Whole Organ Regeneration - How Do Salamanders Do It?

Introduction

This activity introduces and investigates developmental processes that operate within multicellular organisms to maintain structure and function. This activity uses lecture, group discussion, and hands-on laboratory experiments to investigate tissue regeneration. The activity is scheduled over three class periods (~75 minutes each) but you can teach at your own pace.

Activity Outline

Learning Goals

Students will be able to distinguish between homeostasis, development, and regeneration.
Students will be able to discuss why some organisms regenerate tissues and others don't.
Students will be able to develop a model of organ regeneration that considers tissues and specialized cells.
Students will be able to explain the utility of perturbing a structure or process in a scientific experiment.
Students will formulate hypotheses, design experiments, collect and analyze data, and interpret results. By performing experiments, students will gain working knowledge of the scientific process.

Activity Description - Class Period 1

1) The activity begins with a brief introduction to axolotl salamanders and their amazing ability to regenerate whole organs, including limbs, tail, brain, and spinal cord*.

2) Students are paired into groups of 3-4 and each group is asked to develop a recipe/model for limb regeneration, providing a step-by-step description of the overall process. A student in each group writes down the model**.

3) A member of each group reads aloud their model to the whole class and the instructor writes key steps of each model on a chalk/white board, noting similarities and differences among groups***.

4) The instructor provides a more complete lecture about regeneration, walking through the steps of regeneration and integrating information from group models where appropriate****.

5) The instructor provides an overview of the laboratory exercise that will be performed during the next class period*****.

Teaching Notes

* The instructor provides some interesting fun facts about regeneration, for example noting the relative time that it takes for a salamander to regenerate a limb and the capacity to regrow only and exactly tissues that are removed by amputation of the limb or tail. However, the instructor purposely does not go into the details of regeneration, this is left to the imagination of students.

**Free-thinking exercises provide students an opportunity to formulate their own initial ideas, drawing upon any information that comes to mind. Such exercises reveal gaps in logic and knowledge that can be addressed by the instructor as the class works toward a consensus model of regeneration. It helps to emphasize that regeneration is an example of a developmental process that takes time to complete; it doesn't happen instantaneously. Analogous to following a food recipe that involves completing a series of steps, student can similarly develop a step-by-step set of instructions for regenerating a limb.

***Instructors should anticipate that students will offer lots of interesting ideas that may or may not be relevant to regeneration. Be flexible and open, wrong ideas can be cleaned up later when providing an over-view of regeneration. Be sure to collect group models for contemporary and archival reference for future classes.

****The lecture should detail regeneration at a development process level and not at a molecular mechanism level.

Background Information

Regeneration can be thought of as a model/recipe that requires successful completion of a series of steps.

Step 1. Regeneration is triggered by an injury that breaks the protective skin barrier and exposes internal anatomy. Scientists typically perform amputation injuries because they are highly repeatable. (Important Concept: Repeatability and rigor are essential when planning and performing experiments). Injury causes damage and some cells to die and some cells to survive. (Important Concept: Debris from dying cells, signals from surviving cells, and the wound environment provide important cues for initiating regeneration).

Step 2. The open wound caused by an amputation injury is quickly covered by skin cells. In small axolotl embryos, skin cells will cover a tail amputation wound in 30-40 minutes. In adult axolotls, skin cells will cover the wound in 8-12 hours. (Important Concept: Regeneration is faster in smaller/younger animals). During wound closure and extending several days afterwards, the immune system is stimulated to protect the salamander from infection. (Important Concept: Regeneration initially involves a wound healing stage).

