Long-term imaging of living adult zebrafish

Daniel Castranova, Bakary Samasa, Marina Venero Galanternik, Aniket V. Gore, Allison E. Goldstein, Jong S. Park and Brant M. Weinstein
DOI: 10.1242/dev.199667

Editor: Kenneth Poss

Review timeline
Original submission: 30 March 2021
Editorial decision: 4 May 2021
First revision received: 2 November 2021
Accepted: 30 November 2021

Original submission

First decision letter

MS ID#: DEVELOP/2021/199667

MS TITLE: Long-Term Imaging of Living Adult Zebrafish

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I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript with additional experiments and information before we can consider publication. If you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. In particular, reviewers request clarity on the functionality of the technique, with concerns regarding key details that are omitted at several points. They also have concerns over the rate of survival of imaged animals, whether the technique is applicable to longitudinal imaging runs requiring multiple rounds of anesthesia, and whether the imaging itself impacts the biological events under study. I agree these are important points to address.

If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please note that we are happy to extend revision timeframes as necessary. Please also note that Development will normally permit only one round of major revision.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Zebrafish have traditionally been studied at embryonic and larval stages. Adult zebrafish have become an increasingly popular model, prompting the need for improved strategies allowing for long-term live imaging of juvenile and adult zebrafish. This is generally achieved by immobilizing and intubating the fish.
Although there is a detailed published protocol (Xu et al, 2015; PMID:26584446) for upright microscopes, there is no similar protocol for inverted microscopes.
The manuscript from Castranova and colleagues offers a method the authors claim can be applied to any configuration of microscope, while boasting additional safety features and temperature control. The authors demonstrate the utility of their 3D-printed, intubation chamber with impressive imaging of neutrophil recruitment to a skin wound over a 20+ hour period.

Comments for the author

The major limitation of the submission is that, as currently presented, the manuscript offers only an incremental advance over the method of Xu et al., falling short of the journal's criterion that Techniques articles provide "a novel technique, or a sufficiently substantial advance of an existing technique." Furthermore, there was a significant (> 25%) failure rate of the apparatus. Below are suggestions on ways to improve the manuscript.

Major Comments:

- How does the proposed fish intubation method significantly improve upon other published methods, namely the one proposed by Xu et al.?
In general, the manuscript would benefit from a more thorough discussion of the published intubation/imaging methods, including explicit statements of how the proposed method is a significant improvement on available methods. For example Xu et al states that "Our system is specifically designed for use with upright confocal microscopes. However, this system can be adapted for use with inverted microscopes by replacing the bottom of the imaging chamber with optical glass."
- Protocol lacks a clear step-wise explanation of how to carry out methodology.
Although the figure illustrations were helpful to see the final configuration of the intubation rig, it would have been extremely helpful for the methods to be clearly stated in a step-by-step, or sequential manner. In the paper's current state, one might find it difficult to faithfully recapitulate the set up. If there are space limitations, this could be provided in the supplemental materials.

Given that there was not a 100% survival rate of the tested fish, a troubleshooting guide of common factors that contribute to success or failure of the method and fish survival would be helpful to the reader. For example, is there a range of anesthesia concentrations, tubing size or flow rates that correlate to fish size or genetic background? How can the depth of anesthesia and fish health be determined and monitored during the experiment?
- Is the method reliable for experiments requiring multiple imaging sessions?
Two of the seven fish did not survive after a single intubation trial. Many regenerative processes, such as appendage regeneration, may require multiple rounds of imaging with repeated intubation sessions. A researcher may hesitate to perform such an experiment if the fish's survivability is suspect. Please clarify whether all 5 "surviving" fish were fully recovered or just showed basic vital signs after the extended imaging session.

For the 2/7 fish that did not survive, the authors state there was mechanical failure of the intubation setup. This is an unacceptably high failure rate. The authors should provide more extensive testing of the reliability of their method and fish survival. The text should be clarified to say the method requires some limited oversight to ensure the equipment is performing properly.
- Neutrophil experiment lacks control fish The neutrophil recruitment experiment was impressive, but it appears there is a global increase in neutrophil signal/frequency in areas of the fish that did not receive the injury. For instance, comparing Figures 3C and 3F, a large amount of signal accumulates in the uninjured fin region by 18 hours (3F). Was there a significant effect of laser exposure and/or prolonged immobilization during the experiment? Control fish imaged for the same

duration would confirm there was no significant effect of phototoxocity and ensure the mounting conditions did not contribute to changes in fluorescence.
- A more detailed discussion of casper fish The authors emphasize the use of the casper mutant fish, however, the advantages and limitations of this genetic background should be discussed. For example, the reader would benefit from a comparison of the depth of imaging possible in casper mutants compared to zebrafish with wild-type pigmentation. What limitations, if any, of this genetic background should be considered?
- The manuscript should appeal to a broad audience.
The appeal of the manuscript beyond a relatively narrow zebrafish readership is unclear. As written, the first two paragraphs of the introduction contained a very generic discussion of the advantages of zebrafish. Could the authors more succinctly summarize these points and state the broad problem they are addressing earlier (i.e., in vivo imaging of developmental/regenerative processes beyond embryonic stages, which is a general challenge in the field)?
In terms of the methodology, could their imaging approach be applied to aquatic vertebrates beyond zebrafish?

