

Hands-On Classroom Activities for
Exploring Regeneration and Stem
Cell Biology with Planarians

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ABSTRACT

Regeneration has long fascinated humanity, and its documentation has progressed from simple descriptive observations to the intense molecular and cellular investigations of today. The overarching goal of this work is to make the key methods and tools being used in modern regeneration and stem cell biology research accessible to docents and students in the classroom. We have designed a series of experimental activities with accompanying protocols using four inexpensive, commercially available planarian species indigenous to North America: *Girardia* sp., *Dugesia dorotocephala*, *Phagocata morgani*, and *Phagocata gracilis*. These planarians are fast and robust regenerators, and can easily be maintained in the classroom. The activities presented here can be used to guide students through hypothesis-driven experiments, and range from simple manipulations aimed at high school students (e.g., planarian husbandry, feeding, and cutting experiments) to gene expression and protein function analyses suitable for college students. Regeneration time courses, the more complex whole worm in situ hybridizations, and RNA interference for gene knock-down experiments are described for each of the four species. Cumulatively, the suggested methods and experiments will facilitate the exploration of animal regeneration biology and promote curiosity-driven, hands-on application of the scientific method.

Key Words: β -catenin; blastema; *Girardia* sp.; *Dugesia dorotocephala*; odf2; *Phagocata gracilis*; *Phagocata morgani*; planarian maintenance; regeneration; RNA interference; stem cells; tissue-specific marker; whole worm in situ hybridization.

○ Introduction

Today, life is being interrogated at unprecedented levels of resolution spanning molecular interactions to cellular, multicellular, and organismal behavior. A recurring theme in these efforts is the essential role that curious minds observing nature play in the process of discovery and understanding. Because

observation and examination of nature is central to the development of human thought, hands-on approaches are widely regarded as effective classroom methods not only to explain many biological processes, but also to encourage student curiosity. Here, we introduce an age- and ability-scalable, experimental activity program using freshwater planarians that promotes a hands-on approach for students to explore fundamental questions underlying animal regeneration, tissue homeostasis, and stem cell biology.

Regeneration and Stem Cells

Regeneration is the fascinating process that replaces damaged or lost structures in adult organisms. Many animals across the tree of life manifest this remarkable ability, chiefly among them, starfish arm regeneration, fish tail fin regeneration, and salamander limb regeneration (Figure 1). In extreme cases, such as in hydra and planarians, each tissue fragment can regenerate a complete, new organism (Sánchez Alvarado & Tsonis, 2006).

Regeneration has long captured the human imagination. Aristotle reported on the reconstitution of an amputated lizard tail circa 350 BC (Aristotle et al., 1965), and in the eighteenth century, naturalists began to study regeneration systematically by performing scientific experiments. Abraham Trembley, a Swiss naturalist, documented the regenerative capacity of hydra (Lenhoff et al., 1986), and Lazzaro Spallanzani, an Italian priest, biologist, and physiologist, reported the regenerative properties of earthworms, snails, newts, and frogs (Dinsmore, 1991; Tsonis & Fox, 2009). During the early twentieth century, Thomas H. Morgan, an American geneticist and embryologist, wrote

on this topic in his book, *Regeneration* (1901). Researchers suspected and later confirmed that many forms of regeneration often rely upon the activities of persistent and undifferentiated post-birth cells referred to as adult stem cells (Coutu et al., 2011; Randolph,

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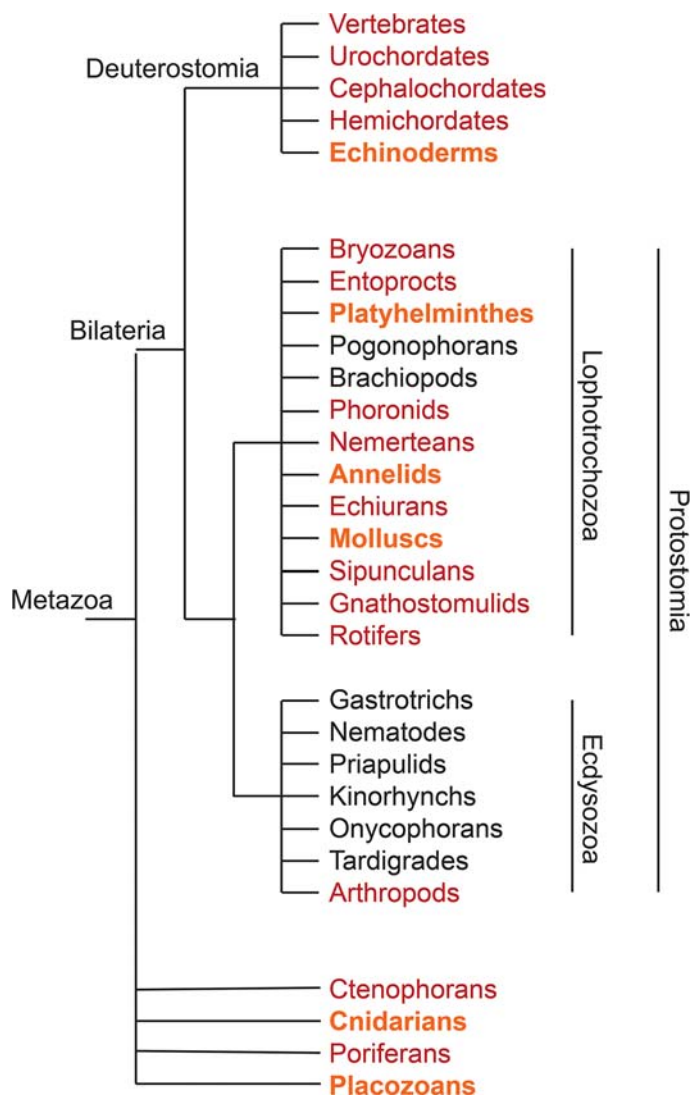


Figure 1. Distribution of regenerative capacities amongst animals. Bold orange: phyla with animals displaying robust regeneration abilities, i.e., Cnidarians (e.g., hydra), Mollusks (e.g., snails), Annelids (e.g., earthworms), Platyhelminthes (e.g., planarians) and Echinoderms (e.g., starfishes). Red: phyla with animals able to regenerate at least one body part, i.e., Poriferans (e.g., sponges), Arthropods (e.g., insects and crustaceans), Urochordates (e.g., sea squirts) and Vertebrates (e.g., fishes and salamanders). Black: phyla that include animals in which regeneration of missing body parts has not been unambiguously documented. A color version of this figure can be viewed online.

1891). These cells (see Glossary) both replenish themselves and differentiate anew into tissue-specific cells that replace those lost to physiological wear and tear and/or injury (Smith et al., 1991).

The presence and proliferation of highly potent stem cells in both vertebrates and invertebrates are frequent mechanisms underpinning animal regeneration (Gurley & Sánchez Alvarado, 2008). Stem cell potency is defined by the ability of these cells to generate different cell types. Four general classes have been described: (1) totipotent stem cells can give rise to any of the varied cell types of extra-embryonic and embryonic tissues of an organism; (2) pluripotent stem cells produce most, but not all cell types; (3) multipotent stem cells produce

only a few different cell types; and (4) unipotent stem cells generate only a single cell type (Stocum, 2001). Like differentiated cells, the morphological and functional features of undifferentiated stem cells can be defined by gene expression activities. The activation and repression of genes is a finely regulated molecular choreography involving transcription factors, i.e., molecules that bind DNA. For instance, we now know that the pluripotency of mammalian embryonic stem cells depends in great part on the activities of four transcription factors, known as OCT4, SOX2, cMYC and KLF4. These proteins regulate the expression of such genes as *Wnt*, *Hedgehog*, *BMP/TGF- β* , and *Notch*, which are essential for the self-renewal, maintenance, and differentiation of stem cells into specific tissue types (Liu et al., 2008). Together, OCT4, SOX2, cMYC, and KLF4 are also known as the Yamanaka factors, because of Dr. Yamanaka's discovery that the presence of these four factors could convert adult differentiated cells into pluripotent stem cells (Takahashi & Yamanaka, 2006). Although very important, these transcription factors are not the only molecules involved in the maintenance of stem cell pluripotency. Scientists are continually studying the role in stem cell biology of previously identified and newly discovered molecules.

