We greatly appreciate the reviewers’ comments on the value and importance of our manuscript and thank them very much for the helpful suggestions. We have made all of the suggested changes to clarify the procedure and we have added alternatives to some of the methods that we presented. We feel that with these changes the manuscript has significantly improved, and we hope that the key issues raised by the reviewers are adequately addressed.

**Reviewers' comments:**

**Reviewer #1:**

*Manuscript Summary:*

This protocol describes a method for time-lapse imaging tail regeneration in zebrafish. This is an increasingly popular model for studying regeneration and this protocol contains several helpful hints that will benefit labs wanting to image fin regeneration or other processes in larval zebrafish.

*Major Concerns:*

Comments/questions about the protocol

**Comment 1**. How long can larval fish be time-lapsed in this manner and remain healthy? Does regeneration proceed at the same rate as in unembedded, free-swimming larvae? It would be helpful if the protocol discussed these issues so that readers could consider potential applications.

**Response 1.** We have now updated the discussion section and included a paragraph starting line 457 explaining that we can image up to 1.5 days with this method but that alternative media, such as Danieau could be used to extend the imaging time. For example, we showed previously (Rieger et al., PLoS Biology 2009) that imaging in this medium was feasible for 48 hours on a confocal microscope. Although not included in the discussion, we have also repeated the imaging procedure using Ringers solution and a 1:1000 dilution of 99 % 2-Phenoxyethanol (P1126, Sigma-Aldrich) as anesthetic. Under these conditions we were able to retain the fish healthy for ~ 60 hours in the imaging chamber at 28 °C, suggesting that this method is more optimal.

In addition, we discussed in more detail our presented method in which we remove the agarose from the tail fin during time-lapse imaging to avoid restraining the fin from regeneration. This paragraph starts at line 467. We found that the fin regenerated about 60 % in 1.5 days, a rate that is similar to a previous study by Kawakami and colleagues (Developmental Dynamics 2004) showing that larvae up to 6 dpf regenerate their fins in about 3 days.

**Comment 2**. The protocol described commands using Zeiss Axiovision software, which is fine, but the software being used is not identified until the end of the protocol. The authors should clarify from the beginning which software they are using.

**Response 2.** We based our initial choice not to add this information on the JoVE style guidelines, which reads:

* Avoid the ….. mention of company brand names before/after an instrument or reagent

We however have now included this information earlier in lines 84/ 85, and added it also to later passages (lines 201/ 202 and 279). We do agree that without this information, it is difficult to follow the protocol.

**Comment 3**. Using a "3.5x objective and a 38x zoom" is an unusual choice for this type of imaging. Why not at least use a 10x or 20x objective? If there is a reason why this objective and magnification were chosen it would be helpful if the authors could elaborate on it.

* 1. **Response 3.** To be less confusing, we now state the following: “3.8) Use a stereomicroscope with time-lapse software. Select an appropriate objective and magnification, which will be used later for time-lapse imaging. Here, we use a 3.5 x, 16 mm working distance objective lens on a Discovery V12 (Zeiss) stereomicroscope. As desired, utilize alternative microscopes and objective lenses.”

**Comment 4**. The authors describe steps to measure the fin length in the Imaris software. It would be useful to at least briefly mention how this could be done in the free software ImageJ, particularly since this protocol is designed as a low-cost implementation.

**Response 4**. We have now included this information in step 7.2.3, which reads: “For fin length measurements, select the “Straight” line tool and draw a line between the desired points to be measured.”

**Comment 5**. The period immediately following amputation is potentially interesting but as detailed in step 6.3.1 the authors do not image during this period but rather wait for the imaging chamber to reach 28C. Could this be alleviated by using prewarmed media?

**Response 5**. This is a very good suggestion. We have now also tested pre-warmed buffer and did not find any adverse effects on the agarose. We now state: “Alternatively, utilize pre-warmed buffer to start imaging after shorter adjustment time.”

**Comment 6**. For clarity on the homemade petri dish imaging chamber, the authors should refer to figure 3 of ref #18.

**Response 6.** We now have added this information.

**Comments on the figures**

1. Unless it's already very clear in the video, a graphic illustrating how larvae are embedded post-amputation could be useful.

**Response**: We have now included a figure shown as Figure 3, which outlines the procedure of amputation, mounting and scraping off of the agarose. We hope this adds sufficient clarification.