Step 4. Below the newly covered wound surface, enzymes are activated to breakdown and remodel the internal environment. These changes are associated with two important developmental processes: de-differentiation and stem cell recruitment. To understand these concepts, we need to think about cells and their life cycles. During a cell's life cycle, it matures from an undifferentiated or stem cell state to a differentiated state. When a cell becomes fully differentiated it performs a specific function (e.g. muscle cell or bone cell) can no longer undergo cell division (mitosis), at least in mammals. During regeneration, signals from the remodeled internal environment instruct some cells to de-differentiate and regain capacity to

divide. Also, signals from the internal environment stimulate stem cells to participate in the regeneration process. (Important Concept: Regeneration in salamanders depends upon cellular de-differentiation and stem cell recruitment, the relative contribution of these processes depends upon the type of organ and even in some cases, the age of the animal).

Step 3. Stem cells and de-differentiated cells migrate to a site just internal to the wound surface and from a cellular mass called a blastema. As the number of blastema cells increase through cell division, new tissue forms and there is noticeable outgrowth beyond the original amputation site. (Important Concept: Tissue outgrowth happens gradually over time. The amount and type of outgrowth can be used to describe the regeneration process).

Step 4. Cells in the blastema have a memory of what tissues they were recruited from. For example, muscle stem cells that are recruited into the blastema differentiate to form new muscle tissue. This step of regeneration is often called pattern formation because newly regenerated tissues start to reform or pattern the regenerating limb. (Important Concept: Regenerating cells differentiate to form the correct, missing cell types).

Step 5. After a limb or tail fully forms it is a miniature replica of a full-sized organ. Over the course of a few additional weeks the tail and limb increases in size, growing to a size that is proportional to the size of the organism. (Important Concept: Cells regenerate organs of the correct size and shape).

Activity Description - Class Period 2

1) The activity begins with a brief introduction to animal research. Much of what we know about life and ourselves comes from experiments using animals. There are strict guidelines to ensure that animals are treated ethically when performing experiments, the type and number of animals must be justified and approved by a regulatory committee that ensures that approved methods are followed when animals are used in experiments. In the case of axolotl tail regeneration, the activity uses a non-feeding embryo stage that does not require an Institutional Animal Care and Use Committee (IACUC) protocol. If the activity used a feeding stage larva or adult, an IACUC protocol would be required to perform the activity*.

2) The instructor provides an overview of the experiment that will be performed by students. Working in groups of 2, students will anesthetize axolotl embryos in a solution of benzocaine, carefully remove and lay embryos out on wet paper towels beside a ruler, and then under a dissecting microscope, perform an amputation that removes 2 mm of the distal tail tip. After performing the amputation, the embryo is placed in a well of a microtiter plate that contains a solution used for axolotl embryo culture. The instructor then demonstrates the procedure to the class**.

3) Students then perform the activity.

4) Students monitor embryos in microtiter plates for recovery, share data, and clean up their work areas.

Step By Step Instructions

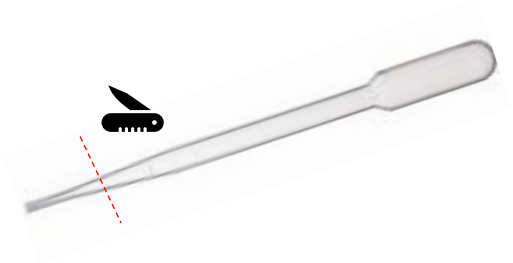
1) Each group receives the following items:

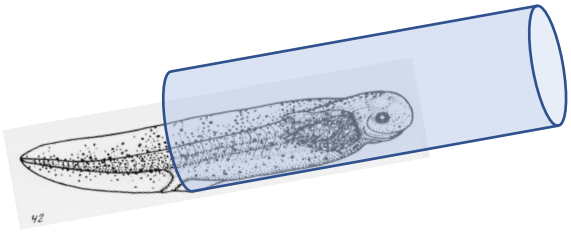
- plastic or glass container that is partially filled with axolotl rearing water (ARW)[1] and contains a few axolotl embryos
- plastic or glass container that is partially filled with 0.02% benzocaine[2]
- 3 ml plastic dropper pipettes
- Scalpel or razor blade
- 15 cm ruler
- Paper towels
- Squirt bottle filled with ARW
- 12 well microtiter plate that is prefilled with either 2 ml of ARW or 2 ml of a chemical that inhibits regeneration
- Dissecting microscope

[1]ARW Recipe: 1.75 g NaCl, 100 mg $MgSO_4$, 50 mg $CaCl_2$, and 25 mg KCl per liter. The water is buffered using NaHCO3 to achieve a pH in the range of 7.1 -7.6.