Planarians as a Model for Regeneration Studies

Planarians are flatworms belonging to the phylum Platyhelminthes. Thousands of species have been described; some live in freshwater, like the species introduced here, others in marine environments, and some on land (Schockaert et al., 2008). Planarians are bilaterally symmetric and contain many complex tissue and organ systems, including photoreceptors (eyes), epidermis, muscle, a bilobed brain, two ventral nerve cords, protonephridia (kidneys), and a digestive system (Figure 2) (Newmark & Sánchez Alvarado, 2002). The blind gut is a highly branched gastrovascular cavity composed of one anterior and two posterior branches. The gut connects to the pharynx, an extendable muscular tube used for feeding and defecation (Figure 2) (Elliott & Sánchez Alvarado, 2012). Planarians are also highly motile and move via the coordinated movement of cilia localized on their ventral epidermis (Elliott & Sánchez Alvarado, 2012).

Planarians have been adopted as a model system for studying regeneration and stem cell biology because they can regenerate an entire organism from virtually any portion of their bodies in a relatively short period of time. This terrific capability depends on an abundant population of adult pluripotent stem cells called neoblasts, which are found throughout the planarian body plan, with the exception of the area in front of the photoreceptors and the pharynx (Figure 3A). The vast majority of neoblasts are found in the mesenchyme, occupying the space between the gut branches (Reddien & Sánchez Alvarado, 2004). After amputation, neoblasts increase their proliferation rate, and their progeny form an unpigmented mass of new tissue called the regeneration blastema, in which cellular differentiation is orchestrated to ultimately restore the missing body parts (Figure 3B) (Reddien & Sánchez Alvarado, 2004). Almost concurrently, the old tissues undergo a remodeling process, which facilitates the functional integration of the newer differentiating blastema to the preexisting anatomy. In the end, this process reestablishes a properly proportioned and functioning new organism (Reddien & Sánchez Alvarado, 2004).

Another interesting characteristic of planarians is their capacity to scale their bodies depending on nutrient availability. Planarians

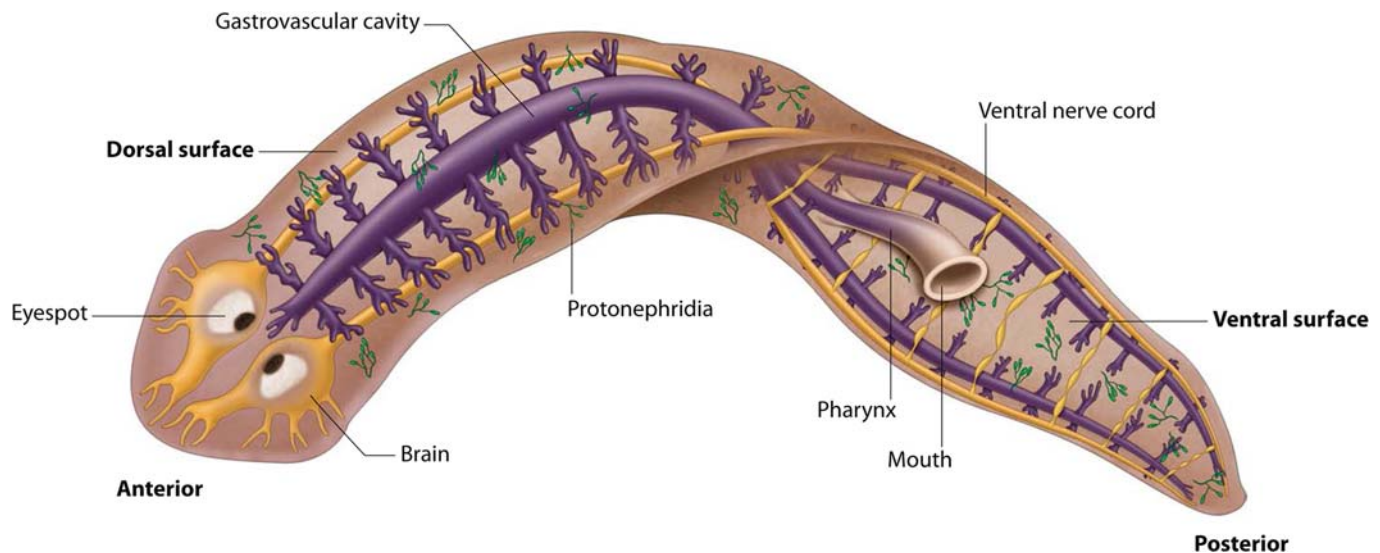


Figure 2. Generic planarian illustrating external and internal anatomy. The eyes, mouth, pharynx, gut, brain, ventral nerve cords, and protonephridia are shown. A color version of this figure can be viewed online.

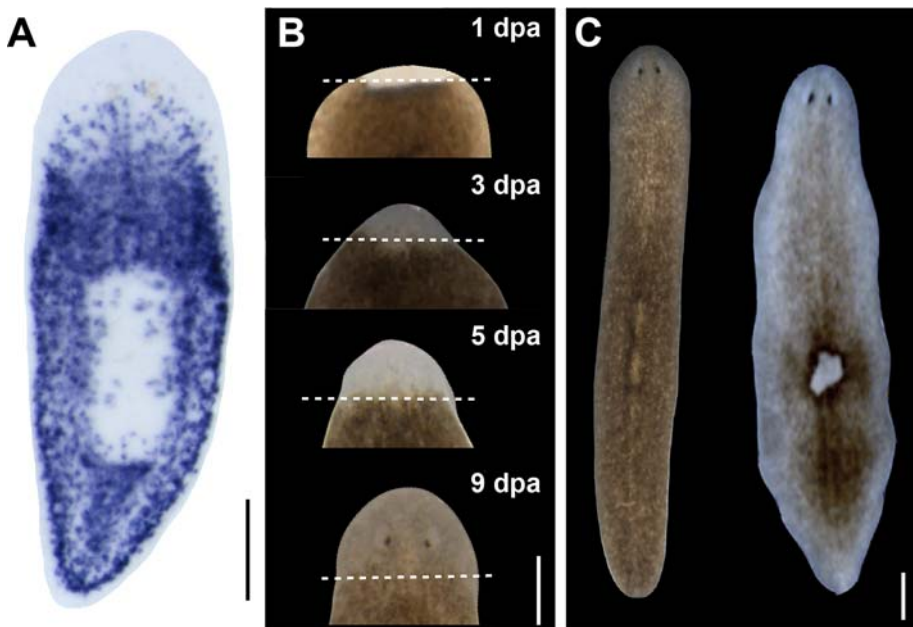


Figure 3. All panels depict the non-commercially available species *Schmidtea mediterranea*. **(A)** Colorimetric whole mount *in situ* hybridization (WISH) detects *piwi-1* gene expression and illustrates planarian neoblast (stem cell) distribution. **(B)** Regeneration time course after head amputation. The unpigmented (white) blastema is already visible close to the wound at 1 day post-amputation (dpa); blastema growth at 3 and 5 dpa; at 9 dpa illustrating the regeneration of eyes in the new head. **(C)** Healthy planarians (left) display an elongated body, uniform brown color, and a smooth body edge, whereas a sick planarian (right) may display a contracted body, a dorsal lesion, and a ruffled body edge. Scale bars = 500 μm . A color version of this figure can be viewed online.

can survive prolonged starvation periods (multiple months), and during this time reduce their size while remaining a functioning, proportioned, and regeneration-capable worm. Besides being caused not by changes in cell size, but rather cell number, this “degrowth”

is fully reversible: once food is available again, the animals regrow to the original size (Newmark & Sánchez Alvarado, 2002).

Currently, the most studied planarian species is *Schmidtea mediterranea*, thanks to many laboratories that optimized molecular and morphological tools (Newmark & Sánchez Alvarado, 2002). However, *S. mediterranea* is not endemic to the United States, and specific regulations are in place for the handling of this species to avoid its accidental propagation in North America. This fact, therefore, limits the routine use of *S. mediterranea* in the classroom.

The aim of this paper is to overcome this limitation by presenting optimized experimental protocols for four North American planarian species, *Girardia sp.*, *Dugesia dorotocephala*, *Phagocata morgani*, and *Phagocata gracilis*. These species can be readily and inexpensively purchased from commercial vendors (Carolina Biological Supply Company or Ward’s Science, see References for urls) and easily maintained in the classroom (Kenk et al., 1972). Here we show results for three different experiments of varying expertise from high school to college levels: (1) amputation strategies and regeneration time courses; (2) analyses of gene expression by whole mount *in situ* hybridization (WISH); and (3) evaluation of gene function using RNA interference (RNAi), that specifically

inhibits expression of a gene of interest. Husbandry information, protocols, lists of required materials, bioinformatic resources, and other tools designed to facilitate experimentation are available at <http://cuttingclass.stowers.org>.

