closed, defining Stage 1. **Panel 2** shows physa with raised radial arches around the oral pole, with inflated tissues below, corresponding to Stage 2 (panels 0 - 2 are views of the oral end). **Panel 3** shows tentacle buds emerging at the oral pole (facing right) of the elongated and inflating physa, now at stage 3. Note rudimentary mesentery elements are visible and the pharynx is forming in the dark area at the oral end. **Panel 4** shows the emergence of true tentacles, as well as transient 'blebs' at the oral pole, defining Stage 4. Linear mesenteries are visible in the inflated physa. The large round mass visible inside the polyp is of unknown origin and will be expelled through the mouth. **Panel 5** shows nearly complete regeneration characterized by more than four pleated mesenteries, a fully formed pharynx and eight or more tentacles, which defines it as Stage 5. (B, C) Aboral views of individual physa illustrate the biradial arrangement and morphology of mesenteries. In this view, pleated mesenteries appear to have a bulge of tissue midway (green arrowhead). A Stage 4 physa has four or less pleated mesenteries, and a stage 5 physa has more than four (C). Mesenteries with or without pleating are indicated by green or yellow arrowheads, respectively. Black arrowhead in (C) points to a pleated mesentery that has extended radially. (D, E) Removal of a mucous sheath from the physa is depicted. The white arrows indicate a mucous sheath surrounding the physa (D) that should be removed as in (E) before scoring regeneration. Sometimes residual tissue (yellow arrows) may be trapped within the sheath, and this also should be removed. Asterisk indicates oral poles, where marked. Red size bars are 0.5 mm in all panels except A5 (1.0 mm). Panels B and C of this Figure have been modified and reprinted with permission from Reference 10. [Please click here to view a larger version of this figure.](#)

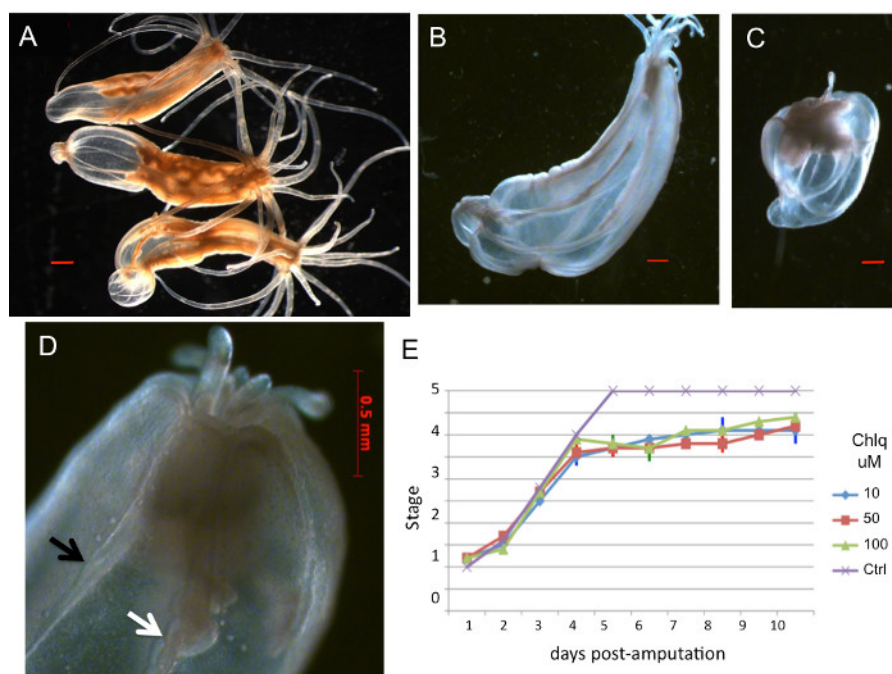


Figure 2. Effects of Chloroquine on Regeneration. To demonstrate an application of the NRSS, we tested the effects of a chloroquine, an inhibitor of autophagy. Physa were amputated and immediately placed in 1/3x ASW containing 0.1% DMSO (control) or chloroquine at 10, 50, 100 μ M. Physa were scored at 24 h intervals using the NRSS. **(A)** Representative endpoint images taken of controls when they reached stage 5. **(B - D)** Representative images of chloroquine-treated physa that reached a "regenerative plateau" of stage 4. Chloroquine caused similar defects in regeneration at all doses tested. The most notable problem was the lack of full regeneration of mesenteries and tentacles. Abnormal body morphology (e.g., stunting) was also occasionally noted **(C)**. Presence (white arrow) and lack (black arrow) of pleats in a chloroquine-treated physa is shown in **D** (tentacles are partially withdrawn). **(E)** The staging data for all physa plotted as a function of time (at 23 °C). [Please click here to view a larger version of this figure.](#)

