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A Layered Mounting Method for Extended Time-Lapse Confocal Microscopy of Whole Zebrafish Embryos

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INTELLIGENT SYSTEMS ENGINEERING

INDIANA UNIVERSITY

Dear Dr. Jewhurst:

Please, find here the resubmission of our manuscript LAYERED MOUNTING METHOD FOR EXTENDED TIME-LAPSE CONFOCAL MICROSCOPY OF WHOLE ZEBRAFISH EMBRYOS by Upahyay et al. We would like to thank the Reviewers and Editor for very valuable comments and we have revised the manuscript accordingly.

The manuscript focuses on a mounting method for extended time-lapse imaging of live zebrafish embryos. The mounting method works for any inverted microscope. Because the procedure on how to set up time-lapse imaging varies from microscope to microscope, I did not include a step-wise description for the imaging in the protocol. Please, see the Rebuttal Letter to Editor's Comments. Please, let me know whether you find this should be included or not.

Two versions of the manuscript are included in the submission, one without track changes, and one with track changes (the latter uploaded as a Supplemental File).

Best regards,

Maria Bondesson

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TITLE:

A Layered Mounting Method for Extended Time-Lapse Confocal Microscopy of Whole Zebrafish Embryos

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KEYWORDS:

zebrafish, embryo, confocal microscopy, time-lapse, mounting, transgenic

SUMMARY:

This article describes a method to mount fragile zebrafish embryos for extended time-lapse confocal microscopy. This low-cost method is easy to perform using regular glass-bottom microscopy dishes for imaging on any inverted microscope. The mounting is performed in layers of agarose at different concentrations.

ABSTRACT:

Dynamics of development can be followed by confocal time-lapse microscopy of live transgenic zebrafish embryos expressing fluorescence in specific tissues or cells. A difficulty with imaging whole embryo development is that zebrafish embryos grow substantially in length. When mounted as regularly done in 0.3-1% low melt agarose, the agarose imposes growth restriction,

leading to distortions in the soft embryo body. Yet, to perform confocal time-lapse microscopy, the embryo must be immobilized. This article describes a layered mounting method for zebrafish embryos that restrict the motility of the embryos while allowing for the unrestricted growth. The mounting is performed in layers of agarose at different concentrations. To demonstrate the usability of this method, whole embryo vascular, neuronal and muscle development was imaged in transgenic fish for 55 consecutive hours. This mounting method can be used for easy, low-cost imaging of whole zebrafish embryos using inverted microscopes without requirements of molds or special equipment.

INTRODUCTION:

The zebrafish has long been a model organism for developmental biology, and microscopy is the major method to visualize embryonic development. The advantages of using zebrafish embryos for developmental studies include small size, optical clarity, rapid development, and high fecundity of the adult fish. The generation of transgenic zebrafish lines expressing fluorescence in certain tissues or cells have allowed for a direct visualization of tissue development in ways not possible with larger vertebrate animals. In combination with time-lapse microscopy, details and dynamics of the tissue development can be readily studied.

A difficulty with imaging zebrafish development is that the embryos grow substantially in length; the embryo extends its length four times within the first 3 days of life[1]. Also, the body of the early embryo is soft, and easily becomes distorted if growth is restricted. Yet, to perform confocal microscopy, the embryo must be immobilized. To keep embryos in a fixed position for confocal time-lapse imaging, they are regularly anesthetized and mounted in 0.3-1% low-melt agarose. This has the advantage of allowing for some growth during imaging for a certain period of time, while restricting movements of the embryo. Parts of the embryo can efficiently be imaged like this. However, when using this method for imaging of the whole embryo for extended time periods, distortions are observed because of restricted growth caused by the agarose. Thus, other mounting methods are required. Kaufmann and colleagues have described an alternative mounting of zebrafish embryos for light sheet microscopy, such as selective plane illumination microscopy (SPIM), by mounting the embryos in fluorinated ethylene propylene (FEP) tubes containing low concentrations of agarose or methylcellulose[2]. This technique produces a superb visualization of embryogenesis over time. Schmid et al. describe mounting of up to six embryos in agarose in FEB tubes for light-sheet microscopy[3] providing visualization of several embryos in one imaging session. Molds have been used to create embryo arrays for efficient mounting of larger numbers of embryos[4]. Masselkink et al. have constructed 3D printed plastic molds that can be used to make silicon casts that zebrafish embryos at different stages can be placed in, enabling mounting in a constant position for imaging, including confocal imaging[5]. 3D printing has also been used to make molds for consistent positioning of zebrafish embryos in 96-well format[6]. Some molds are customized for certain stages and may not permit unrestricted growth for long time periods, whereas other molds are more flexible. Recently, Weijts et al. published the design and fabrication of a four-well dish for live imaging of zebrafish embryos[7]. In this dish, the tail and trunk of anesthetized fish embryos are placed manually under a clear silicone roof attached just above a cover glass to form a pocket. The embryo is then fixed in this position by the addition of 0.4% agarose. This