○ The Models

Characteristic Attributes of Four Commercially Available Planarian Species

Four planarian species (*Girardia sp.*, *D. dorotocephala*, *P. morgani*, and *P. gracilis*) are available from American vendors (Carolina Biological Supply Company or Ward's Science, see References), and each can be readily distinguished based upon a number of easily observable traits (Table 1) (Figure 4) (Kenk et al., 1972).

○ Proposed Activities

Planarians are remarkably well-suited organisms for educational purposes. Not only are their regenerative capacities saliently manifested, but they are also easy to manipulate, inexpensive, and easy to maintain and expand if needed. The animals can be purchased, then amplified by amputation as needed. Here we provide a list with several activities related to planarian regeneration, gene expression, and loss-of-function. The protocols for these experiments are available on the website <http://cuttingclass.stowers.org>, and they do not require sophisticated equipment and/or extensive training. Moreover, activities could also involve the use of planarians for behavioral experiments, histological sample processing, morphological or antibody staining, DNA and RNA purification, protein extraction, and isolation and culture of bacterial strains.

The goals of these activities are to teach: (1) how to maintain a model system, with a rigorous and consistent protocol that guarantees experimental reproducibility; (2) how to set up an experiment with indispensable controls; (3) how to decide which are the quantitative/qualitative data that should be collected; (4) how to make observations and discuss the obtained data; and (5) how to develop an explanation/model that justifies the collected data.

Specifically, in the sections below (specifically “WISH: A Method to Visualize Gene Expression” and “RNAi: Disruption of Gene Function”), the students will acquire knowledge of and abilities in

molecular biology. The section on WISH shows that cell characteristics and functions are defined by specific gene expressions, and that gene expression patterns differ across tissues. The section on RNAi shows that the proteins produced from mRNA do the majority of the work inside the cells, and that the degradation of specific mRNA could seriously change/affect cell functionality.

Maintaining, Feeding, and Amplifying Planarians

Students can participate in the maintenance, feeding, and amplification of their planarians, which requires amputation of large worms and subsequent regeneration of the fragments. Related activities are mentioned in Table 2. “Planarian Maintenance” protocol is available on the website <http://cuttingclass.stowers.org>.

Amputation Strategies and Regeneration Time Courses

Referred to as “almost immortal under the edge of a knife” (Dalyell, 1814), planarians can be cut into fragments, most of which can regenerate into a complete new organism (Newmark & Sánchez Alvarado, 2002). Students can systematically cut the worms and follow the fate of the fragments. Here, time courses of three different amputation strategies are provided (Figures 5–8). This set of experiments demonstrates the robustness and reproducibility of regeneration in planarians. Notably, each species rebuilds missing body part(s) while preserving the orientation of body axes. Related activities are mentioned in Table 2. “Observing Planarian Regeneration” protocol is available on the website <http://cuttingclass.stowers.org>.

WISH: A Method to Visualize Gene Expression

WISH is a technique used to detect expression of any gene of interest in both intact and regenerating worms. The WISH protocol exploits specific, synthetically made riboprobe, whose sequence is complementary to the mRNA of the gene of interest. The riboprobe binds the mRNA, and then specific antibodies detect the mRNA-riboprobe complex. Finally, antibodies are visualized through a chemical

Table 1. Species-specific planarian features.

Name	Size	Head shape and Auricles	Pigmentation
<i>Girardia sp.</i>	6–18 mm long and 1–3 mm wide	Head: Triangular with rounded anterior tip. Auricles: Short and broad.	Dorsal side: Almost uniformly brown with irregularly dispersed white splotches. Ventral side: Lighter than the dorsal side.
<i>Dugesia dorotocephala</i>	30 mm long and 3.5 mm wide	Head: Triangular with rather pointed anterior tip. Auricles: Elongated, sharply pointed, extended laterally, and held elevated while the worm is moving.	Dorsal side: Composed of spots of various shades of brown, white, and black. Ventral side: Lighter than the dorsal side.
<i>Phagocata morgani</i>	14 mm long and 2 mm wide	Head: Rectangular with truncated anterior end and rounded lateral edges. Auricles: Flattened along the head.	Dorsal and ventral sides: White because of the lack of pigmentation.
<i>Phagocata gracilis</i>	8–30 mm long and 1.5–6 mm wide.	Head: Truncated at the anterior end with slightly bulging frontal margin. Auricles: Rounded and protruding laterally at the anterior end of the head.	Dorsal side: Dark brown to almost black. Ventral side: Somewhat lighter than the dorsal side. The pharynx cavity contains multiple pharynges.

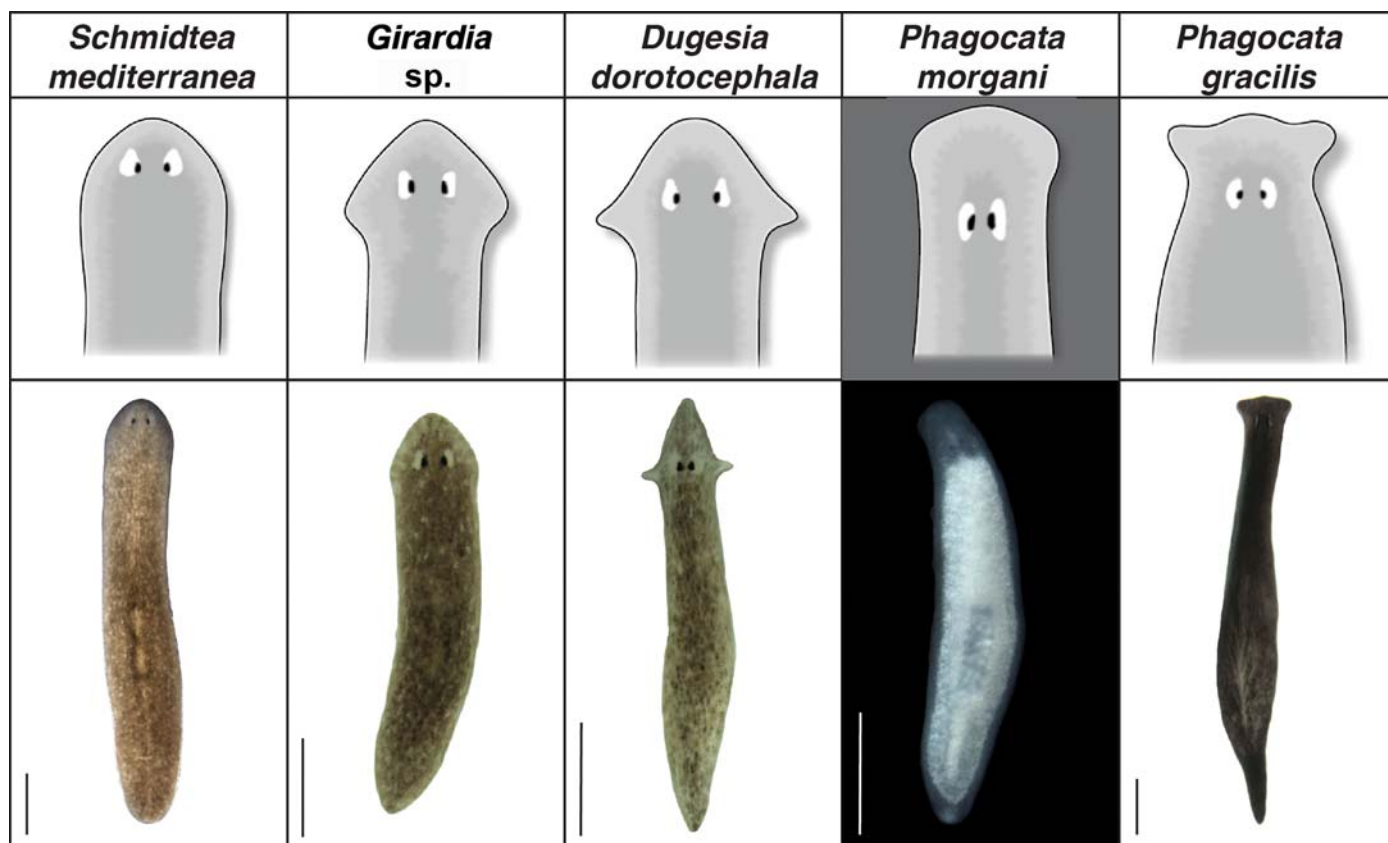


Figure 4. Planarian identification key. The planarian species *S. mediterranea*, *Girardia* sp., *D. dorotocephala*, *P. morgani*, and *P. gracilis* are distinguishable by their head shapes and body pigmentation. Scale bars = 500 μ m. A color version of this figure can be viewed online.