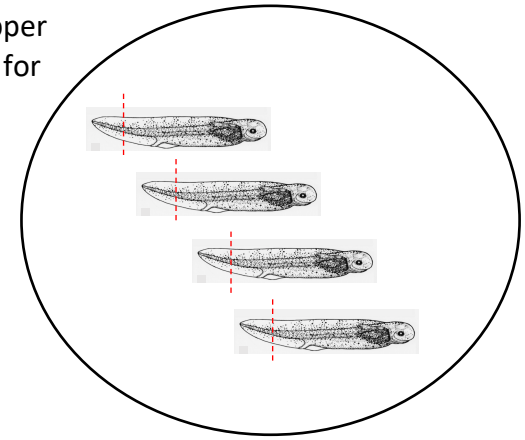
[2]0.02% benzocaine anesthetic: Benzocaine is made by dissolving 0.2 g benzocaine in 5 ml 100% EtOH. This solution is rapidly stirred into 1 liter of ARW.

2) Each well of a microtiter plate is filled with 2 ml of ARW***.
3) A single paper towel is folded in half and laid flat on the dissecting microscope stage. The paper towel is thoroughly wettened with ARW using the squirt bowl.
4) An embryo is carefully sucked up headfirst into a plastic dropper that has been clipped at the end to increase bore size; the embryo is then placed in the benzocaine solution. The embryo is left in the benzocaine solution for 3-5 minutes until non-moving.

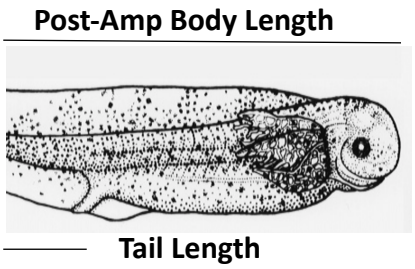




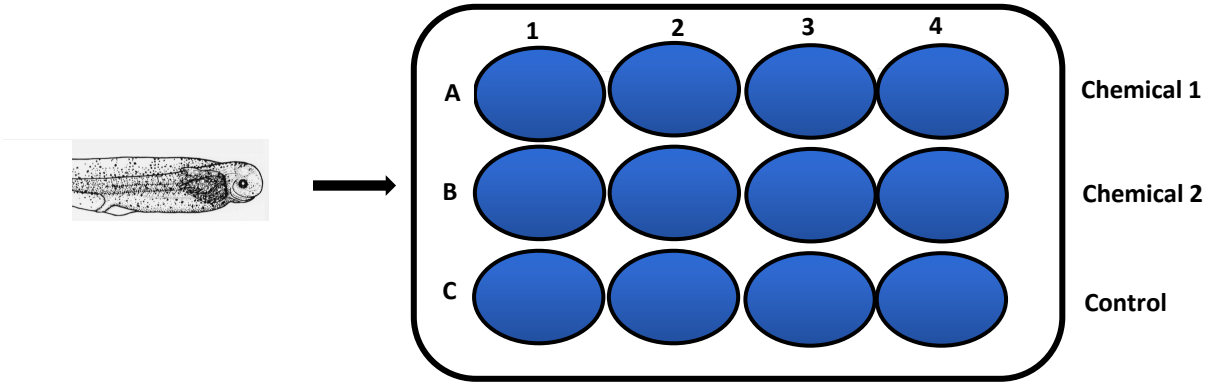
5) After an embryo is sedated, it is sucked into the dropper headfirst and moved to the plastic container with ARW for a brief rinse. It is then sucked into the dropper and carefully positioned horizontally on wet paper towels on the microscope stage****.
6) A ruler is positioned above the embryo to provide a reference for performing the amputation.
7) While looking through the eyepieces of a dissecting microscope, a scalpel is used to amputate 2 mm of the distal tail tip.



