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Capturing tissue repair in zebrafish larvae with time-lapse brightfield stereomicroscopy

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Abstract:	The zebrafish larval tail fin is ideal for studying tissue regeneration due to the simple architecture of the larval fin-fold, which comprises of two layers of skin that enclose undifferentiated mesenchyme, and because the larval tail fin regenerates rapidly within 2-3 days. Using this system, we demonstrate a method for capturing the repair dynamics of the amputated tail fin with time-lapse video bright field stereomicroscopy. We demonstrate that fin amputation triggers a contraction of the amputation wound and extrusion of cells around the wound margin, leading to their subsequent clearance. Fin regeneration proceeds from proximal to distal direction after a short delay. In addition, developmental growth of the larva can be observed during all stages. The presented method provides an opportunity for observing and analyzing whole tissue-scale behaviors such as fin development and growth in a simple microscope setting, which is easily adaptable to any stereomicroscope with time-lapse capabilities.
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Cover Letter

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December 9, 2014

Dear Dr. Nguyen,

Pleased find enclosed a revised version of the manuscript entitled "**Capturing tissue repair in zebrafish larvae with time-lapse brightfield stereomicroscopy**".

Thank you for the rapid proceedings with our manuscript. We have made the requested changes and corrected some additional errors following the final proofreading. The changes are marked with the "track change" function. We have further updated the Equipment and Materials list. We hope that with this final version, the manuscript and video are acceptable for publication. We would like to submit the finalized JoVE video in a separate file, if possible, due to its large size (~1.2 Gb).

Sincerely,

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TITLE:
Capturing tissue repair in zebrafish larvae with time-lapse brightfield stereomicroscopy

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KEYWORDS:
Fin regeneration, Time-lapse imaging, Stereomicroscope, Heated incubation chamber, Post-imaging analysis

SHORT ABSTRACT:
We present a protocol for capturing the dynamics of zebrafish larval tail fin regeneration on a whole-tissue scale using brightfield-based stereomicroscopy. This technique enables capturing the regeneration dynamics with single cell resolution. This methodology can be adapted to any stereomicroscope equipped with a CCD camera and time-lapse software.

LONG ABSTRACT:
The zebrafish larval tail fin is ideal for studying tissue regeneration due to the simple architecture of the larval fin-fold, which comprises of two layers of skin that enclose undifferentiated mesenchyme, and because the larval tail fin regenerates rapidly within 2-3 days. Using this system, we demonstrate a method for capturing the repair dynamics of the amputated tail fin with time-lapse video brightfield stereomicroscopy. We demonstrate that fin amputation triggers a contraction of the amputation wound and extrusion of cells around the wound margin, leading to their subsequent clearance. Fin regeneration proceeds from proximal to distal direction after a short delay. In addition, developmental growth of the larva can be observed during all stages. The presented

method provides an opportunity for observing and analyzing whole tissue-scale behaviors such as fin development and growth in a simple microscope setting, which is easily adaptable to any stereomicroscope with time-lapse capabilities.

INTRODUCTION:

The ability of an organism to orchestrate tissue repair processes after injury is crucial for its survival [1]. While all animals have the capacity to heal their wounds, the extent to which tissues regenerate differs greatly among species. Vertebrate species such as zebrafish, salamanders and frog tadpoles have the remarkable ability to regenerate lost tissues, including their appendages, portions of their eyes, heart, and central nervous system [2-4]. Mammalian species, such as the African spiny mouse and rabbits, are capable of regenerating holes in their pinnae [5-7], and humans and mice regenerate portions of their liver as well as their digit tips during fetal and juvenile stages [8-12]. Although it is not well understood yet why and how certain species regenerate tissues more effectively than others, the presence of similar genetic pathways suggests that these mechanisms may lie dormant in species without great regeneration potential [13,14]. Thus elucidating tissue repair and regeneration mechanisms in species with satisfactory regeneration outcomes will benefit regeneration in humans.

We have chosen the larval zebrafish tail fin as a paradigm to demonstrate its regeneration with time-lapse brightfield stereomicroscopy. The zebrafish larval tail fin is anatomically simple as compared to the more complex adult structures, consisting of a two-layered, infolded epithelium with somatosensory axons innervating the skin that surrounds medially located mesenchymal cells [15]. Despite the anatomical differences, larval tail fin regeneration is somewhat comparable to adult fin regeneration in terms of the molecular signatures and the outgrowth responses [16,17]. As compared to the adult fin, imaging larval tail fin regeneration has however several advantages: 1) larval fin regeneration is completed within just 2-3 days [16], 2) larvae can be mounted in low-melt agarose, and 3) larvae do not require feeding until ~ 5 days post fertilization (dpf) due to the presence of the yolk sac. This makes zebrafish larvae ideal for observing tissue repair dynamics *in vivo.*

The presented method enables the capture of detailed dynamics underlying the early processes of fin regeneration. Many studies have utilized fluorescence-based confocal microscopy to study cellular and subcellular biological processes in embryonic and larval zebrafish. Sophisticated confocal imaging setups are however often not accessible to everyone and highly expensive as compared to other imaging techniques. In contrast, the presented methodology utilizes a Discovery V12 stereomicroscope equipped with Axiovision software and a time-lapse module, thus providing a more affordable alternative to expensive imaging equipment to examine tissue behaviors. We demonstrate that this method can be utilized for imaging tissue regeneration with high temporal resolution at a minimal cost. The implications for this method could extend beyond basic biology to advance mammalian regeneration studies using organ cultures, for therapeutic development through pharmacological and genetic screens, and it can serve as a teaching tool in a classroom setting.