Table 2. Activity suggestions.

A. Section: "Maintaining, Feeding, and Amplifying Planarians"	
Goals: Model maintenance; simple experiment set up; data collection; acquisition of knowledge about planarian ability to deal with the presence or absence of food	What happens to worms starved for one or two months when compared to well-fed worms?
	Do the worms maintain proper body proportions after prolonged feeding and/or starvation?
	Can you see the gut branches if you add food coloring to the food?
	How many days after amputation do worms start eating again? (Note: Do not put too much food in the dish while the worms are regenerating as they may become sick. A sick worm develops dorsal lesions (Fig. 3C) and is no longer able to regenerate.)
B. Section: "Amputation Strategies and Regeneration Time Courses"	
Goals: Model maintenance; experiment set-up; data collection; acquisition of knowledge about planarian ability to regenerate after different amputation strategies and other conditions	Can you visualize all the regeneration steps described in the "Results: Regeneration" section?
	What is the smallest piece of tissue that can regenerate a complete animal?
	Can you produce any regeneration abnormalities from certain types of cuts (such as double-headed or double-tailed worms)?
	What is the behavior of the regenerating fragments over time (i.e., speed of swimming, swimming direction, light avoidance)?
	How might the environment (i.e., light exposure or temperature changes) affect the regenerative responses of these animals?
C. Section: "WISH: A Method to Visualize Gene Expression"	
Goals: Acquisition of basic molecular biology knowledge	How does the distribution of neoblasts correlate with the ability of the different regions of the body to regenerate a new animal?

Table 2. Continued

and abilities; acquisition of knowledge of transcription/translation processes and correlation between gene expression and cell features	Can a ribo probe specific to one species also work with other species?
	Are you interested in a specific gene? Can you find the homologous sequence in a planarian transcriptome database and design specific primers? (Note: Consult the tools and tutorials available on the website http://cuttingclass.stowers.org .)
	How can you clone a gene of interest and design a ribo probe using the suggested sequence or sequences?
D. Section: “RNAi: Disruption of Gene Function”	
Goals: Acquisition of basic molecular biology knowledge and abilities; acquisition of knowledge of both the importance of mRNAs for normal cell functioning and the potential problems caused by missing, truncated or mutated mRNAs/genes.	What is the minimum number of feedings needed to obtain the <i>β-catenin(RNAi)</i> phenotypes?
	Are <i>β-catenin</i> and <i>odf2</i> required for homeostasis, regeneration, or both? (Note: The RNAi-fed worms can be split into two groups: the first for monitoring homeostasis phenotypes in intact worms, and the second for following the regeneration phenotypes after amputation.) Does the phenotype emerge at the same time in both groups, and what might this mean?
	If <i>β-catenin(RNAi)</i> animals are kept alive, how many heads can they develop?
	If <i>odf2(RNAi)</i> animals are kept alive without additional dsRNA feeding, do they ever start to swim straight again?
	Which other genes would you like to try to knock down? What phenotype do you expect? Can you find homologous sequences in planarians and design specific primers using the available transcriptomes? (Note: Consult the tools and tutorials available at http://cuttingclass.stowers.org .)
	Can you clone your own gene and transform the bacteria or synthesize and purify the dsRNA using the suggested sequences or sequences you may be interested in? (Note: Look at the tools and tutorials available at http://cuttingclass.stowers.org .)
	What happens if you try to feed <i>Girardia sp.</i> or <i>D. dorotocephala</i> using the “food with dsRNA” protocol?
	What happens if you try to feed <i>P. morgani</i> or <i>P. gracilis</i> using the “food with bacteria” protocol?
B and C (above): Visualization of Gene Expression (WISH) after Amputation	
Goals: Multiphase experiment set-up; acquisition of ability to correlate different regeneration stages with planarian internal anatomy and gene expression.	What is the internal organization of the worms during regeneration? How are the organs remodeled in the regenerating fragments?
	What is the internal organization of abnormal animals produced by amputation?
C and D (above): Visualization of Gene Expression (WISH) after Disruption of Gene Function (RNAi)	
Goals: Multiphase experiment set-up; acquisition of ability to deeply investigate an RNAi phenotype, looking at planarian internal anatomy and gene expression.	After <i>β-catenin(RNAi)</i> , what is the distribution of axial patterning markers?
	After <i>β-catenin(RNAi)</i> , does the planarian with two heads also have two brains?
	After <i>odf2(RNAi)</i> , are there any neural defects?
	After <i>odf2(RNAi)</i> , are there any muscular defects?

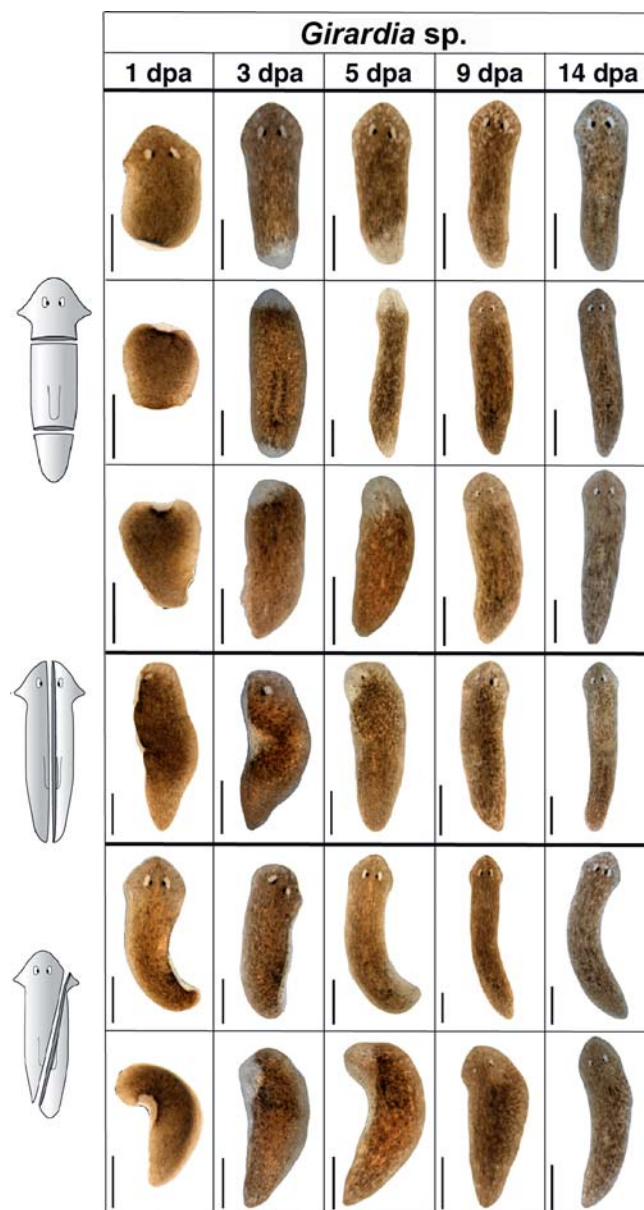


Figure 5. *Girardia* sp. regeneration time courses at room temperature after three amputation paradigms: two transverse amputations; one longitudinal amputation; one oblique cut. Worm fragments were observed 1, 3, 5, 9, and 14 dpa. Immediately after amputation, wound closure is triggered by muscle contractions (darkly pigmented region at the site of injury) followed by the appearance of an unpigmented blastema. Regenerating eyes are visible by 5 dpa. By 9 dpa, the pharynx regenerates and the blastema becomes pigmented. By 14 dpa, regeneration is complete. Scale bars = 500 μ m. A color version of this figure can be viewed online.

reaction that produces blue staining in the cells that contain the mRNA-riboprobe-antibody complex (King & Newmark, 2013). Here, the expression of eleven genes is shown, enabling students to visualize the stem cells (neoblasts), body axis domains, and major organ systems (Table 3) (Figures 9–12). Related activities are mentioned in Table 2. “Whole mount *in situ* Hybridization (WISH)” protocol is available at <http://cuttingclass.stowers.org>.

RNAi: Disruption of Gene Function

To understand the function of a given gene, biologists disrupt its activity and observe the deficiencies (phenotypes) that result. RNAi is the standard technique applied to planarians to dissect gene function (Sánchez Alvarado & Newmark, 1999; Newmark et al., 2003;

Rouhana et al., 2013). The RNAi exploits a specific double-strand RNA (dsRNA), complementary to the mRNA of interest that must be either produced in bacteria or synthesized *in vitro*. The dsRNA is delivered into the worm by either feeding or injection, and diffuses into the cytoplasm of the cells. The dsRNA activates a defense mechanism that induces the degradation of both dsRNA and its complementary mRNA (Agrawal et al., 2003). Thus, the function of any given gene during regeneration can be studied through systematic RNAi knock-down (Hannon, 2002).