8) Students can record at this time the length of the embryo post-amputation or the length of the tail (using the cloaca as a reference point). Alternatively, if microscopes are equipped with cameras, embryo images can be acquired*****.



9) The embryo is picked up with the plastic dropper and moved into a single well of the microtiter plated. The well-position of each embryo in the plate provides a way to reference and separately identify each individual (e.g. A1, A2, A3, A4)
10) Steps 3-9 are repeated until all embryos have been processed.



12 well microtiter plate

Teaching Notes

*Introduce the ethical use of animals in research and encourage student discussion. Alternatives to animal research can be discussed, like the use of cell lines and synthetically grown organ-mimics from cells. It is important to note that regulations are not only meant to ensure the ethical use of animals in research but also that experiments are performed in a rigorous and reproducible manner, so that justifiable numbers of the most appropriate animal are used.

**Embryos are removed from their egg membranes and jelly coat by the instructor before the activity begins. This requires one or two sharp pair of forceps and some patience/skill. Emphasize the fragility of the embryos that the students will be working with, they can be easily damaged if handled in correctly. Embryos are manipulated using disposable plastic droppers that are cut to have a relatively wide bore. Embryos are suctioned into the end of the pipette with fluid and then moved to wet paper towels that are positioned on the stage of a dissecting microscope. Students use razor blades to cut the end of the tail tip; it is important to show them how to properly hold the razor blade to make reproducible cuts without cutting themselves. You will find that some students will have difficulty performing amputations while looking through the dissecting microscope, make sure that the eyepieces have been properly set to clearly see the embryo and a ruler that is positioned slightly above the axolotl on the stage to serve as a reference for amputating only 2 mm of the distal tail tip. A mock experiment can be performed ahead of time to give students practice in performing amputations under a microscope using pieces of cooked spaghetti.

***The instructor will give some groups ARW (control) and some groups a chemical inhibitor of regeneration (treatment) in their microtiter plates. Many chemicals have been shown to inhibit regeneration (Ponomareva et al 2015), especially chemicals that inhibit histone deacetylases (Voss et al 2019). Generally, inhibitors are used at a final concentration of 10 micromolar. If funds are not available to purchase known chemical inhibitors of regeneration, the activity can use an unknown chemical (e.g. aspirin) or simply monitor the regeneration process using only a control condition.

****Instead of processing embryos one at a time, multiple embryos can be processed simultaneously, by laying them in staggard columns as shown in the figure above.

*****Collect data from groups into a spreadsheet that can be shared and used to calculate statistics like the mean and standard deviation. Students can monitor embryos during the week to observe the process of regeneration.

Activity Description - Class Period 3 (6-7 days after Class Period 2)

1) The activity begins with the instructor providing a brief review of the previous two class periods. A week has passed since embryos received tail amputations. Typically, a week is a sufficient length of time for regeneration to complete. The instructor introduces the need to analyze the embryos again and collect regeneration data to complete the experiment. The instructor provides an overview of the activity that will be performed by students. Working in groups of 2, students will anesthetize axolotl embryos in microtiter plates using a solution of benzocaine, carefully remove and lay embryos out on wet paper towels beside a ruler, and then under a dissecting microscope, write a qualitative description of what is observed for each embryo, and measure total body length and tail length. After making observations and recording data, embryos are returned to wells of the microtiter plate*.

3) Students perform the activity described above and then clean up their work areas**.

4) Each group analyzes their data by calculating means and standard deviations and by making a graph that summarizes their results***.

5) Each group shares their findings and results with the class****.

