

Responses to reviewers

I'd like to thank the reviewers for taking the time to provide helpful and constructive feedback on this manuscript. Detailed responses to each reviewer's suggestions can be found below.

Reviewer #1:

1. It would be extremely helpful to the reader if nice pictures of the skin flap; folding and the end product with the stitches were included. There are diagrams but those are not the real thing. It would be a big plus to have real pictures of the different steps along with the diagrams.

I thank the reviewer for pointing this out. While images are not included, each of these specific steps will be clearly delineated during the step-by-step video demonstration so that readers can visualize the steps of the protocol.

2. In fig 1 step 3 there is a little error: there are 2 bones in the zeugopod, the radius and ulna, and not just one as the cartoon displays.

I apologize if this was confusing. The piece of tissue I have drawn protruding in Figure 1 Step 3 was meant to represent both the muscle and underlying bone. I have now added in the exposed radius and ulna to the diagram and labeled the muscle and bone more clearly.

Reviewer #2:

Major Concerns:

1) adding rationale and explanation behind certain steps or in the choice of reagents used in the protocol. Explaining the use of sulfamerazine sodium, why you perform the surgery on ice, what you mean by "axolotl system water" (and would there be alternatives, e.g. Holtfreter's). This will make the protocol more accessible to new users as well as those that have different experimental setups or considerations. 2) having more of this information as well as troubleshooting and caveats at key points in the protocol itself instead of only within discussion portions. I often had critiques of the protocol that were answered much later in the manuscript. I think trying to bring some of that information to key points in the protocol would be helpful.

I thank the reviewer for this feedback. I have now added the rationale and clarified explanations for sulfamerazine solution, surgical recovery on ice, and axolotl system water (e.g. modified Holtfreter's solution) at the beginning of the protocol during the preparation step (Step 1).

Minor points:

- Are there experimental control conditions that should be followed for later quantitative analysis such as a skin flap without suturing.

I thank the reviewer for bringing up this important point. I have now added an additional step to the protocol, step 2.9, to address this (text below from lines 185-191).

“2.9. Perform a limb amputation on the contralateral limb (optional internal animal control) by amputating the limb at mid-zeugopod level with surgical scissors. Push back the muscle tissue with surgical scissors and trim the exposed bone.

NOTE: An internal contralateral limb control can be done to better assess the success of the surgery during step 4 in the same animal. However, normal amputation of the same limb in a separate animal can also be used to serve as a control.”

I have also added the following text to step 4.2 when assessing the success of the surgery (underlined text in lines 224-227).

“If inspecting on the third week post-surgery or later, make sure a blastema has not formed and compare with how the normal control amputated limb (either from the same animal or a different animal) has progressed during regeneration (i.e. whether a blastema has formed).”

Finally, I have added text to the representative results section to include comparisons to control regenerating limbs in lines 234-235 and 242-244, respectively.

“A successful surgery results in no blastema formation in approximately 2-3 weeks depending on the size of the animal, while control regenerating limbs should form a blastema normally.”

“For comparison, researchers should also examine the control regenerating limb which should have a wound epidermis over the amputation plane and form a blastema over 2-3 weeks.”

- Clarification - Are axolotls hydrated during surgery and recovery with only axolotl water or the anesthetic tricaine solution?

The axolotls are hydrated during the surgery with anesthetic tricaine solution and during recovery with sulfamerazine solution. We have now clarified this in the text in line 195.