Here, two RNAi experiments are described: the *β -catenin*(RNAi) illustrates a polarity phenotype, and *odf2*(RNAi) gives rise to a behavioral phenotype (Figure 13) (Gurley et al., 2008; Petersen & Reddien, 2008; Reddien et al., 2005). Related activities are

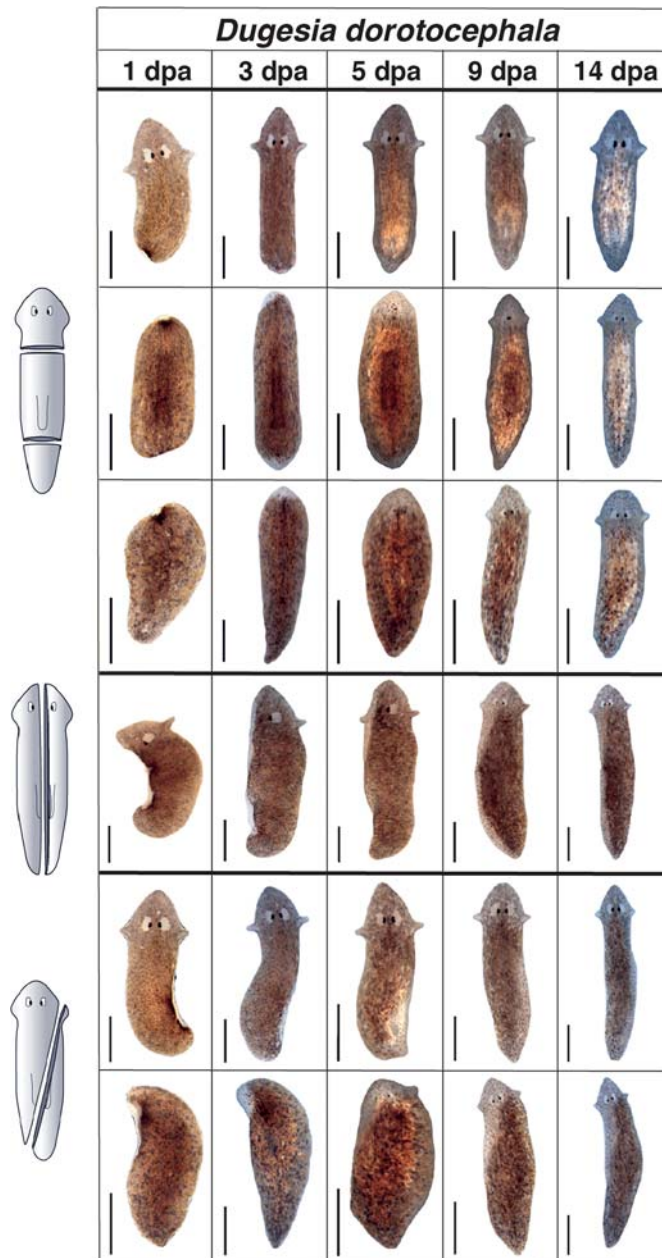


Figure 6. *D. dorotocephala* regeneration time courses at room temperature after three amputation paradigms: two transverse amputations; one longitudinal amputation; one oblique cut. Worm fragments were observed 1, 3, 5, 9, and 14 dpa. Immediately after amputation wound closure is triggered by muscle contractions (darkly pigmented region at the site of injury) followed by the formations of an unpigmented blastema. Regenerating eyes are visible by 5 dpa, and the worm outline is reconstituted. By 9 dpa, the pharynx regenerates, new auricles become visible, and the blastema becomes pigmented. By 14 dpa regeneration is complete. Five days after the third amputation paradigm, only the right auricles are regenerated. This asymmetry is due to the different amount of tissue present on the lateral side of the head. Scale bars = 500 μ m. A color version of this figure can be viewed online.

mentioned in Table 2. “RNA Interference (RNAi)” protocol is available at <http://cuttingclass.stowers.org>.

○ Results

Regeneration

Regeneration is documented for *Girardia* sp. (Figure 5), *D. dorotocephala* (Figure 6), *P. morgani* (Figure 7), and *P. gracilis* (Figure 8)

following three different amputation paradigms: (1) two transverse amputations perpendicular to the anteroposterior (A/P) axis; (2) one lateral amputation parallel to the A/P axis; and (3) one oblique cut made at an approximate 45° angle to the A/P axis.

- **Wound healing:** Following amputations, the first important step is the rapid contraction of the wound that reduces exposure of internal tissues to the environment (Figures 5–8). Specifically, after the second amputation paradigm, the contraction of the body causes the formation of a curved, U-like body shape,

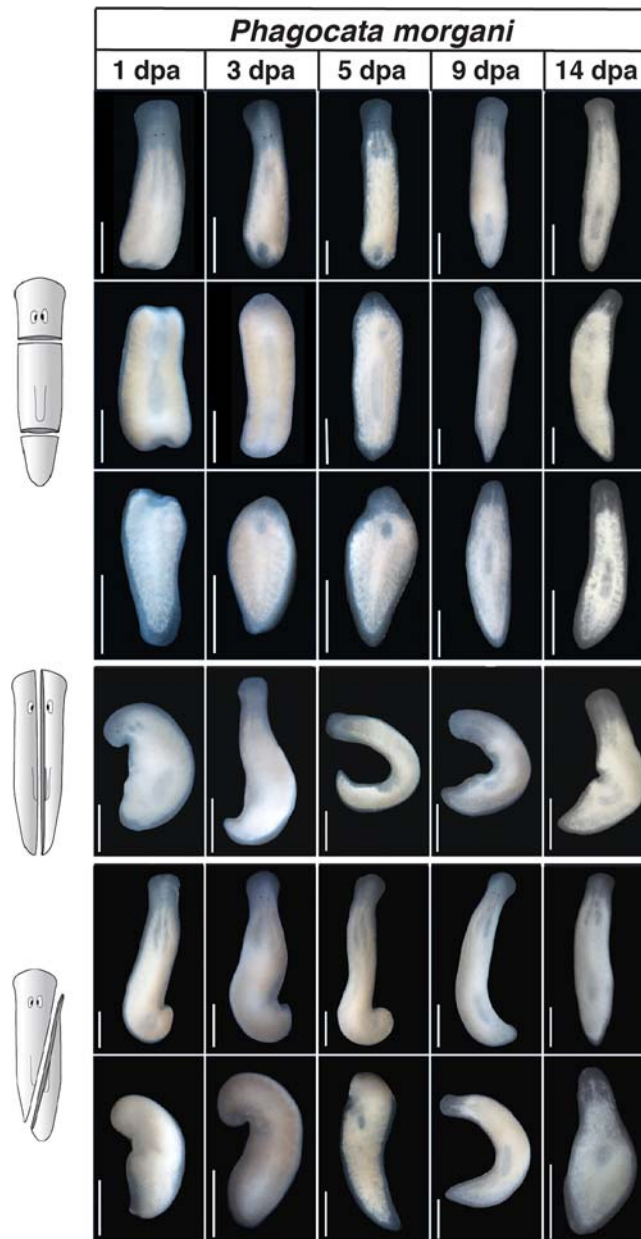


Figure 7. *P. morgani* regeneration time courses at room temperature after three amputation paradigms: two transverse amputations; one longitudinal amputation; one oblique cut. Worm fragments were observed 1, 3, 5, 9, and 14 dpa. Immediately after amputation, wound closure is triggered via muscle contractions. The white color of the worms makes the observation of blastema formation challenging, but at 3 dpa, a transparent area of new growing tissues is distinguishable. The regeneration of the pharyngeal cavity corresponds to the transparent area in the middle of the planarian body (3 dpa). At 5 dpa, the worm outline is reconstituted, and regenerating eyes are visible since 9 dpa. *P. morgani* is slightly slower in regenerating than *Girardia sp.* and *D. dorotocephala* because at 14 dpa the body shape, scale, and proportion are not yet completely reestablished, and the eyes are significantly smaller than the original one. Scale bars = 500 μ m. A color version of this figure can be viewed online.

and the fragments swim in a circle. Similarly, after the third amputation paradigm, the musculature contraction causes the formation of a curl where the tissue is thinner, and also in this case, the posterior fragment cannot swim straight.