mounting allows for the imaging of the about 2 mm long posterior part (trunk and tail) of the embryo, and as up to 12 embryos can be mounted per well, the method allows for the imaging of multiple samples. Similarly, Hirsinger and Steventon recently presented a method where the head of the fish is mounted in agarose, while the tail can freely grow, and this method also efficiently facilitates imaging of the trunk and tail region of the embryo[8].

This article describes a layered mounting method for zebrafish embryos that restrict the movements of the embryos while allowing for unrestricted growth. The advantages of this mounting method are that it is a low-cost, fast and easy method to mount embryos of various stages for imaging using any inverted microscope. The mounting permits long-term imaging of the whole body (head, trunk and tail) during embryo development. To showcase the usability of this method, whole embryo vascular, neuronal and muscle development was imaged in transgenic fish. Two embryos per session, at two wavelengths in 3D were imaged by time-lapse microscopy for 55 consecutive hours to render movies of tissue development.

PROTOCOL:

The animal work presented here was approved by the Institutional Animal Care and Use Committees (IACUCs) of the University of Houston and Indiana University.

1. Fish husbandry

NOTE: Work with vertebrate models requires an IACUC approved protocol. It should be conducted according to relevant national and international guidelines.

1.1. Maintain adult zebrafish as described in previously published literature[9].

1.2. In the afternoon, place adult zebrafish in breeding tanks. Breed males to females at a ratio of 1:2.

2. Preparation of solutions

2.1. Make a stock solution of 1% low melt agarose in embryo media (E3: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, adjusted to pH 7.0). Aliquot the stock solution into 1.5 mL tubes and store them at 4 °C.

2.2. Make a stock solution of 4% (w/v) Tricaine (MS-222) in distilled water. Store at 4 °C in a dark bottle.

CAUTION: Tricaine is toxic and should be weighed and dissolved in a fume hood.

2.3. Make a stock solution of 20 mM N-phenylthiourea (PTU) in distilled water. Store at -20 °C.

3. Preparation of embryos

3.1. After mating, harvest embryos in E3 in a Petri dish and incubate them at 26.5 °C for about 28 h before mounting.

NOTE: This slows down the development of the embryos so that the embryos are approximately at 30 somite stage at the beginning of imaging.

3.2. Anesthetize embryos in 0.16-0.20% tricaine in E3. To inhibit pigmentation, add 200 µM N-phenylthiourea (PTU).

3.3. Dechorionate the embryos using forceps under a dissecting microscope. Using two forceps, grip and gently pull the chorion apart to release the embryo.

4. Mounting in agarose

NOTE: The developed mounting method requires two different concentrations of low-melt agarose in E3 with 0.02% Tricaine and PTU as needed. The first agarose solution contains an optimal concentration of agarose at which the distortion and motility are at a minimum. The optimization is described in step 5 below.

4.1. Heat the agarose solutions for the two layers (concentration defined in step 5 below and 1%) to 65 °C. Let the agarose cool down to approximately 30 °C just before mounting so that the embryo is not harmed by the heat. For mounting, use 35 mm glass bottom dishes with a No. 0 cover glass bottom. The cover glass attached to the bottom of the dish creates a 10 mm shallow (approx. 1.2 mm deep) well, in which the embryo is to be placed.

NOTE: In this case, the concentration with the least motility and distortions were between 0.025 to 0.040% agarose.

4.2. Gently place a dechorionated embryo with one of its lateral sides toward the bottom of the dish using a glass pipette or micropipette. If using a micropipette, cut the outer part of the tip to increase the size of the opening to fit the embryo (**Figure 1A**). Carefully remove any remaining E3 with a micropipette.

4.3. Add the first agarose solution to the small well created by the cover glass attached to the bottom of the dish to cover the embryo (Layer 1) (**Figure 1B**). Ensure that the agarose covers the small well but will not overflow it.

4.4. Cover the small well with a cover glass (22 mm x 22 mm) (**Figure 1C**) to create a narrow agarose filled space with the embryo between the two cover glasses.