Minor Comments:
- Safety
The bonus safety features to prevent overflow seem more than sufficient to ensure water does not escape from the top, but it still seems suspect that if the grease seal around the chambered coverglass (or the coverglass itself) fails, the microscope hardware would be catastrophically flooded. Is there any failsafe or advice for avoiding this seemingly possible scenario?
- Can the intubation rig fit most upright microscopes?
The authors claim this rig can be "adapted to any microscope system". However the high walls on the rig pose a problem for upright microscopes. The objectives would likely be physically blocked by the walls while attempting to lower onto the sample. Please make clear how “universal” this rig is, and what might be need to be adjusted for different scope configurations.
- Consider removing Supplementary Movie 2
This movie could be removed as it does not convey anything that can’t be stated with text alone. Also, the recovered fish is swimming at a very reduced rate compared to the pre-intubation condition. This might raise more questions rather than offering the reader assurance that the intubated fish was fully recovered.
- Figure 2 organization
The diagrams for Figure 2 are excellent, however it would be beneficial to arrange A-D in a manner that represents the order for assembling the rig. For example, 2D introduces sealing off the chambered coverglass with silicone grease, but that would likely be one of the first steps in setting up the rig.
Again, it would be helpful to have a clear, stepwise procedure laid out for the reader to follow. This paper does a fantastic job showing the final product, but more information on how to assemble the rig would be greatly appreciated.
- Description of scale removal wound
The type of zebrafish injury performed is not articulated clearly in its current state. How deep was the wound? “Scale removal by abrasion” should be reworded to indicate the exact nature of the injury, relative to the more commonly used technique of plucking individual scales with forceps.

Reviewer 2

Advance summary and potential significance to field

The manuscript by Castanova et al presents an experimental setup for the long-term imaging of adult zebrafish. This is an extension of a previous method by Xu et al 2015 and is adapted for inverted microscopes. The tool could be useful to a large number of groups. The authors provide clear instructions and explanations on how to build the chamber and demonstrate its use in following neutrophil migration toward a wound. The paper is clearly written. Overall, this method paper would be of interest to developmental biologists.

Comments for the author

I only have one major suggestion to improve the paper.
Main comment:
The authors do not discuss the possibility that keeping a fish anaesthetized for a long-time could alter the biology of interest. This possibility should be acknowledged. It would also be important for the authors to discuss possible controls to alleviate this concern. Even if fish stay alive it is not obvious that tissue functions are not altered in some ways.

Minor comment:
Cox et al Current Biology 2018 achieved about 24 hours of continuous imaging of regenerating zebrafish scales. This paper should be cited.

Reviewer 3

Advance summary and potential significance to field

The authors designed a new long-term adult zebrafish imaging method for inverted microscopes with multiple safety features to protect the microscope system. The current protocol is a step forward compared previous methods published in the past. The submitted article has a good novelty and importance for the zebrafish imaging community and it is suitable to publish in Development 'Techniques and Resources' section.

Comments for the author

Major Comments:
The paper is generally well written and structured. Nevertheless, the manuscript needs some details in regards to the experimental design: 1) The authors designed a 96 well plate holder on the stage, but they did not mention the purpose for this design. 2) A total of 7 adult zebrafish were imaged in a long-term experiment in the self-designed chamber. 5 fish lived for 20 hours. However, 2 fish died after 10 hours in the chamber because of a clogged inflow tube blocking water inflow and that two outflow tubes shifted down too far causing a low water level in the chamber. The authors didn't discuss the improvement and solutions to strengthen the reliability and increase fish survival during the experiment. 3) Fish are sensitive to water temperature and the temperature fluctuation could potentially impact the experimental results. In the system the authors mentioned a temperature monitoring system and the temperature adjustment by a heat block.
However, during a long-term/overnight experiment, it might be important to monitor the temperature continuously. The authors need to add some details on describing how they maintain water temperature in the system.