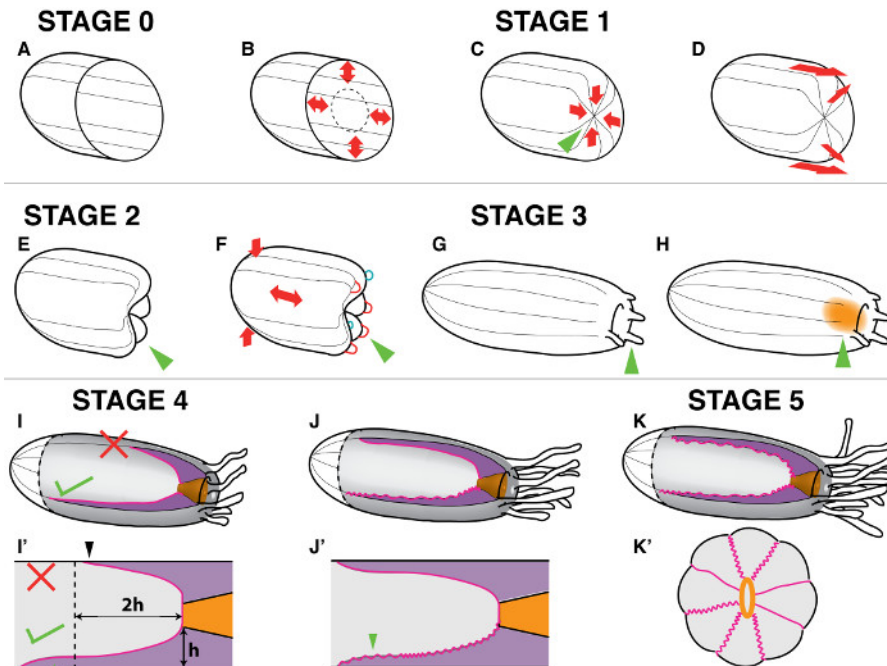


Figure 3. Summary of key features of the NRSS stages. This diagram shows the key morphological changes that define each stage in the NRSS. Direction of movements are indicated by red arrows and features by green arrowheads. Stage 0 depicts open wound just after cutting (A), and t). The wound edges undergo closure radial contraction toward the center (B). Stage 1 is characterized by full closure of the wound (C) and elevation of the arches between oral-facing ridges (D); the center of the oral surface (arrowhead) is depressed. Stage 2 has a pronounced arching of the oral surface (arrowhead, E); the physa begins to elongate and become narrow (F) with tentacle buds and blebs visible at the apex of the arches (arrowheads). Stage 3 has stable tentacle buds (arrowhead, G) and the regenerating pharynx may be seen as a density at the oral pole (orange mass, arrowhead in H). Stage 4 shows the presence of linear and unpleated mesenteries (I) that should be at least twice as long as they are tall at junction of pharynx (I'). Four or fewer mesenteries may be pleated at their inner edge — the mesenterial filament (J, J'). Stage 5 is characterized by more than four pleated mesenteries (K), which can be best assessed by viewing from the aboral end (K'). Re-print with permission from Reference 10. [Please click here to view a larger version of this figure.](#)

Discussion

Use of *Nematostella* as a model of wound healing and regeneration is becoming increasingly popular. Thus, it is important to be able to visualize the morphological patterns of a particular protocol before effective cellular and molecular analyses can be assigned and compared. *Nematostella* have a high degree of regenerative "flexibility", being able to reform almost any missing structure amputated at any location, at post-planula stages of life. Thus, various investigators have examined regeneration resulting from amputation or wounding in different regions of polyps, at different ages and sizes⁷⁻¹⁸.

The staging system described here follows the transformation of a morphologically uniform section of physa, amputated from the aboral end of an adult, to an anatomically complete juvenile polyp lacking only reproductive tissue (we have not yet determined when these regenerated juveniles become sexually mature). This approach examines regeneration from a simple rudiment of tissue to one of near maximum complexity. Including five physa as a minimum group size is recommended to normalize for potential individual variability and survivorship. Of course, this number can be adjusted to suit the particular experimental goals but the NRSS protocol allows 2 mL of medium per physa to negate a potential 'volume effect' like that which has been reported to affect regeneration in hydra²³.