PROTOCOL:

Zebrafish (*nacre* strain) were bred and raised according to established protocols. All efforts were made to minimize suffering, using 0.4 mM Tricaine for anesthesia and 1 mM Tricaine for euthanasia. Zebrafish embryos and larvae were handled in strict accordance with good animal practice as approved by the appropriate committee (MDI Biological Laboratory animal core IACUC number 13-20). This study was approved by the National Human Genome Research Institute Animal Care and Use Committee, MDIBL Institutional Assurance # A-3562-01 under protocol # 14-09.

Note: The imaging procedure that captures fin regeneration in larval zebrafish is summarized in the following steps:

1. Raising of zebrafish to larval stages

1.1) Collect the eggs and place approximately 50 eggs into a 100 x 25 mm Petri dish containing 0.03% Instant Ocean salt in deionized water supplemented with 0.00004% methylene blue. Incubate overnight in a 28.5 °C incubator.

1.2) The 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).

Note: Medium such as Ringers [18], Hanks [18], E2 [19], E3 [20], and Danieau [21] may be preferred.

1.3) Add fresh embryo medium to the dish. If using pigmented strains, optionally add 0.2 mM 1-phenyl-2-thiourea (PTU), as PTU will prevent melanogenesis and thus pigmentation of the larvae. Let the embryos further develop in the incubator until 2 days post fertilization or any other desired larval stage.

2. Preparation of the imaging chamber

2.1) Method 1: Imaging chambers made from PVC or Teflon tubing (Figure 1)

Note: This method is similar to Concha and Adams (1998) [22].

2.1.1) Acquire drinking-water grade plastic or Teflon tubing from a hardware store with a 25 mm outer and a 20 mm inner diameter. Cut the tubing to make rings of approximately 10 mm thickness with an even surface on each side. Use > 200 grit sandpaper to smoothen the edges.

2.1.2) Clean the rings with warm water and 70% ethanol and let them air dry.

2.1.3) With a pipette tip, apply silicon grease to one half of a ring and attach the ring to a 75 mm x 25 mm glass cover slip. Alternatively, use a 3 mL syringe filled with silicon

grease instead of a pipette tip.

Note: Because it is difficult to insert the silicon grease into the 3 mL syringe, add the silicon grease to a 30 mL syringe first and use this for filling the 3 mL syringe.

2.2) Method 2: Preparation of a Petri dish as an imaging chamber.

2.2.1) Acquire 35 or 60 mm diameter Petri dishes with a glass coverslip attached to the lid (Figure 2A). Alternatively, as shown in Figure 3 of Distel & Koester (2007) [23], drill an opening small enough to hold a coverslip into the lid of a small Petri dish and apply silicon grease to the outside with a 3 mL syringe. Using a clean pipette tip, carefully attach a round or square coverslip of desired thickness to the outside (Figure 2B).

2.3) To ensure that the agarose being used for mounting stays firmly attached during the imaging procedure, attach a fine plastic mesh inside the ring. First, cut the mesh made of window screen obtained from a hardware store, into the size of the inner ring diameter using fine scissors with an angle. Then cut a small rectangle > twice the size of the larva into the middle of the mesh (Figure 1, 2).

2.4) Apply four small dots of non-toxic silicon grease to the interface between the cover slip and the chamber ring (Figure 1A).

2.5) Use forceps to attach the mesh firmly to the bottom of the glass coverslip.

3. Mounting and imaging of the pre-injured larva (this step is optional)

Note: This step is suitable for comparisons between the amputated and regenerated fin length, as the amputation plane after fin regeneration is not recognizable in zebrafish larvae.

3.1) Prepare a 0.5-1.2% low-melt agarose solution in embryo medium for immobilization of the specimen.

3.2) Heat the agarose in a microwave and transfer the liquid agarose into 1.5 mL tubes that are pre-warmed to 42 °C in a heating block.

3.3) Let the hot agarose cool down to 42 °C, which can be maintained over several weeks at this temperature. Try to avoid pipetting the larvae into agarose above 42 °C, as this will be detrimental to the animals.

3.4) Anesthetize several larvae using 10 mL of 0.4 mM Tricaine (pH 7) in embryo medium in a 60 mm diameter Petri dish. Prepare the Tricaine according to the Zebrafish Book Recipes [18].

3.4.1) Alternatively, use 10 mL of a 1:1000 dilution of 99% 2-Phenoxyethanol in a 60 mm diameter Petri dish. Poke the larvae with a capped microloader pipette tip to assess

their touch response before proceeding. Use only non-responsive larvae.

3.5) Transfer one larva into the 42 °C agarose solution using a glass Pasteur pipet. Do not transfer excessive liquid into the agarose, otherwise the agarose will be too diluted and not solidify.

3.6) Discard the remaining liquid from the pipet and transfer the larva with one drop of agarose into a small Petri dish (35 or 60 mm diameter). Position the larva on its side for imaging of the tail fin.

3.7) Allow the agarose to solidify. Assess the agarose with a plastic pipette tip; the tip will immerse into the agarose if it is too liquid. Once the agarose has solidified, a small indentation will be visible upon touching with a pipette tip. Following solidification, add Tricaine solution and proceed to the stereomicroscope that will be used later for time-lapse imaging.

Note: This step may also be performed by placing the anesthetized larva on a Petri dish coated with 1.5% agarose in embryo medium.