2. Fig 4A: the "length" and "area" text and graphics should be different colors for clarity.

**Response**: We have now changed the color in the fin area graph to red.

3. Fig 4E: I'm not sure I understand why the authors chose fold-change for their quantification. Plotting the absolute length might be more useful for some questions.

**Response**: The complication with measuring larval fin regeneration is that the amputation plane is not always at the same position and is not visible after regeneration, as it is in adult fins. Therefore, we chose to subtract the post-amputation length from the pre-amp length to obtain the length of the amputated fin portion for comparisons with the regenerated fin portion. However, a recent publication by R. Mateus and colleagues (PLoS One 2012) shows that regardless of the amputation plane, fins regenerated within a similar time regardless of the anterior-posterior position at which the amputation was performed. Thus, this suggests that different amputation planes along the AP axis stimulate differing rates of regeneration. Consistently, a comparison showing the pre-amputated fin length compared with the endpoint regeneration length as suggested by the reviewer is indeed an improvement over our original graph type. We have therefore changed this graph and now show the absolute fin lengths compared between the pre-amputated and regenerated fin.

4. Is it possible to include a movie legend? It would be useful to know what the timepoints are for the movie and how long a time it represents post-amputation. Labels on the movie indicating the "purse-string" effect and extruded cells would be helpful.

**4. Response:** We have now added a movie legend starting at line 426, and we have refined the movie and display the suggested information.

*Minor Concerns:*

Minor comments on text

1. Line 94: "Nacre" should be lower-case italics according to standard nomenclature.

**Response:** We have changed this.

2. Line 212: what is 0.03% embryo medium?

**Response:** This was an oversight. We now state 0.03% Instant Ocean salt in deionized water (termed embryo medium).

3. Line 222: should refer to the earlier step(s) used to create imaging ring.

**Response:** We have now added this information.

*Additional Comments to Authors:*

N/A

**Reviewer #2:**

As far as they go, the methods presented are fine. I have a couple of points.

**Comment:** The authors use embryo medium made of 0.03% Instant Ocean salt solution. I strongly discourage this, as it is unbuffered and can exhibit wildly variable pH. This is particularly important if adding pharmacological inhibitors. We have noticed issues with death and fin defects during timelapse recording due to pH changes induced by tricaine itself (and I'm unclear if the tricaine stock the authors used is buffered). I would recommend that the authors add that both the tricaine stock is appropriately buffered (as per the zebrafish book: <http://zfin.org/zf_info/zfbook/chapt10.html#wptohtml63>) and also recommend using a buffered embryo medium such as E2 (<http://zebrafish.org/documents/protocols/pdf/Fish_Nursery/E2_solution.pdf>).

I think it is important that the community standardize such things.

**Response 1.**  We appreciate this comment. We indeed adjusted the pH of our tricaine stock solution to 7.4, and the final solution shows pH 7. Based on this suggestion and **comment 1 of reviewer 1**, we have decided to repeat the imaging procedure using Ringers solution and 2-phenoxyethanol as anesthetic, which showed that both are well tolerated by larval zebrafish. With these two conditions altered, we now see that larval health was significantly improved and the larvae survived for a minimum of 60 hours. We have decided to add this information in the discussion section and also reference a previous publication (Rieger et al, PLoS Biology 2009) in which we kept the larvae alive for 48 hours using Danieu solution and tricaine.

We also now refer to these other media in the protocol, which reads as follows:

“1.2) Next morning remove the dead embryos with a glass pipette and rinse the eggs in a strainer with 0.03% Instant Ocean salt in deionized water (termed embryo medium). Medium such as Ringers and Hanks (http://zfin.org/zf\_info/zfbook/chapt10.html#wptohtml28), E2 (<http://zebrafish.org/documents/protocols/pdf/Fish_Nursery/E2_solution.pdf>), E3 (Nuesslein-Volhard and Dahm 2002; Zebrafish: A practical approach) and Danieau (The Zebrafish: 2nd Edition, R. Koester and S. Fraser) may be preferred.

“3.4) The tricaine stock should be buffered and prepared based on (<http://zfin.org/zf_info/zfbook/cont.html#cont10>). Alternatively to tricaine, use a 1:1000 dilution of 99% 2-Phenoxyethanol, and for embryo medium utilize buffered media such as Ringers, Hanks, E2, E3 and Danieau solution.”