- **Blastema formation and regeneration of new anatomy:** The next step during regeneration involves the formation of a blastema, which is a special type of tissue found in many regeneration contexts. It consists of a single-layered epidermis surrounding an internal mass of proliferating and differentiating cells

able to regenerate all missing tissues (Newmark & Sánchez Alvarado, 2000). In all species, the blastema is unpigmented (white) and it gradually increases in size as new anatomy is rebuilt (Figures 5–8). The most obvious anatomical feature that emerges from the blastema during regeneration are the eyes, which increase in size and pigmentation over the time.

- **Tissue remodeling:** The process of integrating newly regenerated structures with preexisting anatomy through tissue remodeling is an important step. Tissue remodeling is most evident in the

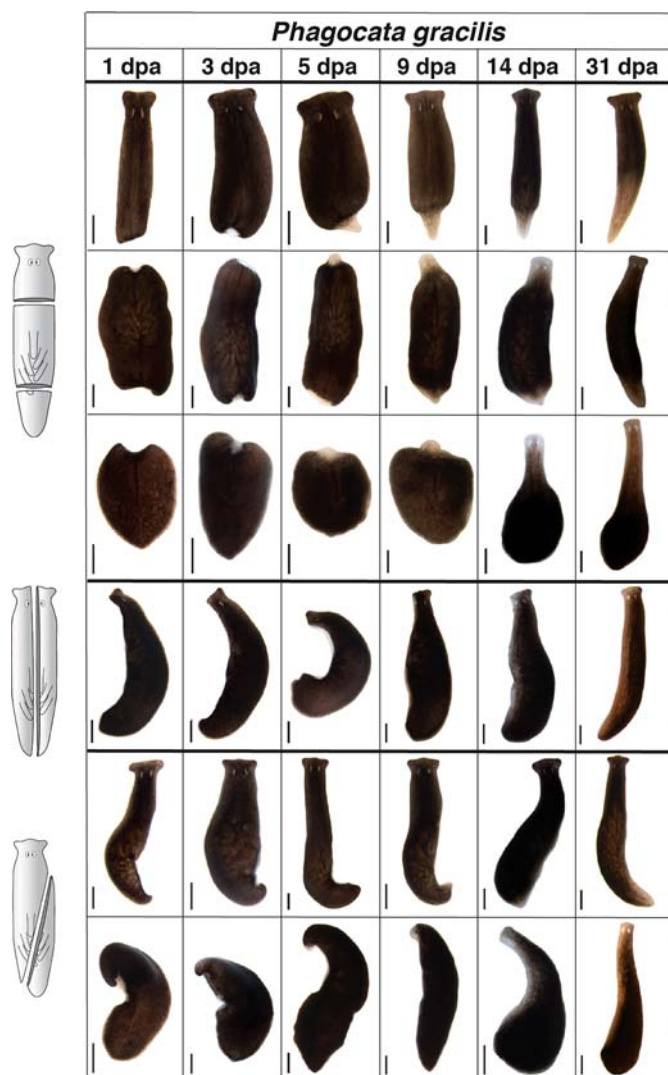


Figure 8. *P. gracilis* regeneration time courses at room temperature after three amputation paradigms: two transverse amputations; one longitudinal amputation; one oblique cut. Worm fragments were observed 1, 3, 5, 9, 14, and 31 dpa. Immediately after amputation, wound closure is triggered via muscle contractions, and soon after, an unpigmented blastema forms. Regenerating eyes are visible from 9 dpa. The dark color of *P. gracilis* does not allow monitoring the regeneration of the pharyngeal cavity and multiple pharynxes. *P. gracilis* has significantly slower regenerative capacity than other planarians and almost no ability to remodel and resize its old tissues. At 14 dpa the regenerated tissues remain less pigmented than the original fragment. At 31 dpa, body pigmentation has almost recovered, but body plan scale and proportion has not been fully reestablished. Scale bars = 500 μm . A color version of this figure can be viewed online.

case of the pharynx after the first amputation paradigm. The new pharynx appears in the blastema, and eventually becomes positioned in the new animal's midsection as the scale and proportion of the body is reestablished. Although the preexisting pharynx is in the central fragment, it shrinks in size over the course of tissue remodeling to properly scale for the size of the new animal body. The pharynx can be easily seen and monitored from the dorsal side of the worms.

- **Completion of regeneration:** Regeneration is considered complete when the blastema becomes fully pigmented, the body proportions are reestablished, and the animal is capable of eating. *Girardia* sp., *D. dorotocephala*, and *P. morgani* (Figures 5–7) complete regeneration in 14 days at room temperature. In contrast, *P. gracilis* (Figure 8) requires two more weeks to fully

regenerate. Notably, during the entire regeneration process, the polarity of the axes is preserved, as is the ability of the fragments to swim.

WISH: Localization of Gene Expression

The WISH protocol, through the use of riboprobes, detects and stains the cells that are expressing a gene of interest. In this paper, markers for planarian body axes and organs were selected and used to illustrate their anatomy and distribution (Table 3).

- **Stem cells:** *piwi-1* is a gene expressed specifically in neoblasts and is most commonly used to visualize the planarian stem cells (Reddien et al., 2005). Neoblasts are spread throughout the body of all four planarian species. *Girardia* sp. (Figure 9), *D. dorotocephala* (Figure 10), and *P. morgani* (Figure 11)

Table 3. Function and expression localization of genes used for the WISH protocol.

Name	Function	Tissue specific expression	Citation
<i>piwi-1</i>	Protein that promotes stem cell maintenance and differentiation.	stem cells (neoblasts)	Reddien et al., 2005
<i>pc2</i>	Enzyme whose activity is the proteolytic processing of prohormones to mature neuropeptides.	nervous system	Collins et al., 2010
<i>opsin-like</i>	Light-sensitive protein with the ability to convert photons of light into electrochemical signal.	eyes	Sánchez Alvarado & Newmark, 1999
<i>porcupine-like</i>	Protein involved in the pathway of lipid metabolism.	gut branches	Gurley et al., 2008
<i>laminin-like</i>	Fibrous protein present in the basement membrane of epithelia.	pharynx	Cebrià & Newmark, 2007
<i>innexin</i>	Transmembrane protein involved in the formation of channels permeable to ions and small molecules.	protonephridia (kidney)	Oviedo & Levin, 2007
<i>collagen</i>	Elongated fibril mostly found in fibrous tissues, such as muscle tissue.	muscle	Witchley et al., 2013
<i>ifb</i>	Cytoplasmic intermediate filament involved in mechanical strength and structure attachment in epidermal cells.	dorsoventral (D/V edge)	Molina et al., 2011
<i>slit</i>	Secreted midline protein with repulsive activity that prevents some cells to cross the midline.	midline	Cebrià et al., 2007
<i>sfrp-1</i>	Secreted frizzled-related protein and modulator of wnt signaling.	anterior end	Gurley et al., 2008
<i>frizzled-like</i>	Wnt receptor involved in the maintenance of the posterior tissues.	posterior end	Gurley et al., 2008

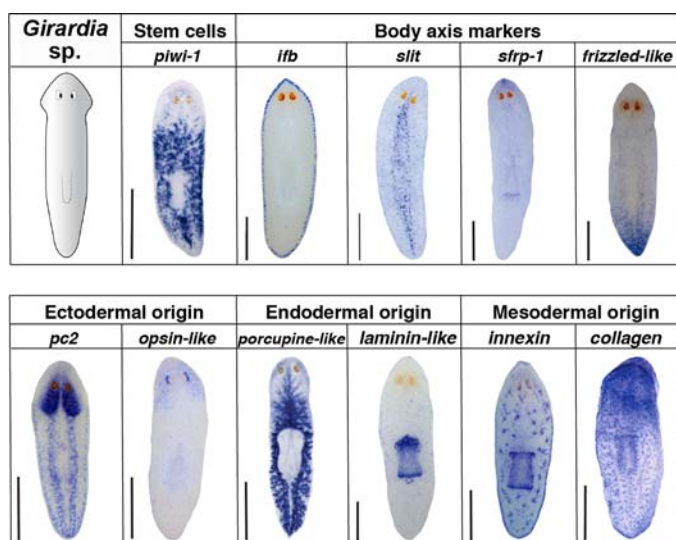


Figure 9. Colorimetric WISH of *Girardia sp.* genes illustrating planarian anatomy: *piwi-1* (neoblasts), *ifb* (D/V edge), *slit* (midline), *sfrp-1* (anterior end), *frizzled-like* (posterior end), *pc2* (nervous system), *opsin-like* (eyes), *porcupine-like* (gut branches), *laminin-like* (pharynx), *innexin* (protonephridia), and *collagen* (muscle). Scale bars = 500 μ m. A color version of this figure can be viewed online.