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, use a 3.5 x, 16 mm working distance objective lens on a stereomicroscope. As desired, utilize alternative microscopes and objective lenses but choose a proper magnification to account for potential xy-drift and growth of the fin during the imaging procedure.

3.9) Select the camera detection mode on the microscope.

3.10) In the software, select the “live” mode to view the larva on the screen.

3.11) Open the “Properties” window to automatically detect the brightness.

3.12) Manually adjust the contrast at the microscope trans-illumination base.

3.13) Move the larva out of the field of view and select the Shading Correction feature to minimize background noise.

3.14) Position the larva back into the field of view and take a snapshot. Save the image.

3.15) Remove the agarose from the larva by first scraping the agarose off the head. This way the larva can be slipped out of the agarose by gently pulling the head away from the remaining agarose with a capped microloader pipet tip or an insect pin.

3.16) With a glass Pasteur pipet transfer the larva into fresh Tricaine solution.

4. Amputation assay

4.1) Prepare a 1.5% agarose solution using embryo medium and pour a thin layer into a Petri dish. Let the agarose solidify.

4.2) Under a stereomicroscope, place the larva sideward onto the solidified agarose and amputate the tail fin with a 23-gauge syringe needle with slight pressure (Figure 3A).

5. Mounting the larva for time-lapse imaging

5.1) Proceed as described in steps 3.1 – 3.5 (Figure 3B).

5.2) Transfer a drop of 0.5-1.2% liquid agarose at 42 °C containing the larva into the imaging chamber ring (step 2.1), orient the larva and let the agarose solidify. Fill the ring with Tricaine solution. Alternatively, if the Petri dish chamber (step 2.2) is utilized, mount the larva onto the lid coverslip and fill the lid with Tricaine solution.

5.3) To allow proper wound healing or tissue regeneration to occur, carefully scrape off the agarose surrounding the distal tail fin using a capped microloader pipette tip or an insect pin. Try not to injure the fin repeatedly (Figure 3C).

5.4) Decant the Tricaine solution containing the removed agarose and fill the chamber ring with fresh Tricaine solution.

5.5) Apply silicon grease to the top of the chamber ring and attach a 75 mm x 25 mm glass slide. Try to avoid air pockets in the chamber, as they will interfere with brightfield imaging and desiccate the larva over time.

5.6) If using a Petri dish as an imaging chamber, apply silicon grease to the top rim of the bottom chamber and fill the bottom chamber with Tricaine solution. Carefully decant the Tricaine solution in the lid and turn the lid over to immerse the larva into the Tricaine solution of the bottom chamber at a slight angle to avoid air pockets. The chamber will be sealed due to the silicon grease.

6. Time-lapse imaging

6.1) Assemble a heated incubation chamber as described in [23,24] (Figure 4).
Place the incubation chamber around the microscope and turn on the heat. Adjust the temperature to 28 °C for about 10 - 20 min or until the temperature has stabilized.

6.2) Open the front of the heated incubation chamber and place the imaging chamber onto the microscope stand with the coverslip facing upward toward the objective.

6.3) Position the larval fin in a way that 2/3 of the field of view remains unoccupied. This ensures the capture of the growth and regeneration of the fin over the course of the imaging procedure without having to reposition the larva.

6.3.1) Adjust the mounted larva to 28 °C for ~ 30 min prior to starting the time-lapse recording to avoid changes in brightfield intensity or potential shifts of the agarose. Alternatively, utilize pre-warmed buffer to start imaging after shorter adjustment time.

6.4) To set up the time-lapse recording, open the 6D Multidimensional Acquisition window in the Axiovision software and select the z-stack and time-lapse option.

6.5) (Optional) In the z-stack tab and Slice Mode, select the slice thickness and then select the Start/Stop mode.

6.6) Define the upper and lower position of the stack.

6.7) In the time-lapse tab, select the interval and the duration of the movie and then start the movie by pressing the start button. We found that 30 minute intervals are sufficient and this interval does not generate excessive data; however shorter intervals may be used.

6.8) Check the position and z-stack dimensions during the first hour if the larva was not pre-adjusted. If necessary, reposition the larva again after a day, as the larva may have shifted.

6.9) Save the file at the end of the time-lapse recording and proceed with the post-processing and quantifications using available image analysis software, such as Imaris [25] or the open source software packages Image J [26] and Fiji [27].

7. Data analysis

7.1) Determining the fin length.

7.1.1) Open the time-lapse movie in the imaging software and save the files in the proprietary file format to enhance software performance.

7.1.2) Select the orthogonal view to display individual sections as projected stacks. For drift correction, under the Fiji menu, select Plugins, then Registration, and “Correct 3D drift”. This will open a Fiji window and perform the drift corrections. Correct rotational drift in the Imaris Spots function. Alternatively, install the StackReg and TurboReg plugins in Image J and import to Fiji. Choose the desired transformation algorithm in the StackReg plugin.

7.1.3) To measure distances (*e.g.*, wound diameter or fin length), select the “Add New Measurement Points” option in the upper left tool bar.

7.1.3.1) Under “Configure list of visible Statistics Values” in the bottom left menu, select the statistics values to be displayed.

7.1.3.2) In the “Line Mode” under the settings tab select “Pairs (AB, CD ...)”.

7.1.3.3) In “Labels Properties” select “Name” and “Distance”, to display the distance between points A and B next to the measurement line.

7.1.3.4) Switch to the “Edit” mode and hold down the shift button to select the first point at the end of the notochord. Then use the same configuration to select the second point at the distal fin margin.

7.1.3.5) Under the “Statistics” tab, select the disk button (Export All) at the lower right to display and export the distance in the image.