**Comment:** It seems convoluted to mount, image, de-mount, cut the fin, re-mount, excavate the agarose, timelapse image. It is not apparent to me why the authors do not simply mount, image, cut the fin and excavate the agarose, timelapse image. There may be a good reason, but if this is also an option, it should be presented.

**Response 2.** This is indeed correct. However, as now discussed starting at line 447, in our experience it is rather difficult to amputate a fin properly on a glass coverslip with a syringe needle without tearing the tissue apart. Therefore the mounting and demounting procedure is justified if a precise growth rate needs to be determined. We did however add a phrase in parenthesis after the title in step 3 stating that imaging of the pre-injured larva is optional, and in step 3.7 we now state that this procedure can be performed by imaging the fin after placing the anesthetized larva onto a Petri dish coated with 1.5 % agarose in embryo medium.

**Reviewer #3:**

The manuscript entitled "Capturing tissue repair in zebrafish larvae with time-lapse bright-field stereomicroscopy" by Lisse et al. presents a simple and straightforward method to perform time-lapse analysis on wild type and injured zebrafish larvae using bright-field stereomicroscopy. The manuscript and the accompanying movie read very clearly, the method is well demonstrated and easy to recapitulate. The presented technique can be used for a wide variety of experimental questions and will be a highlight in a lab course and undergraduates und graduate students. Hence, it will be a valuable addition to the collection of JoVE Protocols. Prior to publishing I recommend to add, change or discuss a few minor issues:

**Comment** 1) In the second paragraph of the introduction (line 66-76) embedding in low-melt agarose is introduced and later demonstrated in the protocol section. Recently though, agarose as mounting medium has been suggested to be only suboptimal (Kaufmann et al., 2012. Development 139:3242-3247) and the authors mention themselves in the discussion that agarose embedding could impede growth. Thus the manuscript should point out possible alternatives for embedding embryos such as methyl cellulose, plasma clots (Langenberg et al. 2003) or FEP tubes (Kaufmann et al., 2012).

**Response 1.** We have now added a paragraph in the discussion section starting line 467, in which we mention these alternatives.

The paragraph reads like this:

“To avoid defects in fin regeneration, we removed the agarose from the tail fin prior to imaging. Our data shows that within 1.5 days the fin has regenerated to about 60 %. This regeneration rate is consistent with a previous study defining 3 days as an average time for tail fin regeneration in zebrafish larvae up to 6 dpf 16. Alternative methods to agarose could however be utilized to mount the fish for imaging. For example, thin plasma clots 26 or fluorinated ethylene propylene (FEP) tubes coated with methylcellulose and filled with very low agarose concentrations (0.1%) have been recommended for light sheet microscopy 27 and may be suitable for our presented method. However, we do not recommend methylcellulose and 0.1 % agarose, as they require that the specimen are mounted at the bottom of the chamber due to the lack of solidification of these media. Very high concentrations of methylcellulose will moreover generate air pockets based on our experience, and these may interfere with the imaging procedure. If these media are preferred with using the bottom chamber, it is important that an appropriate working distance between the objective lens and the specimen is present. It should be noted that methylcellulose as a mounting medium is recommended only for up to 1 day, as it may interfere with larval health 27.

**Comment 2**) In the introduction of the protocol larvae from the nacre strain are used (line 94) while later PTU is added (line 113). I thought that nacre mutant larvae lack melanophores and do not require PTU treatment or is the protocol written for wild type larvae then this should be made clear.

**Response 2.** We have changed the text and now state that this step is optional if using pigmented strains. This was an oversight given that we do not use PTU for *nacre* fish.

**Comment 3**) In the imaging section the stereomicroscope is introduced, but as specific settings are mentioned which may not be available for every stereomicroscope it should be specified here (line 186-188), what kind of stereomicroscope (company, model) was used for the work presented here.

**Response 3.** We have added this information but as suggested in comment 2 to reviewer 1, the JoVE style guidelines state to avoid company brand names before and after instruments. However, we also agree that this leads to confusion if a specific instrument is utilized.

**Comment 4**) Embedding zebrafish embryos in agarose in a Petri dish is performed by flipping the lid with the embryo hanging down from the top in a drop of solidified agarose (5.6). Will gravity result in a slow downward drift of the embryo over time? This could cause focus shifts along the z-axis. The authors should discuss this point and offer some advice how to deal with such potential shift during image recording or data analysis.