Minor comments
1. INTRODUCTION Paragraph 1: The last sentence, 'Finally, and perhaps most importantly, the optical clarity of zebrafish embryos and early larvae makes it possible to carry out very high-resolution optical imaging of all developing organs and tissues, including those deep within the animal'. To echo with the topic about long-term imaging of living adult zebrafish, perhaps the authors can start to mention the transparent casper (roy orbison and nacre double mutant) adult zebrafish here and then followed by more genetic modification techniques on zebrafish model in paragraph 2.
2. INTRODUCTION Paragraph 2: Second sentence introduces 'ENU mutagenesis' without a proper annotation. N-Ethyl-N-Nitrosourea (ENU).
3. INTRODUCTION Paragraph 3: The last sentence: 'Furthermore, there are tissues that do not exist during early embryonic and larval development such as the newly described intracranial lymphatic vascular network that can only be imaged and studied in juvenile and adult stage fish.' After reading this sentence, I expect to see a case study on 'intracranial lymphatic vascular network' by using this system. However, in the result, the author performed a imaging study on neutrophil recruitment to a scale removal wound. Therefore, it might be helpful to introduce the advantage of regeneration/immune studies in zebrafish in the introduction.

4. MATERIALS AND METHODS, Fish Preparation: the authors perhaps need to specify the age of the fish used in the experiment part. (months?)
5. MATERIALS AND METHODS, Image Acquisition: The authors wrote ‘DSred2’ in ‘Image Acquisition’
section, while the transgenic line in ‘Fish Husbandry and Fish Strains’ section is ‘Tg(lyz: DsRed2)’. It is better to be uniform in both sections.
6. The annotation for Casper transgenic line should be uniform. For example, the ‘casper (roy, nacre double mutant (White et al., 2008))’ in MATERIALS AND METHODS, ‘Fish Husbandry and Fish Strains’ section is different from the ‘pigment-free casper (roy orbison and nacre double mutant)’ in RESULTS AND DISCUSSION, Paragraph 4.
7. In Figure 3, scale bar: To show the micrometer, the authors used ‘um’ instead of ‘μm’.
8. Supplemental Table 1: The item name should be uniform. For example, ‘Intubation Tubing’ with capital ‘Tubing’ is different from ‘Overflow tubing’ using ‘tubing’. Please check other item names.
9. REFERENCES: It is better to have a uniform format for the page. For example, the third reference, ‘Fazio M., Ablain, J., Chuan, Y., Langenau, D. M. and Zon, L. I. (2020). Zebrafish patient avatars in cancer biology and precision cancer therapy. Nat Rev Cancer 20, 263-273.’, used 263-273, while the sixth reference, ‘Hwang W. Y., Fu, Y., Reyon, D., Maeder, M. L., Tsai, S. Q., Sander, J. D., Peterson, R. T., Yeh, J. R. and Joung, J. K. (2013). Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat Biotechnol 31, 227-9.’, used 227-9.
10. REFERENCES: ‘Yan, C., Brunson, D. C., Tang, Q., Do, D., Iftimia, N. A., Moore, J. C., Hayes, M. N., Welker A. M., Garcia, E. G., Dubash, T. D. et al. (2019). Visualizing Engrafted Human Cancer and Therapy Responses in Immunodeficient Zebrafish. Cell 177, 1903-1914 e14.’ The paper title of this reference should be uniform with others, using lowercase.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:

Zebrafish have traditionally been studied at embryonic and larval stages. Adult zebrafish have become an increasingly popular model, prompting the need for improved strategies allowing for long-term live imaging of juvenile and adult zebrafish. This is generally achieved by immobilizing and intubating the fish. Although there is a detailed published protocol (Xu et al, 2015; PMID:26584446) for upright microscopes, there is no similar protocol for inverted microscopes. The manuscript from Castranova and colleagues offers a method the authors claim can be applied to any configuration of microscope, while boasting additional safety features and temperature control. The authors demonstrate the utility of their 3D- printed, intubation chamber with impressive imaging of neutrophil recruitment to a skin wound over a 20+ hour period.

Reviewer 1 Comments for the Author:

The major limitation of the submission is that, as currently presented, the manuscript offers only an incremental advance over the method of Xu et al., falling short of the journal’s criterion that Techniques articles provide “a novel technique, or a sufficiently substantial advance of an existing technique.” Furthermore, there was a significant (> 25%) failure rate of the apparatus. Below are suggestions on ways to improve the manuscript.

Major Comments:

- How does the proposed fish intubation method significantly improve upon other published methods, namely the one proposed by Xu et al.? In general, the manuscript would benefit from a more thorough discussion of the published intubation/imaging methods, including explicit statements of how the proposed method is a significant improvement on available methods. For example, Xu et al states that “Our system is specifically designed for use with upright confocal microscopes. However, this system can be adapted for use with inverted microscopes by replacing

the bottom of the imaging chamber with optical glass."