7.1.3.6) Repeat the measurements at selected times by moving the slider below the image to the right. Instead of creating new Measurement Points, the previous ones can be repositioned by first selecting the point with the left mouse button, then simultaneously pressing the shift and the left mouse button at the new position.

7.1.4) Alternatively to the Measurement Points option, utilize the slice viewer to measure distances. In the slice view mode, scroll to a desired position and click on the first and second position with the left mouse button. The distance will be displayed. This option however does not allow for data export.

7.2) Determining fin length and area in ImageJ.

7.2.1) Open the time-lapse movie in ImageJ using a plugin that recognizes the .zvi file format. Alternatively, load in a QuickTime file or tiff sequence.

Note: If using an uncompressed tiff file format, the image dimensions do not need to be specified.

7.2.2) If opening a different file format without the file information, select “Set Scale” under the “Analyze” menu to first define the image distance and unit. In the “Set Scale menu”, type in the “Distance in pixels”, below type in the “Known distance” for the pixel value (this can be obtained by measuring the number of pixels on a scale bar that was added to the image, then click Measure to obtain the result), and the “Unit of length” (typically “µm”). Then click OK.

7.2.3) For fin area measurements select the “Freehand Selection” tool in the tool bar and outline the fin area by holding the left mouse button down while drawing along the outline of the fin. For fin length measurements, select the “Straight” line tool and draw a line between the desired points to be measured.

7.2.4) Click the “Measure” option under “Analyze” to display the area and length of the fin. Repeat this step as often as necessary for multiple time points of the movie.

7.3) Using statistics software the data can be graphically displayed.

REPRESENTATIVE RESULTS:

The presented technique is suitable to elucidate tissue repair dynamics in response to amputation. The movie demonstrates that amputation of the fin initially triggers a purse-string effect, characterized by contractions via actin-myosin cables that are present in the fin-fold [28] (Figure 5 A,B). Concomitantly, cells are extruded from the wound (see movie). The contraction may thus be a means to expel cells that are likely destined to undergo cell death. Our results further show that the developmental growth of the larva occurs independently of regeneration (movie), whereas fin regeneration does not initiate until about 14 hours post amputation as measured by fin length and area over the time course of 36 hours following amputation (Figure 5C, D). The total regenerative fin growth after 1.5 days was about 60% of the original fin length (Figure 5 E). Taken together, these results demonstrate that amputation triggers fin contraction, extrusion of cells from the wound, and a temporally delayed regenerative response. While extruded cells are likely destined to undergo cell death, the nature of these cells needs to be further clarified.

Figure 1: Imaging chamber ring assembly

(**A**) Shown is a plastic ring that is attached to a coverslip with silicon grease. A plastic mesh is attached to the inside of the chamber with four small dots of silicon grease. (**B**) The chamber containing the mounted larva is filled with Tricaine solution and a glass slide is attached to the top. (**C**) A mounted 2 day-old larva (arrow) is shown at higher magnification to depict its size in relation to the mesh.

Figure 2: Imaging chamber assembly made from Petri dishes

(**A**) Shown is a commercial glass top glass bottom petri dish with a plastic mesh attached to the glass coverslip. (**B**) Shown is a self-constructed Petri dish chamber with a hole drilled into the lid and a coverslip attached from the outside with silicon grease. The mesh and larva are mounted inside the chamber containing Tricaine solution. To seal the chamber, silicon grease is applied to the upper, outer rim of the bottom chamber and the top lid attached.

Figure 3: Scheme of amputation and mounting of a larva for imaging

(**A**) For amputation, place an anesthetized larva onto an agarose-coated Petri dish and amputate the tail fin with a syringe needle. (**B**) For mounting, transfer the larva with a transfer pipette into a 1.5 mL tube filled with 42 °C liquid agarose and pipette a drop containing the larva into the imaging chamber, orient the fish and cover the solidified agarose with embryo medium. (**C**) Scrape off the agarose from the tail fin using a capped microloader pipette tip or similar tool and replace embryo medium with fresh medium. (**D**) Image the tail fin under a stereomicroscope.

Figure 4: Self-constructed heated incubation chamber

(**A-C**) Shown is a heated incubation chamber made of cardboard, bubble wrap and Velcro. A wired dome heater (originally designed for chicken egg incubation) is attached to the chamber using aluminum tape.

Figure 5: Fin regeneration dynamics
(**A**) Scheme of the utilized tail fin amputation assay and quantification method to determine the fin length (red arrow) and area (red outline of the fin). (**B**) Tail fin amputation initially triggers contraction of the fin, followed by regenerative tissue outgrowth. The fin also undergoes developmental growth, as evidenced by the lateral size increase. (**C**) Shown is the fin length as a function of time, revealing a linear regenerative growth starting at ~14 hpa. (**D**) Quantification of the fin area reveals an initially decrease in size, which can be attributed to the contraction of the fin. After ~ 14 hours, the fin size increases at a linear rate. (**E**) Comparison of the fin length before amputation and after 36 hours shows ~ 60% regrowth. Scale bar: 100 µm
Abbreviations: pre-amp, pre-amputation; hours post amputation, hpa; regen, regeneration; amp, amputation

Movie: Fin regeneration over the time course of 36 hours.
Shown is a tail fin of a 2.5-day old larva during the course of regeneration. Starting 30 minutes post amputation regenerative growth is imaged in 30 minute intervals on a stereomicroscope using a 3.5x objective lens.