Step by step instructions

1) Each student group receives the following items:

- plastic or glass container that is partially filled with 0.02% benzocaine
- 3 ml plastic dropper pipettes
- 15 cm ruler
- Paper towels
- Squirt bottle filled with ARW
- 12 well microtiter plate with embryos from previous week
- Dissecting microscope

2) Students pipette 2 ml of benzocaine solution into each microtiter plate well that has an embryo.
3) A single paper towel is folded in half and laid flat on the dissecting microscope stage. The paper towel is thoroughly wettened with ARW using the squirt bowl.
4) After sedated and no longer motile, embryos are moved to wet paper towels on the microscope stage. Students qualitatively describe the outcome of regeneration for each embryo and measure total body length and tail length with a ruler.
5) Embryos are returned to wells of the microtiter plate containing benzocaine.
6) Quantitative data are entered into a spreadsheet for analysis and graphing.

Teaching Notes

*Students can record the length of the embryo or the length of the tail (using the cloaca as a reference point). Alternatively, if microscopes are equipped with cameras, embryo images can be acquired.

**Students will return microtiter plates with embryos to the instructor. Prolonged exposure to benzocaine is an approved method of euthanasia for axolotls; embryos can be disposed of the following day by pour them along with the benzocaine solution down a sink drain. Follow with lots of water, allow tap to run for 2 min or more to dilute benzocaine thoroughly.

***Students should record their data in a notebook before transferring results to a spreadsheet, such as Excel, which can be used to calculate means, standard deviations, make graphs, and perform simple statistical tests, such as Student's t-test. Simple statistical tests provide a way to introduce sampling theory and data analysis for the purpose of testing hypotheses. If the activity included both control and treatment conditions, the students could use Student's t-test to determine if the means differed significantly given the conditions/samples that were compared. If the treatment alters regeneration, this finding can lead to discussions about the presumptive effect of the chemical on a regeneration process.

****All of the student's data can be accumulated into a single file to analyze the data and compare results across groups. The instructor can lead a discussion to address experimental outcomes. It is likely that some embryos will not show perfect regeneration of the distal tail tip, which should look like the tail-tip prior to amputation. The sources of among embryo variation in regeneration outcome, including quantitative differences, and non-regenerative and asymmetrical outcomes can be discussed as sources of technical or biological variation. For example, there may be variation in how students perform amputations; some may amputate more or less than 2 mm of the distal tail tip, others may cause amputation injuries that may heal more slowly or not at all. Variation is expected in experimental results; it is good scientific practice to think about sources of variation that can affect experimental results. Be sure to collect group data for reference when teaching future classes.

Literature Cited and Additional Reading

Ponomareva LV, Athippozhy AT, Thorson JS, Voss SR. 2015. Using *Ambystoma mexicanum* (Mexican Axolotl) embryos, chemical genetics, and microarray analysis to identify signaling pathways associated with tissue regeneration. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology* 178:128-35.

Baddar NW, Dwaraka VB, Ponomareva LV, Thorson, JS, Voss, SR. 2021. Chemical genetics of regeneration: Contrasting temporal effects of $CoCl_2$ on axolotl tail regeneration**.** 250:852-865.

Baddar NW, Chithrala A, Voss SR. 2018. Amputation-induced ROS signaling is required for axolotl tail regeneration. *Developmental Dynamics* 248:189-196.

Voss SR, Ponomareva LV, Dwaraka VB, Pardue KE, Baddar NW, Rodgers AK, Woodcock MR, Qiu Q, Crowner A, Blichmann D, Khatri S, Thorson JS. 2019. HDAC regulates transcription at the outset of axolotl tail regeneration. *Scientific Reports* 9:675.

Voss SR, Smith JJ, Timoshevskaya N, Ponomareva LV, Thorson JS, Veliz-Cuba A, Murrugarra D. 2021. HDAC titration of transcription and axolotl tail regeneration. *Frontiers in Cell and Developmental Biology* 9:767377.