> While we agree with the reviewer that Xu *et al*. did a nice job of introducing intubation methods for the zebrafish, and we have cited them accordingly, our study represents a ground-up redesign that addresses a variety of technical challenges and concerns and provides a major new advance for this technique. This new method for inverted microscopes involves a complete redesign of the imaging chamber and associated equipment, not simply "replacing the bottom of the chamber with optical glass" (which it is not at all clear could be readily accomplished with the Xu *et al*. methods anyway). We included multiple levels of newly designed safety features to prevent water overflow onto microscope optics (**Fig. 2**), implemented a method for tight control of temperature that maintains good oxygenation of the media (**Supp. Fig. 2**), and incorporated pulse dampening of inflow water to eliminate motion of the imaged sample (**Supp. Fig. 3**). We designed a 3-D printable, easily adaptable imaging chamber and provide CAD files for printing the chamber, a comprehensive materials list (**Supp Table 1**), and a detailed, easy-to-follow guide (**Supp. Fig. 1**) to allow any lab to implement and adapt this method. We also demonstrate the usefulness of our new methods via extensive new experiments showing that neutrophil recruitment to wounds is not significantly affected by overnight intubation with or without confocal imaging (**Supp. Fig. 5**), and that fish can be repeatedly intubated and imaged on multiple days (**Fig. 4**, **Supp. Movie 2**). We also demonstrate the broader utility of this method for many different aquatic species by showing that we can intubate and image cavefish in addition to zebrafish (**Supp. Fig. 6**, **Supp. Movie 3**). With the additional improvements to our methods in this revised version of the manuscript (many suggested by this reviewer- thank you for the helpful and constructive suggestions!) we believe this will be the "gold standard" for intubation and imaging of aquatic species.

- Protocol lacks a clear step-wise explanation of how to carry out methodology. Although the figure illustrations were helpful to see the final configuration of the intubation rig, it would have been extremely helpful for the methods to be clearly stated in a step-by-step, or sequential manner. In the paper's current state, one might find it difficult to faithfully recapitulate the set up. If there are space limitations, this could be provided in the supplemental materials.

> In response to this excellent suggestion we have added a detailed step-by-step guide showing how to assemble the intubation system (**Supp. Fig. 1**). We also added additional information to help the reader implement the new pulse dampener (**Supp. Fig. 3**) and water sensor (**Supp. Fig. 4**).

Given that there was not a 100% survival rate of the tested fish, a troubleshooting guide of common factors that contribute to success or failure of the method and fish survival would be helpful to the reader. For example, is there a range of anesthesia concentrations, tubing size or flow rates that correlate to fish size or genetic background? How can the depth of anesthesia and fish health be determined and monitored during the experiment?

> Carrying out extended overnight general anesthesia on any living vertebrate animal is very challenging (it is extremely rare, and risky, to keep a person under general anesthesia for 24 hours!), and the survival rate in our overnight adult time-lapse imaging experiments is comparable to survival rates we have observed in overnight time-lapse imaging experiments with zebrafish embryos and larvae, which are much more tolerant of anesthesia. We carried out trial experiments to determine the anesthetic dose we use, which carefully straddles the fine line between maintaining adequate anesthesia and ensuring immobility for high-resolution imaging and maintaining high survival. We have also noted in our manuscript a number of things that can improve survival, such as preventing excessive pressure on the ovaries in females and ensuring that fish are at least partially "woken up" before being de- intubated. The most effective way we have to monitor fish health during our experiments is to observe the strength of blood flow, which is readily observed using our high-resolution imaging. We would also note that we have obtained 100% survival in shorter 3.5 hour intubation/imaging runs, even when these are repeated multiple times over consecutive days (**Fig. 4**, **Supp. Movie 2**), demonstrating that our method is highly effective for maintaining fish survival during the shorter intubations most labs would probably use.

- Is the method reliable for experiments requiring multiple imaging sessions? Two of the seven fish did not survive after a single intubation trial. Many regenerative processes, such as appendage regeneration, may require multiple rounds of imaging with repeated intubation sessions. A researcher may hesitate to perform such an experiment if the fish's survivability is suspect. Please clarify whether all 5 "surviving" fish were fully recovered or just showed basic vital signs after the extended imaging session. For the 2/7 fish that did not survive, the authors state there was mechanical failure of the intubation setup. This is an unacceptably high failure rate. The authors should provide more extensive testing of the reliability of their method and fish survival. The text should be clarified to say the method requires some limited oversight to ensure the equipment is performing properly.

> In response to the reviewers comments, we have carried out an additional 7 short term (3.5 hour) and 26 overnight intubation/imaging experiments. We obtained survival rates of 100% for our shorter- term runs and 77% for our overnight runs. As noted above, the survival rate for overnight time-lapse imaging is comparable to what we have observed for overnight time-lapse imaging of zebrafish embryos and larvae, and we believe this represents a remarkable achievement considering how much more sensitive adults are to anesthesia. Fish returned to our aquaculture system after overnight intubation resumed feeding and were alive and healthy several weeks post-intubation. As discussed further below, we also carried out extensive additional controlled and quantitated experiments (**Supp. Fig. 5**) demonstrating that neutrophil recruitment to wounds is not significantly altered by overnight intubation and imaging.
>
> Importantly, in direct response to the concern the reviewer notes above regarding survival of fish subjected to "multiple rounds of imaging with repeated intubation sessions," we added additional experiments showing that our method could be used for multiple rounds of shorter-term (3.5 hour) intubation/imaging on consecutive days with 100% survival (**Fig. 4**, **Supp. Movie 2**). Thus, for shorter intubation/imaging experiments our method is very reliable for ensuring fish survival. We also addressed the reviewers concerns about ensuring the equipment was set up and maintained properly by adding a detailed user's guide (**Supp. Fig 1**) that includes a large number of troubleshooting tips and tricks to help avoid potential mechanical issues.