4.5. Place a layer of 1% agarose solution on top of the cover glass all over the bottom of the dish (Layer 2) (**Figure 1D**). As this layer solidifies, it holds the cover glass in place.

4.6. Fill the remaining portion of the dish with E3 containing 0.02% Tricaine to keep the system hydrated (Layer 3) (**Figure 1E**).

NOTE: In this setup, the cover glass and 1% agarose protect the bottom layer from getting diluted.

5. Optimization of agarose solution for layer 1

5.1. To identify the optimal concentration of agarose for Layer 1, use a multiscale grid search approach. Mount embryos in increasing concentrations of agarose ranging from 0.01% to 1% followed by time-lapse imaging of embryo growth restriction and motility in the field of view. Identify the concentrations where both the distortion and motility are at a minimum.

5.2. To optimize the concentration of agarose further, mount the embryos using a finer range of concentrations of agarose (e.g., between 0.025 and 0.040% agarose) depending on the concentration found to be best in step 5.1 (e.g., 0.025%, 0.028%, 0.031%, etc.).

NOTE: In our laboratory, the optimal agarose concentration was around 0.03%.

6. Time-lapse imaging

6.1. Perform time-lapse imaging for the whole embryo or parts of it for up to 55 h. For optimal embryo growth and development, use a microscope stage with an incubator set at 28.5 °C.

NOTE: This mounting method works for any inverted microscope with time-lapse functionality for fluorescence and bright field imaging.

REPRESENTATIVE RESULTS

Development of the mounting method

The main aim of this work was to develop a low-cost mounting technique for time-lapse imaging of zebrafish development for extended periods of time. The layered mounting method was developed to allow for full growth of the fragile zebrafish embryo body, while restricting its movements. If the agarose concentration of layer 1 is too high, the embryos will become distorted and curved (**Figure 2**). Embryos grown at 0.1% and 0.5% agarose have shortened tails, distorted fins, and curved heads. On the contrary, if the agarose concentration is too low, the embryos will move out of the field of view during time-lapse microscopy, even though they are anesthetized, as the growing tail swings out from the embryo body and causes it to move. In our hands, the optimal agarose concentration varied between 0.028% and 0.034% agarose between different batches of agarose. Mounting below 0.025% agarose did not provide enough resistance for the embryo to stay in the field of view.

Extended time-lapse imaging of vascular, neuronal and muscle development

After optimizing the mounting method described above, time lapse confocal microscopy images were captured over a span of 55 h. 10x and 20x objectives with 0.45NA and 0.75NA,

respectively, were used to capture images around 40 z-plane optical slices with a 11 µm step size. For whole embryo imaging, 3 frames of view were merged with 10% overlap. Two embryos were imaged per session, and the imaging of each embryo for each time point took approximately 20 minutes. Images were rendered by the microscope software and movies generated by converting the confocal images (nd2 files) to uncompressed avi format. This conversion was done after making maximum intensity projections on the z-axis and sequencing the 2D projections as video at the rate of 5 frames/second.

For the time-lapse imaging, we used live transgenic zebrafish expressing GFP or RFP in different tissues. An advantage of using transgenic fish with endogenous fluorescence for time lapse imaging is that the fluorescence molecules, such as GFP and RFP, are produced continuously in the live embryo, and, thus, it does not easily photo bleach. First, embryos of a cross of *Tg(kdr1: EGFP)mitfa*$^{b692/b692}$ [10] and *Ubi-zebrabow* [11] were imaged. In these embryos, RFP is expressed in all cells of the embryo, which allows for visualization of the general embryo structure. GFP is expressed in the endothelial cells of the vasculature. Double transgenic embryos were imaged for 55 hours from approximately 30 somite stage to visualize vascular development in the head and body (**Figure 3** and **Supplementary Movie S1**) using a 10x objective with 0.45 NA. Two embryos/session were imaged with z-stacks and time-lapse in a loop so that after imaging the first embryo at two different wavelengths, the second one was subsequently imaged and then the first one again. **Figure 3** shows the intersegmental vessel (ISV) sprouting, development of subintestinal vessels and head vasculature, and caudal vein plexus condensation together with trunk extension. Imaging of the whole embryo with the vasculature shows that ISV sprouting starts in between somites and grows dorsally up to the point of the neural tube, where the ISVs takes a different path and sprouts in a direction over to the next anterior somite boundary.