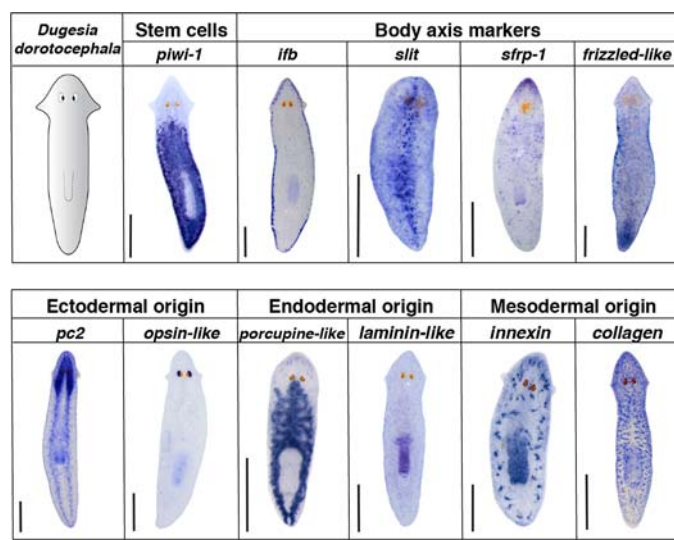


Figure 10. Colorimetric WISH of *D. dorotocephala* genes illustrating planarian anatomy: *piwi-1* (neoblasts), *ifb* (D/V edge), *slit* (midline), *sfrp-1* (anterior end), *frizzled-like* (posterior end), *pc2* (nervous system), *opsin-like* (eyes), *porcupine-like* (gut branches), *laminin-like* (pharynx), *innexin* (protonephridia), and *collagen* (muscle). Scale bars = 500 μ m. A color version of this figure can be viewed online.

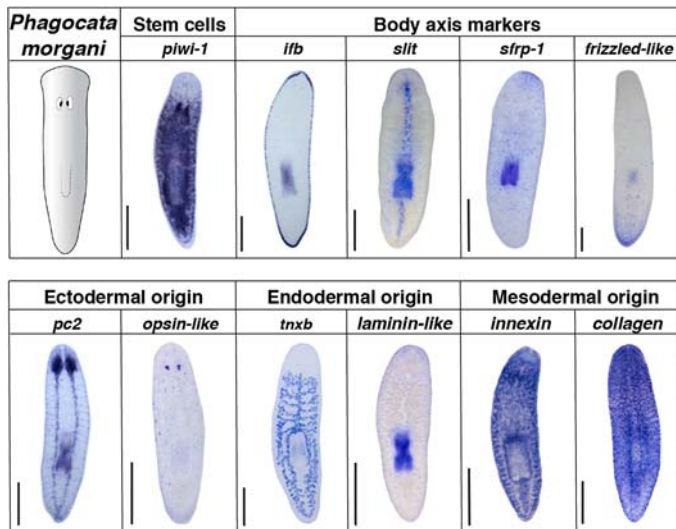


Figure 11. Colorimetric WISH of *P. morgani* genes illustrating planarian anatomy: *piwi-1* (neoblasts), *ifb* (D/V edge), *slit* (midline), *sfrp-1* (anterior end), *frizzled-like* (posterior end), *pc2* (nervous system), *opsin-like* (eyes), *tnxb* (gut branches), *laminin-like* (pharynx), *innexin* (protonephridia), and *collagen* (muscle). Scale bars = 500 μ m. A color version of this figure can be viewed online.

neoblasts are distributed everywhere except in the pharynx and the most anterior part of the worms. In contrast, *P. gracilis* (Figure 12) neoblasts appear to be more broadly distributed with particular enrichment in the brain.

- **Boundaries and axes:** The expression of the genes *ifb* and *slit* define the mediolateral (M/L) axis. *ifb* defines the edge of the worms, while *slit* delineates the midline of the animals (Figures 9–12) (Molina et al., 2011; Cebrià et al., 2007). Additionally, *sfrp-1* and *frizzled-like* represent good markers of the anterior and posterior ends of the planarian, respectively (Figures 9–12) (Gurley et al., 2008).
- **Nervous system:** The central nervous system (CNS) is composed of two cephalic ganglia that form the brain, and two ventral nerve cords that reach the tip of the tail (Figure 2). The CNS can be visualized by the expression of the gene *prohormone convertase 2* (*pc2*) (Figures 9–12) (Collins et al., 2010). *opsin-like*, coding for a light-sensitive protein, is specifically expressed in the eye's photoreceptor cells (Figures 9–12) (Sánchez Alvarado & Newmark, 1999).
- **Digestive system:** The *porcupine-like* (Figure 9, 10, 12) and *tnxb* (Figure 11) expression patterns reveal the three main branches of the planarian gut (one projecting anteriorly and two posteriorly) and its complex ramification (Gurley et al., 2008; Vu et al., 2015). Expression of *laminin-like* defines the pharynx, a muscular feeding tube connected to the gut (Figures 9–12) (Cebrià & Newmark, 2007). *Girardia sp.*, *D. dorotocephala*, and *P. morgani* typically possess one pharynx (Figures 9–11). However, *P. gracilis* possesses multiple pharynges (Figure 12).
- **Excretory system:** Regulation of water/salt balance, or osmoregulation, in planarians is maintained by the excretory system, which can be visualized with the protonephridia marker *innexin* that stains the flame cells (Oviedo & Levin, 2007).

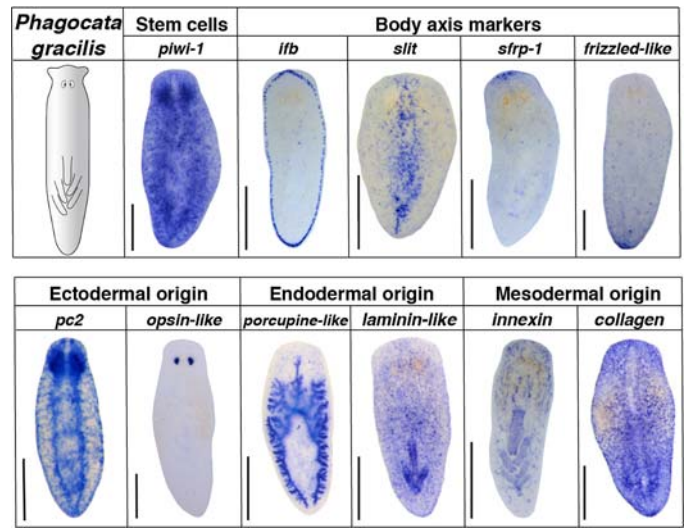


Figure 12. Colorimetric WISH of *P. gracilis* genes illustrating planarian anatomy: *piwi-1* (neoblasts), *ifb* (D/V edge), *slit* (midline), *sfrp-1* (anterior end), *frizzled-like* (posterior end), *pc2* (nervous system), *opsin-like* (eyes), *porcupine-like* (gut branches), *laminin-like* (pharynx), *innexin* (protonephridia), and *collagen* (muscle). Scale bars = 500 μ m. A color version of this figure can be viewed online.

The protonephridia are the single functional units of the excretory system homologous to the nephridia of vertebrate kidneys. The planarian protonephridia are tubular structures spread throughout the entire body of the worms (Figures 9–12).

- **Body wall musculature:** The muscular system expresses collagen and a higher concentration of muscle cells (area with more intense signal) can be observed in the head (Witchley et al., 2013). Muscle cells are also spread in the posterior part of the planarians and in the pharynx (Figures 9–12). *Girardia sp.* shows an intense staining in the pre-pharyngeal region (Figure 9), whereas *P. morgani* has more concentrated muscle cells in the anterior part of the body (Figure 11).

RNAi: Disruption of Gene Function

RNAi specifically degrades mRNA for genes of interest, revealing the function of the targeted gene product through the phenotypes that emerge in the absence of its protein. Here, β -catenin (in each species) and *odf2* (only in *P. morgani*) genes were targeted for RNAi. After three feedings, the animals were cut perpendicularly to the A/P axis into three fragments and observed during their regeneration.