- Neutrophil experiment lacks control fish. The neutrophil recruitment experiment was impressive, but it appears there is a global increase in neutrophil signal/frequency in areas of the fish that did not receive the injury. For instance, comparing Figures 3C and 3F, a large amount of signal accumulates in the uninjured fin region by 18 hours (3F). Was there a significant effect of laser exposure and/or prolonged immobilization during the experiment? Control fish imaged for the same duration would confirm there was no significant effect of phototoxocity and ensure the mounting conditions did not contribute to changes in fluorescence.

> We thank the reviewer for this constructive suggestion. In response to these comments we have now added an extensive additional set of carefully controlled and quantitated new experiments (**Supp. Fig. 5**) showing conclusively that neutrophil recruitment is not significantly impacted by overnight intubation using our methods, or by overnight imaging.

- A more detailed discussion of casper fish. The authors emphasize the use of the casper mutant fish, however, the advantages and limitations of this genetic background should be discussed. For example, the reader would benefit from a comparison of the depth of imaging possible in casper mutants compared to zebrafish with wild-type pigmentation. What limitations, if any, of this genetic background should be considered?

> In response to these comments we have added the following new paragraph to our discussion:
>
> *"Using pigment free casper fish was important to this work because although blood vessels in the scales can be imaged, imaging any structures below the skin becomes very difficult with normally pigmented fish. Researchers using casper fish should be aware that there may be physiological differences between casper fish and wild-type fish, as has been demonstrated in the hair cells in the lateral line (Holmgren and Sheets, 2021)."*

- The manuscript should appeal to a broad audience. The appeal of the manuscript beyond a relatively narrow zebrafish readership is unclear. As written, the first two paragraphs of the introduction contained a very generic discussion of the advantages of zebrafish. Could the authors more succinctly summarize these points and state the broad problem they are addressing earlier (i.e., in vivo imaging of developmental/regenerative processes beyond embryonic stages, which is a general challenge in the field)? In terms of the methodology, could their imaging approach be applied to aquatic vertebrates beyond zebrafish?

> In response to these comments we have carried out additional new overnight intubation and imaging experiments using Mexican cavefish *(Astyanax mexicanus)*, demonstrating the applicability of our method to other aquatic vertebrates (**Supp. Fig. 6**, **Supp. Movie 3**). As suggested by the reviewer we also "retooled" our introduction text to better introduce the broader problem of *in vivo* imaging in later developing and adult aquatic vertebrates, not just zebrafish, and made our discussion of the advantages of the zebrafish model more succinct.

Minor Comments:

-Safety. The bonus safety features to prevent overflow seem more than sufficient to ensure water does not escape from the top, but it still seems suspect that if the grease seal around the chambered coverglass (or the coverglass itself) fails, the microscope hardware would be catastrophically flooded. Is there any failsafe or advice for avoiding this seemingly possible scenario?

> In response to this comment we would note that one of the water safety features, the electronic water sensor, is actually designed to detect and immediately shut the water pump off in the presence of even small amounts of water outside of the chambered coverglass (see **Fig. 2B**). Under normal circumstances water is actually only present inside the chambered coverglass, and the seal around the chambered coverglass is actually not in fact even being used to prevent leakage. In the event water does infiltrate areas of the imaging chamber outside of the chambered coverglass, the electronic water sensor would shut water flow off and prevent any further leakage that might possibly occur due to seal failure.
>
> Having said this, it is nevertheless still important to create a water-tight seal between the chambered coverglass and the 3D printed chamber as a safety measure. We investigated using waterproof double-sided tape, or epoxy, but we found that silicone grease was the best option. After switching out the chambered coverglass a few times, a nice layer of grease forms inside the 3D printed chamber. This, combined with adding additional grease to the chambered coverglass each time makes for a tight seal, although as we note in our user guide it is important to check carefully to ensure a good seal before proceeding with the intubation and imaging.

- Can the intubation rig fit most upright microscopes? The authors claim this rig can be "adapted to any microscope system". However, the high walls on the rig pose a problem for upright microscopes. The objectives would likely be physically blocked by the walls while attempting to lower onto the sample. Please make clear how "universal" this rig is, and what might be need to be adjusted for different scope configurations.

> In response to these comments we have edited the text to more clearly state that our chamber is designed for inverted systems.

- Consider removing Supplementary Movie 2. This movie could be removed as it does not convey anything that can't be stated with text alone. Also, the recovered fish is swimming at a very reduced rate compared to the pre-intubation condition. This might raise more questions rather than offering the reader assurance that the intubated fish was fully recovered.