DISCUSSION:
The presented method allows for observing wound healing and tissue regeneration in living zebrafish larvae with *in vivo* time-lapse imaging on a brightfield stereomicroscope, using a comparatively simple set-up. This procedure requires certain important aspects that we have tested, which will optimize the outcome: 1) Low agarose concentrations (~0.5%) will minimize growth impediments of the continually growing larval zebrafish, 2) Removal of the agarose around the fin is important not to obscure the healing process, 3) Trapping the agarose in a plastic mesh retains the agarose and animal in a stable position throughout the procedure, and 4) A proper temperature-controlled environment, which is essential for larval viability. We have adapted a heated incubation chamber [23,24], which utilizes bubble wrap that is taped onto cardboard, and a wired dome heater to control the temperature and proper air circulation with minimal fluctuations during the imaging procedure. This simple and cost-efficient chamber can be prepared to fit any microscope. A similar heated incubation chamber has been also utilized for imaging mice and chick development [24,29].

We suggest that pre-amputated larvae are mounted for a pre-amputation image, demounted for amputation, and remounted for time-lapse imaging. Although it is feasible to perform these steps in a single step in the final imaging chamber, in our experience we found that amputating the tail fin on a glass coverslip is not optimal, as it tears the tissue and does not result in a clean cut. The agarose-based amputation method using a syringe needle was originally described by Kawakami and colleagues (2004) [16] and is also, in our experience, ideal to perform the amputations. Thus, the rather complicated series of steps that we presented is well justified and ensures an optimal regeneration outcome.

We showed that larval zebrafish at 2 dpf can be imaged up to 1.5 days in agarose and Tricaine solution. We used pH-optimized Tricaine (pH7) solution prepared with Instant

Ocean salt, which does not interfere with the specimen's health for the presented imaging period. We previously however also demonstrated that using Tricaine in Danieau medium permits time-lapse imaging of 2.5 dpf larval zebrafish on a confocal microscope for at least 2 days [30]. Thus, optimal buffer conditions can extend larval health and the length of imaging. Alternatively, lower Tricaine concentrations may be used for anesthesia, or 2-phenoxyethanol, which we found is well tolerated during larval and adult stages at 28 °C for at least 60 hours.

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 [31] 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 [32] 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 [32].

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) [31] 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.

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 or Autoquant X3 could be utilized for this purpose. Alternatively, mathematical algorithms (described in Tadrous[33]) 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.

A clear advantage of the presented imaging method is that it is rapidly adaptable to any stereomicroscope equipped with a CCD camera and time-lapse software and offers a low-cost alternative to more expensive confocal imaging systems. While this method does not utilize fluorescence for cell detection, it can be extended for such applications by utilizing an automated system for shutter control and post-imaging deconvolution software [34]. This would enable users to further observe wound repair and regeneration processes with single cell or subcellular resolution over longer time periods.

The optical clarity and ease with which embryonic and larval zebrafish can be handled, and the adaptability of this method to any stereomicroscope makes it suitable for teaching basic vertebrate biology in a classroom setting. This method can provide students with a better understanding of the basic biological processes underlying tissue repair and regeneration. Other biological processes that have been captured with a similar method are zebrafish embryonic development [23,34] and cardiac function (unpublished). This method also offers the possibility for monitoring wound repair and regeneration in larvae that have been genetically and pharmacologically manipulated.

DISCLOSURES:
The authors have nothing to disclose.

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Imaging chamber ring assembly
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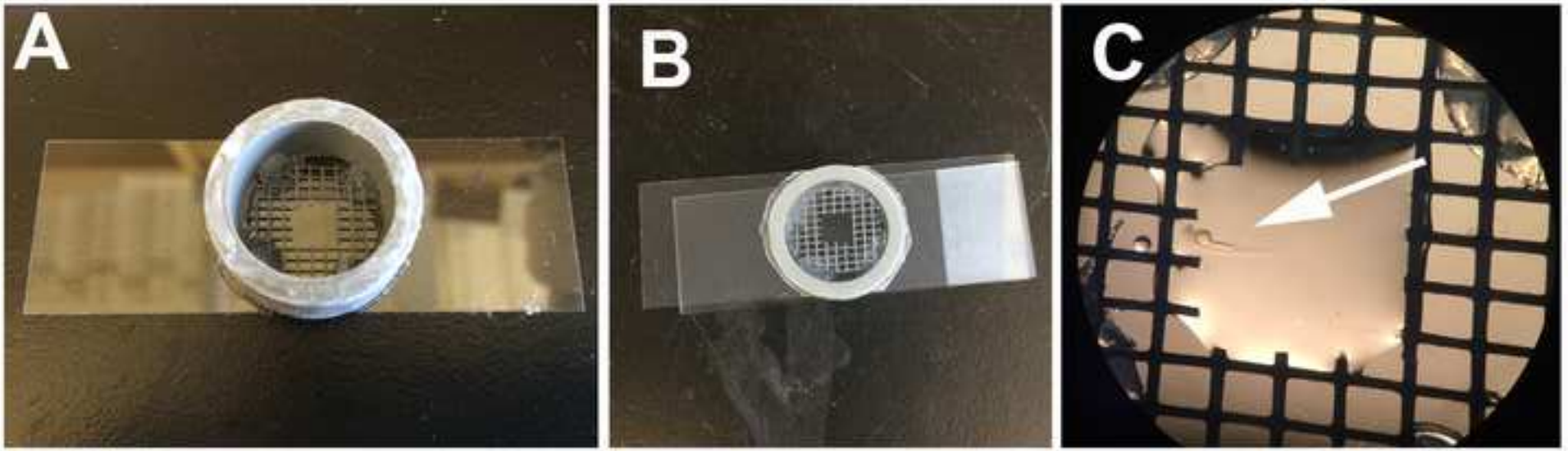