Upon silencing the β -catenin gene, each species displays a disruption in regeneration polarity. Each fragment regenerates an ectopic head from posterior wounds instead of a tail, showing a “double-head” or “Janus” phenotype (Figure 13A). Furthermore, uninjured animals of each species subjected to β -catenin(RNAi) will likely become anteriorized, converting their tails into a second head and sprouting ectopic heads along the body edge, as has been observed in *S. mediterranea*. This phenotype indicates that β -catenin is required for the specification and maintenance of posterior tissues in planarians (Gurley et al., 2008; Petersen & Reddien, 2008; Adell et al., 2009).

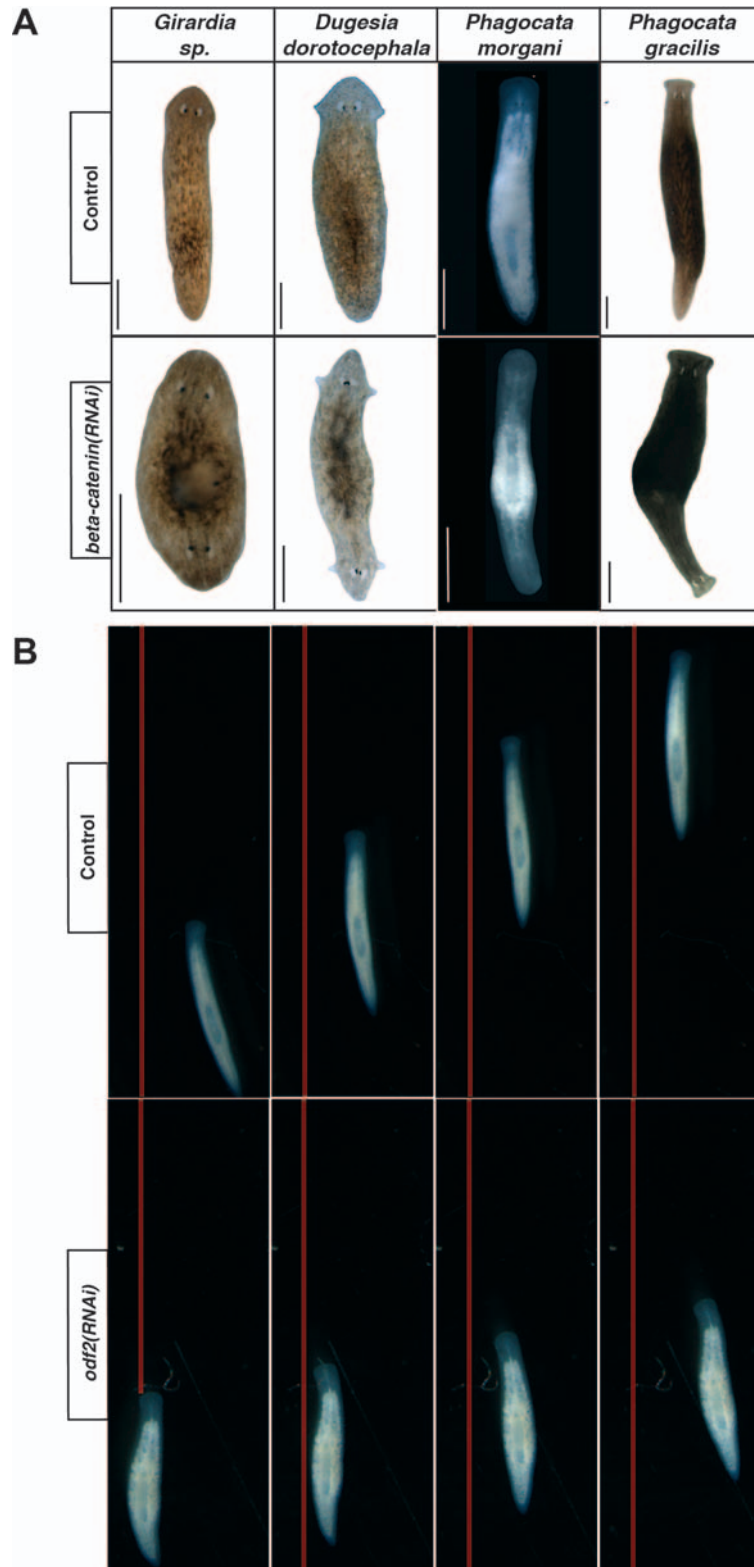


Figure 13. (A) Live images of control and *β-catenin(RNAi)* planarians following pre- and post-pharyngeal amputation for each species: *Girardia sp.*, *D. dorotocephala*, *P. morgani*, and *P. gracilis*. An ectopic head regenerated from the posterior wound is visible in *Girardia sp.* (phenotype visible at 5 dpa in the trunk, at 7 dpa in the head, and at 11 dpa in the tail), *D. dorotocephala* (phenotype visible at 11 dpa in the trunk, at 13 dpa in the head, and at 15 dpa in the tail), *P. morgani* (phenotype visible at 12 dpa in each fragment), and *P. gracilis* (phenotype visible at 18 dpa in each fragment) after *β-catenin(RNAi)* treatment. **(B)** Single frames from a video of control and *odf2(RNAi)* planarians of the species *P. morgani* at 10 dpa. Controls swim roughly parallel to the red line, whereas *odf2(RNAi)* planarians swim off to the right due to disruption of ventral cilia orientation. Scale bars = 500 μm. A color version of this figure can be viewed online.

Upon silencing of the *odf2* gene, all fragments display a behavioral phenotype. The worms appear morphologically normal, except that instead of swimming forward, they move toward their right side (Figure 13B). This “sidewinder” phenotype is caused by the misorientation of the cilia on the ventral side of the animals whose beating allows their normal locomotion. This phenotype shows that *odf2* is involved in the orientation of the ventral cilia, and when it is interfered with via RNAi, the direction of animal locomotion is misoriented (Reddien et al., 2005).

○ Conclusion

Collectively, the information and experimental manipulations presented here provide students with a set of carefully thought out and controlled, curiosity-driven, hands-on experiences in science. The activities are composed of questions, experimental approaches to answer them, data collection, and data analyses, allowing students to apply the scientific method to solve complex biological problems using planarians, invertebrates that are routinely used in biomedical research laboratories. Students will also gain an in-depth knowledge of key topics in the rapidly moving fields of regeneration and stem cell biology, allowing them to better understand and critically evaluate experiments in regenerative medicine and stem cell technologies. Ultimately, we hope this teaching paper will spark student excitement about science in general, and the field of regeneration and stem cell biology in particular.

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Transcriptomes are deposited with the TSA (NCBI). Accession numbers: GEHB00000000 (*Girardia sp.*), GEIG00000000 (*Dugesia dorotocephala*), GEKK00000000 (*Phagocata morgani*) and GEGP00000000 (*Phagocata gracilis*). Funding for this project was provided by Howard Hughes Medical Institute and Stowers Institute for Medical Research.

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Appendix 1. Glossary.

Auricles	Chemosensory organs usually located on the lateral anterior margin of the planarian head.
Blastema	A group of proliferating and differentiating cells that coalesce at a wound to give rise to a new body part.
Ectoderm	The outermost of the three primary germ layers of an embryo; forms the epidermis and nervous system.
Endoderm	The innermost of the three primary germ layers of an embryo; forms the gut and associated structures.
Mesoderm	The middle of the three primary germ layers of an embryo; forms several structures, such as the connective tissue and the excretory system.
Polarity	The asymmetric distribution of specific features in a cell, tissue, or organism that determines axial and/or spatial orientation.
Protonephridia	The excretory system of the worms. Like the kidney, it is a system of branching tubules that maintain the osmoregulatory balance of the body. Each protonephridia is a single unit of the system composed of a multiciliated flame cell, connected to an epithelial duct (tube) that opens externally through pores in the animal’s epidermis.

Appendix 1. Continued

Remodeling	The reorganization of cells and tissues in the preexisting tissue of a regenerating fragment that ultimately reestablishes proper body proportions in the new animal.
Riboprobe	Fragment of RNA, synthesized <i>in vitro</i> , whose nucleotide sequence is complementary to the target mRNA. The nucleotides of the riboprobe are specifically labeled with small molecules (e.g., digoxigenin, dinitrophenol, or fluorescein) for the riboprobe detection and localization in cells and tissues.
RNAi	A technique that uses double-stranded RNA molecules to inhibit the expression of a specific gene, and thus examines the effect of its absence.
Stem cell	An undifferentiated cell that is capable of dividing and giving rise to more stem cells and to differentiated, tissue-specific cell types.
Totipotent cell	A cell with the potential to give rise to all the cell types of the body and extra-embryonic tissues.
Transcriptome	The sum of all RNA molecules expressed in a cell, tissue, organ, or organism in a specific moment and condition.