> In response to these comments we have modified **Supp. Movie 4** (formerly **Supp. Movie 2**). In the original movie we had imaged the fish very shortly after being removed from intubation, and it had not fully "woken up" yet, accounting for its reduced swimming rate. In the new movie we waited slightly longer after intubation to allow the fish to fully wake up, and it

accordingly shows more natural swimming behavior. In addition to before and after intubation images **Supp. Movie 4** also now shows the intubation tube being inserted into the fish's mouth and the wakeup procedure when fresh water is pumped through the system, making this supplemental movie more useful and informative than the previous one.

- Figure 2 organization. The diagrams for Figure 2 are excellent, however it would be beneficial to arrange A-D in a manner that represents the order for assembling the rig. For example, 2D introduces sealing off the chambered coverglass with silicone grease, but that would likely be one of the first steps in setting up the rig. Again, it would be helpful to have a clear, stepwise procedure laid out for the reader to follow. This paper does a fantastic job showing the final product, but more information on how to assemble the rig would be greatly appreciated.

In response to these comments we have reorganized **Fig. 2** as suggested, and we have added a new **Supp. Fig. 1** that provides highly detailed, step by step instructions on how to set up the rig.

- Description of scale removal wound. The type of zebrafish injury performed is not articulated clearly in its current state. How deep was the wound? "Scale removal by abrasion" should be reworded to indicate the exact nature of the injury, relative to the more commonly used technique of plucking individual scales with forceps.

In response to these comments we have added a more detailed description of the cutaneous wounding technique in our Materials and Methods section (pg. 10):
"A small wound was made around the trunk mid-line above the anal fin using a 4 mm dissecting knife (Fine Science Tools # 10055-12). A few scales were removed by scraping the tip of the knife along the side of the fish from dorsal to ventral, gentle scraping was continued until minor damage to the skin was done, causing a very small amount of blood to be seen around the wound site."

Reviewer 2 Advance Summary and Potential Significance to Field:

The manuscript by Castanova et al presents an experimental setup for the long- term imaging of adult zebrafish. This is an extension of a previous method by Xu et al 2015 and is adapted for inverted microscopes. The tool could be useful to a large number of groups. The authors provide clear instructions and explanations on how to build the chamber and demonstrate its use in following neutrophil migration toward a wound. The paper is clearly written. Overall, this method paper would be of interest to developmental biologists.

Reviewer 2 Comments for the Author:

I only have one major suggestion to improve the paper.

Main comment: The authors do not discuss the possibility that keeping a fish anaesthetized for a long- time could alter the biology of interest. This possibility should be acknowledged. It would also be important for the authors to discuss possible controls to alleviate this concern. Even if fish stay alive it is not obvious that tissue functions are not altered in some ways.

We agree with the reviewer that it is important to acknowledge that long-term anesthetization and intubation could alter the biology of interest of specific processes being studies. We have now explicitly done so in both our results and discussion sections:

(pg. 6) "However, we cannot exclude the possibility that prolonged intubation and imaging may cause other physiological changes within the fish, and appropriate controls must always be included depending on the process being studied."

(pg. 8) "It is also important to note once again that while we have shown neutrophil recruitment is not appreciably affected by overnight intubation and imaging, prolonged intubation and imaging may cause other physiological changes within the fish, and appropriate controls must always be included depending on the process being studied."

However, to address the concern of reviewer 1 and this reviewer about neutrophil recruitment in particular, we carried out an extensive additional set of carefully controlled and quantitated new experiments (**Supp. Fig. 5**) conclusively demonstrating that neutrophil recruitment is not significantly impacted by overnight intubation and overnight imaging.

Minor comment: Cox et al Current Biology 2018 achieved about 24 hours of continuous imaging of regenerating zebrafish scales. This paper should be cited.

We thank the reviewer for pointing out this oversight - we now cite this paper in our introduction.

Reviewer 3 Advance Summary and Potential Significance to Field:

The authors designed a new long-term adult zebrafish imaging method for inverted microscopes with multiple safety features to protect the microscope system. The current protocol is a step forward compared previous methods published in the past. The submitted article has a good novelty and importance for the zebrafish imaging community and it is suitable to publish in Development 'Techniques and Resources' section.

Reviewer 3 Comments for the Author:

Major Comments: The paper is generally well written and structured. Nevertheless, the manuscript needs some details in regards to the experimental design:

1) The authors designed a 96 well plate holder on the stage, but they did not mention the purpose for this design.

We have added the following text to pg. 5 of our results section to clarify this point: "and one with four supports extending out from the base of the chamber body designed to fit in a stage designed to hold 96-well plates (Fig 1F) because there are several microscope and stage companies that manufacture stages or inserts designed to fit the 96 well plate dimensions."