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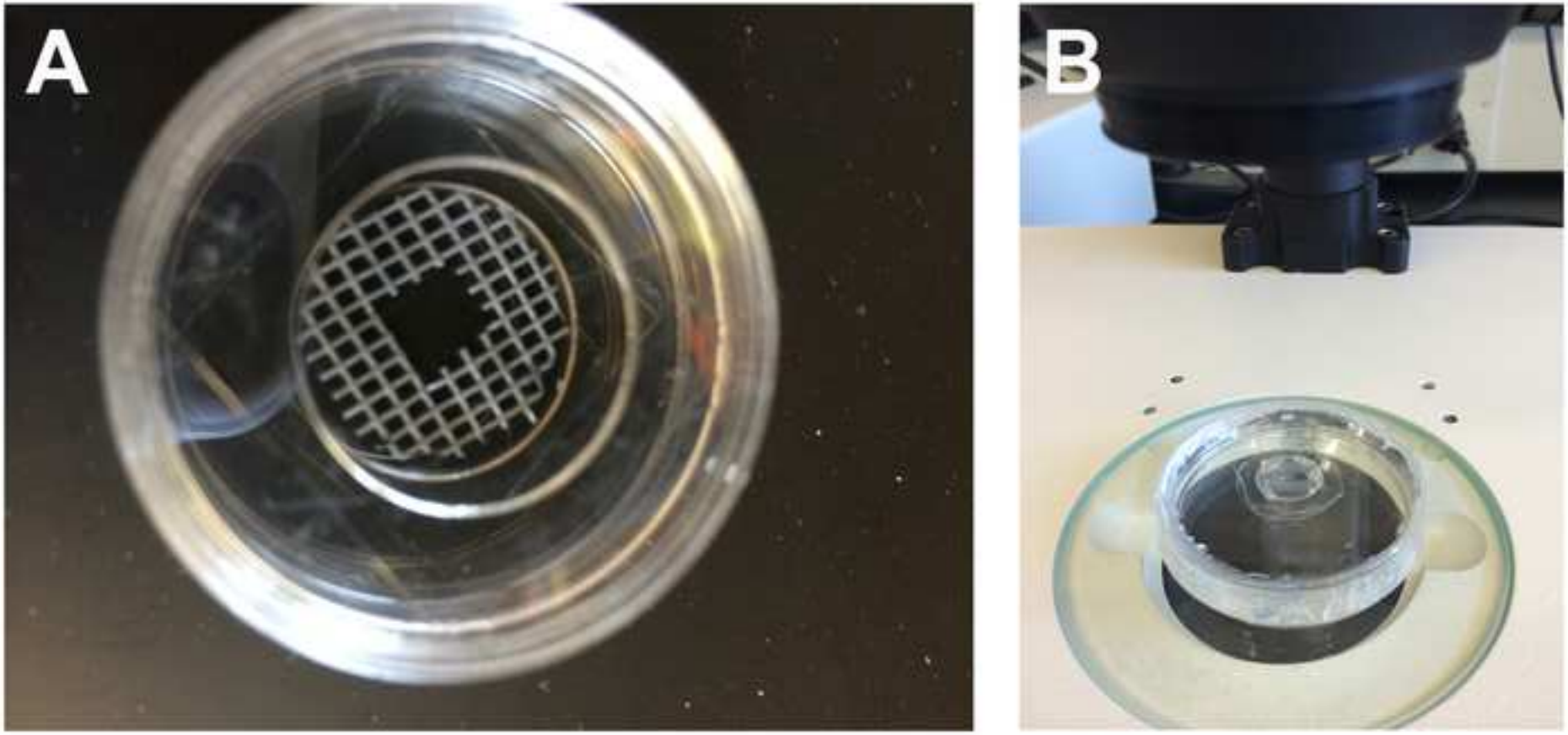