Other approaches to study *Nematostella* regeneration have used adults bisected mid-body or at the oral end, or juvenile 4-tentacle stage polyps bisected mid-body^{7,11-18}. Two studies have examined regeneration in physa liberated by natural fission^{16,18}, and approaches using amputated physa have recently emerged with the NRSS^{8,9}. Each of these varied approaches has its own merits and can address unique questions about regenerative processes occurring under different amputation or wounding regimes, and among differently aged animals. The NRSS protocol for physa amputation and scoring, shown in the present study, generates a relatively uniform set of physa for systematic study and avoids variation in the physa size, tissue composition, and subsequent progression of regeneration observed with natural transverse fission^{18,24}. Although physa amputation corresponds somewhat to the natural mode of asexual reproduction in *Nematostella*, molecular differences have been noted between regeneration resulting from natural fission and amputation at mid-body or at the physa^{16,18,24}. Whether physa produced by the method of amputation described here or natural fission show such differences remains to be determined.

There are a few issues that are critical for success with the rearing amputation, and scoring techniques described here. Polyps selected for amputation should be maintained at the same temperature, matched for physa size, nutritional history and if possible age (although the latter has not been systematically tested to determine if there is an age-effect on variation in stage progression). Obtaining an open wound with a sharp sterile scalpel having a curved blade is important for morphological observations of wound healing between Stage 0 and Stage 1. When the physa is inflated and the curved blade of the scalpel is rocked over the adult tissue, it is amputated in one motion and can be quickly transferred

to treatment well from cutting dish. Amputations that include mesentery or that are the result of extensive physical manipulation should be discarded.

Practice staging untreated physa to get a sense of the individual variation in the population being tested. Variation between individual physa is least for the earliest stages. For example, all physa are in Stage 0 at 0 dpa. In the same way all physa reach Stage 1 in synchrony at 1 dpa. The appearance of Stage 2 may be harder for the observer to discern because 'inflation' is a relative condition that is achieved, however, by 2 dpa with little variation. The appearance of true tentacles marks progression to Stage 3. The regenerating tentacles may be obscured by the appearance of a membranous "cocoon" that impedes visualization of the tentacle bud beneath. If the membranous covering has not been previously removed, it should be done so now. Removal of the membrane with fine tipped tweezer will liberate the regenerating physa. The distinction between Stage 4 and 5 is the number of pleated mesenteries. Stage 4 has four or fewer pleated mesenteries, and Stage 5 has five to eight pleated mesenteries. While pleating can be observed in a lateral view, the exact number of pleated mesenteries is better observed with an aboral view.

One challenge in studying adult *Nematostella* regeneration from a physa rudiment, and indeed with tissues cut from other amputation sites, is the varied clarity of the living tissues. The bulb of the physa is relatively clear in the intact adult, but it becomes rather opaque due to tissue contraction after amputation. Clarity returns gradually (Stage 2) once the wound closes (Stage 1) and the animal begins to inflate, but even then the region around the wound site where tissues and structures are actively regenerating remains somewhat obscured by dense tissues (especially Stage 3). Increased inflation usually accompanies Stage 4 and 5. Fixation followed by optical clarification will almost certainly resolve what is happening at the oral end, but more informative may be live, tissue-specific transgenic reporters that can be monitored for fluorescence and more easily visualized^{15,25-30}.

An amputated physa obviously cannot feed since it lacks tentacles, mouth and mesenteries (which harbor digestive glands), thus requiring regeneration of missing body structures to be accomplished by mobilizing nutrient reserves from non-food sources. The physa can potentially accomplish this is by autophagy, in which cytoplasm, organelles and other cellular components are engulfed intracellularly and processed by a lysosome-dependent mechanism to produce energy and compounds for anabolic processes³¹⁻³³. We find that treating physa with the lysosome inhibitor, chloroquine, causes abnormal regeneration of mesenteries and tentacles, and general body morphology, indicating that autophagy is required for normal regeneration of oral and body structures. Autophagy regulates stem cell functions³⁴⁻³⁶, and plays essential roles in regeneration in *Hydra*, planaria, and zebrafish³⁷⁻⁴¹. Further analysis is required to understand how autophagy influences *Nematostella* regeneration at the cellular and molecular levels, but our first pass experiment shows the utility of using the NRSS as a fast screening method for small molecules that might affect regeneration.

The genetic, molecular and cellular processes that regulate regeneration in *Nematostella* are only in a rudimentary stage of understanding, but this emergent model for regeneration has a growing repertoire of tools for genomic and gene expression analysis. With its annotated genome, a plethora of regional and tissue specific genetic markers, and robust methods for transgenesis, mutagenesis, histology and microscopy, *Nematostella* promises to reveal mechanisms governing anthozoan cnidarian regeneration and uncover whether its regenerative processes are similar or unique among cnidarians and metazoans in general.

Disclosures

The authors have nothing to disclose.

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