2) A total of 7 adult zebrafish were imaged in a long-term experiment in the self-designed chamber. 5 fish lived for 20 hours. However, 2 fish died after 10 hours in the chamber because of a clogged inflow tube blocking water inflow and that two outflow tubes shifted down too far causing a low water level in the chamber. The authors didn't discuss the improvement and solutions to strengthen the reliability and increase fish survival during the experiment.

In response to these and other reviewer comments we have now carried out an additional 7 short term (3.5 hour) and 26 overnight intubation/imaging experiments. We obtained survival rates of 100% for our shorter-term runs and 77% for our overnight runs. The survival rate for the more challenging overnight time-lapse imaging is comparable to what we have observed for overnight time- lapse imaging of zebrafish embryos and larvae, and we believe this represents a remarkable achievement considering how much more sensitive adults are to anesthesia. Fish returned to our aquaculture system after overnight intubation resumed feeding and were alive and healthy several weeks post-intubation. As discussed further below, we also carried out extensive additional controlled and quantitated experiments (**Supp. Fig. 5**) demonstrating that neutrophil recruitment to wounds is not significantly altered by overnight intubation and imaging.

We have also added additional experiments showing that our method could be used for multiple rounds of shorter-term (3.5 hour) intubation/imaging on consecutive days with 100% survival (**Fig. 4**, **Supp. Movie 2**). Thus, for shorter intubation/imaging experiments our method is extremely reliable for ensuring fish survival.

We also addressed now added a detailed user's guide (**Supp. Fig 1**) that includes a large number of troubleshooting tips and tricks to help avoid potential mechanical issues and avoid pitfalls that reduce the likelihood if fish survival.

3) Fish are sensitive to water temperature and the temperature fluctuation could potentially impact the experimental results. In the system, the authors mentioned a temperature monitoring system and the temperature adjustment by a heat block. However, during a long-term/overnight experiment, it might be important to monitor the temperature continuously. The authors need to add some details on describing how they maintain water temperature in the system.

> In response to this useful suggestion we carried out an additional set of overnight intubation experiments where we continuously recorded the water temperature in the imaging chamber (new **Supp. Fig. 2**), showing that temperature fluctuations were no great than 2 degrees Celsius, and in most case were less. It is worth noting that the room this imaging was performed in had much larger fluctuations in air temperature, showing that our system is able to maintain stable water temperatures despite varied air temperatures.

Minor comments

1. INTRODUCTION Paragraph 1: The last sentence, 'Finally, and perhaps most importantly, the optical clarity of zebrafish embryos and early larvae makes it possible to carry out very high-resolution optical imaging of all developing organs and tissues, including those deep within the animal'. To echo with the topic about long-term imaging of living adult zebrafish, perhaps the authors can start to mention the transparent casper (roy orbison and nacre double mutant) adult zebrafish here and then followed by more genetic modification techniques on zebrafish model in paragraph 2.

> In response to comments from both this reviewer and reviewer 1 we have substantially rewritten our introduction.

2. INTRODUCTION Paragraph 2: Second sentence introduces 'ENU mutagenesis' without a proper annotation. N-Ethyl-N-Nitrosourea (ENU).

> The reference to ENU has been removed in the re-writing of the introduction.

3. INTRODUCTION Paragraph 3: The last sentence: 'Furthermore, there are tissues that do not exist during early embryonic and larval development such as the newly described intracranial lymphatic vascular network that can only be imaged and studied in juvenile and adult stage fish.' After reading this sentence, I expect to see a case study on 'intracranial lymphatic vascular network' by using this system. However, in the result, the author performed a imaging study on neutrophil recruitment to a scale removal wound. Therefore, it might be helpful to introduce the advantage of regeneration/immune studies in zebrafish in the introduction.

> As suggested, our introduction now also mentions the immune system and regeneration as processes that need to be studied in juvenile and adult zebrafish:
>
> *"There are also many tissues and processes that do not exist during early embryonic and larval development such as the newly described intracranial lymphatic vascular network (Castranova et al., 2021), a fully functional adult immune system, and adult regeneration processes that can only be imaged and studied in juveniles and adults."*

4. MATERIALS AND METHODS, Fish Preparation: the authors perhaps need to specify the age of the fish used in the experiment part. (months?)

> As requested, we now note in our materials and methods that fish "between 6 and 18 months" were used in our experiments.

5. MATERIALS AND METHODS, Image Acquisition: The authors wrote 'DSred2' in 'Image Acquisition' section, while the transgenic line in 'Fish Husbandry and Fish Strains' section is 'Tg(lyz: DsRed2)'. It is better to be uniform in both sections.

> As requested, we now use Tg(lyz: DsRed2) in both locations.

6. The annotation for Casper transgenic line should be uniform. For example, the ‘casper (roy, nacre double mutant (White et al., 2008))’ in MATERIALS AND METHODS, ‘Fish Husbandry and Fish Strains’ section is different from the ‘pigment-free casper (roy orbison and nacre double mutant)’ in RESULTS AND DISCUSSION, Paragraph 4.