Scheme of amputation and mounting of a larva for imaging
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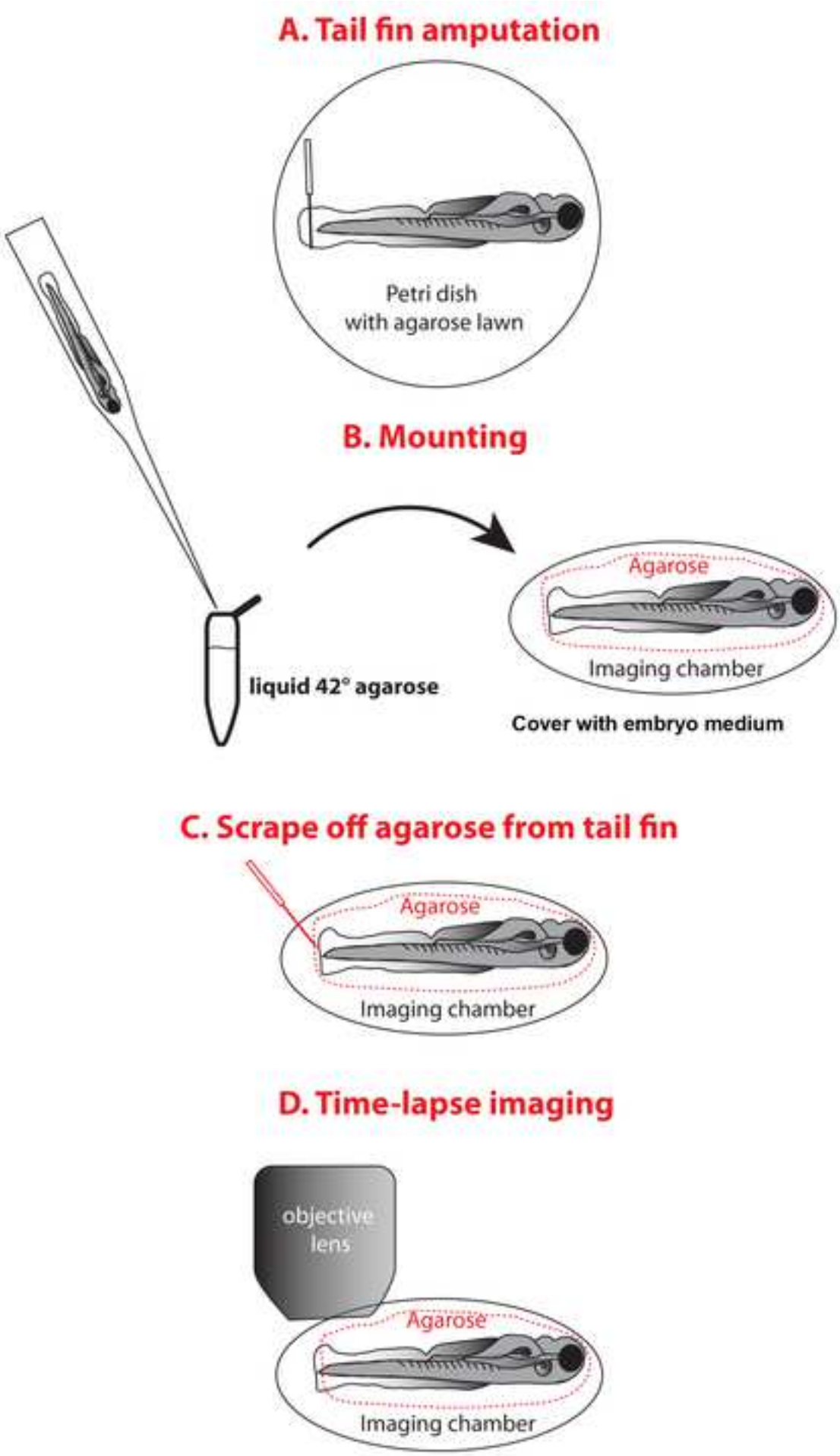

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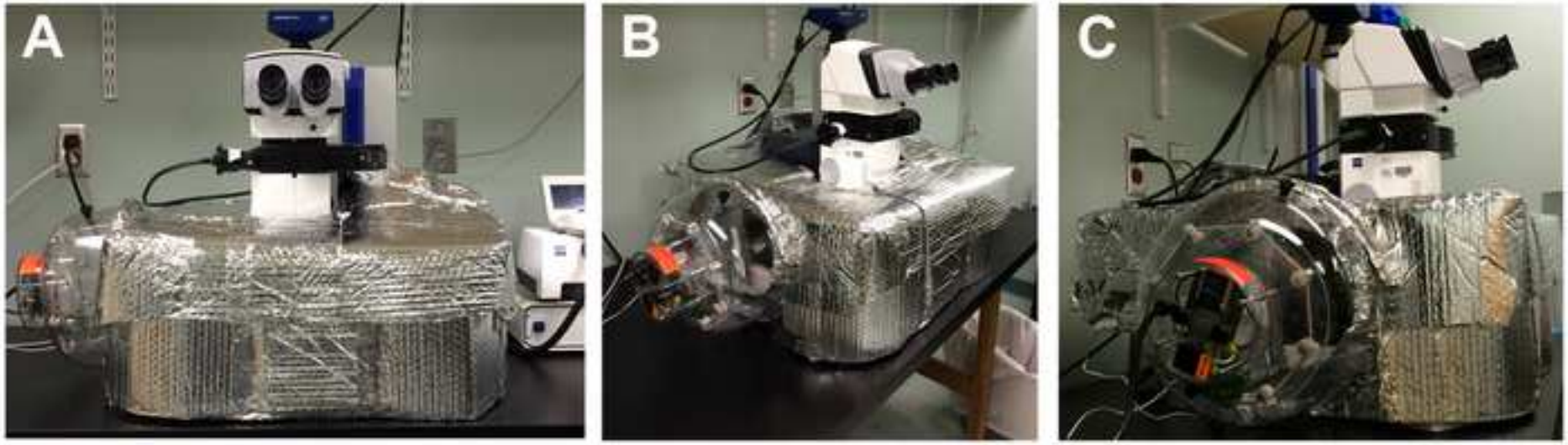