**Response 4.** We have added a discussion section to further discuss this issue starting at line 484. The paragraph reads like this:

“Mounting the specimen in the lid may result in a slow gravitational downward drift. It is therefore recommended to image multiple sections at each time point, which can either be projected into a single plane or only images that are in the focal plane may be extracted for assembling the final movie. Imaging the specimen at the bottom chamber could be an alternative methodology to avoid potential downward drift. Plasma clots could be useful to avoid drift, as the plasma will stick to the outer enveloping layer (EVL, periderm) 26 and therefore may stabilize the specimen. This however needs to be tested, as well as how long larval zebrafish can be maintained in plasma clots without interfering with larval health or fin regeneration.”

**Comment 5**) line 260, a software module and settings are mentioned which are difficult to follow if the provider is not mentioned. Based on later explanations Zeiss Axiovision is being used but it might be good to already mention this here.

**Response 5.** We have now added this information for clarity (now on lines 268/ 269).

**Comment** 6) For the Imaris software the provider Bitplane should be mentioned (line 276) and for ImageJ/Fiji the URL should be provided.

**Response 6.** We have now added this information for clarity.

**Comment 7**) To me it was not clear how the image data is processed. During image capturing z-stacks are being recorded, but then under point 7.1.2 only one section from each individual stack is being used to generate a time-lapse movie? Thus the 3D-information is being lost? How are the individual images chosen? How is it possible to correct for drifts of the fin along the z-axis? This part would need a bit more information in order to be able to understand and follow the procedure. Could deconvolution be used?

**Response 7.** We now added a paragraph to the discussion section explaining that we utilized single images from each stack and the reasoning for that approach was to capture the full thickness of the fin if potential z-drift occurs. We also discuss alternatives as suggested. The paragraph reads as this:

“Our movie was assembled utilizing individual sections (26 µm) of a recorded z-stack, which covered the full thickness of the fin (~10 µm) and which accounted for potential z-drift of the fin during the imaging procedure. In order to retain 3-D information, it is also possible to project z-stacks into single images. Because this may result in blurriness of the image, brightfield deconvolution may be desired. Software, such as Deconvolve (<http://www.deconvolve.net/>) or Autoquant X3 (<http://www.mediacy.com/index.aspx?page=AutoQuant>) could be utilized for this purpose. Alternatively, mathematical algorithms (described in Tadrous 2010 28) can be applied for obtaining a point-spread function of high signal-to-noise ratio (SNR). Obtaining a high SNR represents one of the major hurdles in brightfield deconvolution. Although this method requires high contrast and thin sample thickness, it would be appropriate for imaging of the tail fin due to its reduced width.”

**Comment 8**) line 350 that cells are being extruded because they are destined to die is a far-reaching conclusion, because the fate of these cells have not been analyzed. I would be more careful here in saying that these cells are likely destined to undergo cell death and that the nature of the cells being extruded needs further clarification.

**Response 8.** We have now added a sentence stating the following: **“**While extruded cells are likely destined to undergo cell death, the nature of these cells needs to be further clarified.”

**Comment 9**) line 75 "require" instead of "required"

**Response 9.** We have made that change.

**Reviewer #4:**

*Manuscript Summary:*

The authors describe a simple tail fin transection removal model for studying its regeneration in zebrafish, and a simple low cost set up for time=lapse imaging of the regenerating fin. The protocol is clear and details provided are sufficient for anybody who wants to set up this assay in their own laboratory. And this is in particular would benefit "science in the class room" activity.

*Major Concerns:*

The low melting agarose used to immobilize the larva for imaging might hinder the regeneration outgrowth of the tail fin. Authors should compare the regenerated fin with and without agarose embedding to show that there is no difference. But I suspect that there might be a difference. Perhaps authors should modify this protocol, remove the agarose from the tail fin tip after immobilizing the larva.

**Response:** Our protocol utilizes a method in which we scrape off the agarose from the tail fin prior to imaging. We realize however that this was not very clearly conveyed and we have now added an additional figure, shown as Figure 3, to provide an overview of the procedure. We also added a paragraph in the discussion section to further elaborate on this method. This paragraph starts at line 467 and states that the fin regenerated about 60 % in 1.5 days, which is a rate that is similar to previous findings described by Kawakami and colleagues (Developmental Dynamics 2004) showing that larvae up to 6 dpf regenerate their fins in about 3 days.

*Minor Concerns:*

no

*Additional Comments to Authors:*

no