As requested, we changed the reference in the results to casper (roy, nacre double mutant (White et al., 2008)) to match the methods reference.

7. In Figure 3, scale bar: To show the micrometer, the authors used ‘um’ instead of ‘μm’. This has been corrected.

8. Supplemental Table 1: The item name should be uniform. For example, ‘Intubation Tubing’ with capital ‘Tubing’ is different from ‘Overflow tubing’ using ‘tubing’. Please check other item names.

This has been corrected.

9. REFERENCES: It is better to have a uniform format for the page. For example, the third reference, ‘Fazio, M., Ablain, J., Chuan, Y., Langenau, D. M. and Zon, L. I. (2020). Zebrafish patient avatars in cancer biology and precision cancer therapy. Nat Rev Cancer 20, 263-273.’, used 263-273, while the sixth reference, ‘Hwang, W. Y., Fu, Y., Reyon, D., Maeder, M. L., Tsai, S. Q., Sander, J. D., Peterson, R. T., Yeh, J. R. and Joung, J. K. (2013). Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat Biotechnol 31, 227-9.’, used 227-9.

This has been corrected. References are in uniform format.

10. REFERENCES: ‘Yan, C., Brunson, D. C., Tang, Q., Do, D., Iftimia, N. A., Moore, J. C., Hayes, M. N., Welker, A. M., Garcia, E. G., Dubash, T. D. et al. (2019). Visualizing Engrafted Human Cancer and Therapy Responses in Immunodeficient Zebrafish. Cell 177, 1903-1914 e14.’ The paper title of this reference should be uniform with others, using lowercase.

This has been corrected.

Second decision letter

MS ID#: DEVELOP/2021/199667

MS TITLE: Long-Term Imaging of Living Adult Zebrafish

AUTHORS: Daniel Castranova, Bakary Samasa, Marina Venero Galanternik, Aniket V Gore, Allison E Goldstein, Jong S Park, and Brant M Weinstein
ARTICLE TYPE: Techniques and Resources Report

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks. I also ask that you incorporate the minor requested changes from Reviewer #1 into a final version that you send to the journal.

Reviewer 1

Advance summary and potential significance to field

The authors thoroughly addressed all of the previous reviewers’ comments.
Overall, the manuscript is much improved. The Mexican Cavefish imaging and step-by-step assembly guides were particularly nice additions that will broaden adoption of this method by the field.

Comments for the author

Minor comments on the text:
- Line numbers would have facilitated reviewer comments
- Page 5, please consider removing/revising the word “quickly” from the sentence “...the four stage supports can be quickly modified using Google Sketchup” as this depends on the users familiarity with the software
- Page 8, please consider removing/revising the word “extraordinary” from the sentence “The extraordinary steadiness and reproducible...”
- Page 9, Under “Water flow” - “buffered tricaine” - for experimental reproducibility, please provide a supplier/catalog number and detail how the tricaine was buffered
- Page 11, allele names for roy and nacre are missing. Please consider citing the original papers describing these mutations.
- Page 13, Fig 4 legend- “Tg(lyz:DsRed2)NZ50, Tg(mrc1a:eGFP)y251” - should be italicized with alleles in superscript
- Fig 4B is missing a scale bar
- Please proofread the manuscript carefully - a number of typos and run on sentences were noted, some of which are listed below:
- Page 3, typo at “]use expression”
- Page 3, typo at “Stides in adult zebrafish have”
- Page 4, typo at “2015),which was”
- Page 4, “we describe a newly redesigned method designed for inverted microscopes” consider revising to “we describe a redesigned method for inverted microscopes”
- Page 5, the sentence beginning “We have provided two different versions of the chamber design” is quite long. Please consider revising
- Page 6, “wound area doesn’t” should read “wound area does not”
= Page 11, typo at “Iphone XR” and “Sonny α 6400 mirrorless”
- 3-D and 3D are both used. Please use one for consistency and define at the first use
- casper is inconsistently italicized in the text
- Label on Supp Movie 4 “tircaine-free water” (@30s) should read “tricaine-free”

Reviewer 2

Advance summary and potential significance to field

This paper presents a method for long-term imaging of living adult zebrafish which will be a great resource for the field

Comments for the author

The authors have satisfactory addressed my previous comments

Reviewer 3

Advance summary and potential significance to field

The method paper by Castanova et al. will be useful for the community of biologists interested in the study of immunology, angiogenesis, and tissue regeneration by imaging long-term living adult zebrafish. Overall, IÂ’m satisfied with the authorÂ’s responses and the additional experiments. The imaging work presented in this manuscript is incredibly detailed and beautifully represented as it is typical from the Weinstein lab. The added step-by-step guides and troubleshooting tips from Supplementary Figure. 1 will help readers to overcome pitfalls.

Comments for the author

There is no concern to adress