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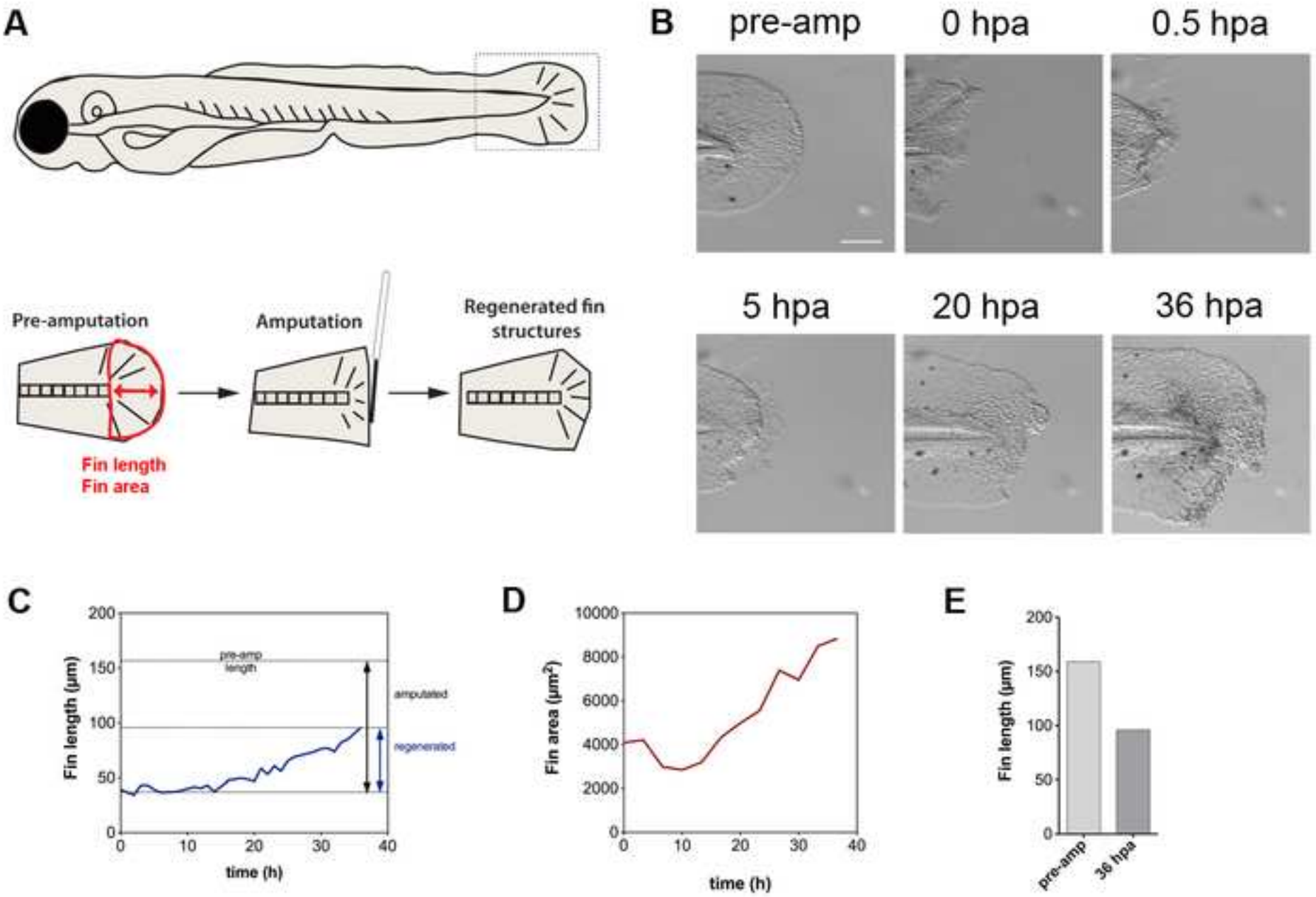

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Reagents

Bullseye Agarose (MidSci, Cat. No. BE-GCA500)
Low-melt agarose (Fisher BioReagents, Cat No. BP1360-100)
1-phenyl-2-thiourea [Alfa Aesar, Cat No. L06690]
Instant Ocean Aquarium Salt (Pet store)
Methylene Blue (0.1% solution) (Sigma, Cat. No. M9140)
Tricaine (Ethyl 3-aminobenzoate methanesulfonate, Sigma-Aldrich, Cat. No. E10505)
2-Phenoxyethanol (Sigma-Aldrich, Cat. No. 77699)
Petri Dish 35 x 15 mm (BD Falcon, Cat. No 351008)
Petri Dish 60 x 15 mm (BD Falcon, Cat. No 351007)
Petri Dish 100 x 25 mm (BD Falcon, Cat. No 351013)
5.75 inch boroschillate glass pipets (Fisher)
35 mm Glass Top Glass Bottom Dish (MatTek Corporation, Cat No. D35-20-0-TOP) Glass: 0.085-0.
Superfrost/Plus microscope slides (Fisherbrand, Cat No. 12-550-15)
Glass coverslips (Electron Microscopy Services, Cat No. 72191-75)
Glass coverslips (Warner Instruments, Cat. No. CS-18R15)
Phifer Phiferglass Insect Screen Charcoal - 48" (Home Depot)
DOW CORNING® HIGH VACUUM GREASE
Microloader pipette tips 20 µl (Eppendorf, Cat. No. 930001007)
Fine Scissors - Sharply Angled Up (Fine Science Tools, Cat. No. 14037-10)
3 mL Luer-Lok™ disposable syringe (BD, Cat. No. 309657)
60 mL Luer-Lok™ disposable syringe (BD, Cat. No. 309653)
23-gauge syringe needles (BD, Cat. No. 305145)
Dumont #5 Forceps (Fine Science Tools, Cat. No. 11295-00)

Equipment
LabDoctor Mini Dry Bath (MidSci)
Zeiss Discovery.V12 compound microscope
Zeiss Plan Apo S 3.5X objective
Zeiss AxioCam MRm
Zeiss Axiovision software, Release 4.8.2SP1 (12-2011)

.115mm

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Author(s): Thomas S. Lisse, Elizabeth A. Brochu, Sandra Rieger

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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.

3.1) **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