Next, embryos of a cross of *Tg(mnx:GFP)mitfa*$^{b692/b629}$ and *Ubi-zebrabow* were imaged for 55 hours approximately from the 30 somite stage to visualize motorneuron development (**Figure 4** and **Supplementary Movie S2**). *Tg(mnx:GFP)* had first been crossed to *mitfa*$^{b692/b692}$ (both from Zebrafish International Resource Center at the University of Oregon, OR) to produce Tg*(mnx:GFP)mitfa*$^{b692/b629}$. The motorneuron axons sprout from the ventral neural tube over the somites towards the ventral side of the embryo. Unlike the intersegmental vessels that sprout in between the somites, the axons sprout over the middle over the chevron-formed somites. The sprouting starts towards the anterior end of the neural tube, in a straight angle from the neural tube, and spreads posterior. By the somite/yolk interface, sprouting changes direction to anterior and posterior sprouting. Note the innervation of the developing heart by the anterior neurites.

Also, embryos of a cross of *Tg(kdr:enl.memRFP)mitfa*$^{b692/b692}$ (cross of *Tg(kdr:enl.memRFP* and *mitfa*$^{b692/b692}$) and *Tg(mnx:GFP)mitfa*$^{b692/b692}$ were imaged. In the former embryos, the red vascular fluorescence was not visible until 36 hours post fertilization (hpf), and therefore we started the imaging at a later time point at 2 dpf. We imaged a part of the trunk, dorsally to the yolk sac extension, using higher magnification (20x objective). This movie shows that the embryos lay still enough for good imaging quality at higher magnification. The co-development of the dorsal sprouting of motorneuron axons in relation to the position of intersegmental

vessels was followed (**Figure 5** and **Supplementary Movie S3**). In these movies, the finer details of ventral axon sprouting are visible, as well as dorsal axon sprouting from the neural tube to a point where neuronal axons and intersegmental vessels co-migrate. The caudal vein plexus condensation is also clearly shown. Note that as a neurite sprout is missing toward the posterior end of the tail (in the position of the 12th neurite from the right side), the closest posterior neuron extends toward the anterior part of the embryo to cover the area between neurites 11 and 13.

Finally, a cross of *HGn39b* [12, 13] and *Ubi-zebrabow* was imaged. *HGn39b* is a zTRAP line with the GFP insert in the activator of heat shock protein ATPase homolog 1 (AHA1) gene (http://kawakami.lab.nig.ac.jp/ztrap/faces/insertion/Insertions.jsp?name=HGn39B) and it expresses GFP in the muscles of the somites (**Figure 6** and **Supplementary Movie S4**). As somite numbers increase, the somites also extend in length and width. This movie also nicely shows heart development in red fluorescence from the *Ubi-zebrabow* fish line.

FIGURE LEGENDS

Figure 1: Description of mounting method. (**A**) Add the zebrafish embryo to the small well created by the glass bottom in the 35 mm dish. (**B**) Add agarose layer 1 to the small well to cover the embryo. (**C**) Carefully place a cover glass over the small well. (**D**) Add agarose layer 2 on the whole bottom of the 35 mm dish. (**E**) Add E3 to the dish. (**F**) Schematic drawing of a cross section of the mounting set up. (**G**) Microscope image (5x objective) of the zebrafish embryo in the final montage.

Figure 2: Embryo growth restriction and developmental delay in different concentrations of agarose. Embryos were mounted in different agarose concentrations and their size and development imaged at 48 hpf. Images captured by a fluorescence microscope equipped with a digital microscope camera (2.5x objective) and the accompanying microscope software.

Figure 3: Visualization of the development of vasculature. Cross of *Tg(kdr1: EGFP)mitfa$^{b692/b692}$* and *Ubi-zebrabow* imaged from about 30 somite stage for 55 h on a confocal microscope. Vasculature in green and all other cells in red. Scale bar 500 µm.

Figure 4: Visualization of the development of neurons and neurite sprouting. Cross of *Tg(mnx:GFP)mitfa$^{b692/b629}$* and *Ubi-zebrabow* imaged from about 30 somite stage for 55 h on a confocal microscope. Motorneurons in green, all other cells in red. Scale bar 1,000 µm.

Figure 5: Visualization of the co-development of neurons and vasculature. Cross of *Tg(kdr:enl.memRFP)mitfa$^{b692/b692}$* and *Tg(mnx:GFP)mitfa$^{b692/b692}$* imaged for 55 hours from about 2 dpf on a confocal microscope. Vasculature in red, motorneurons in green. Scale bar 500 µm.

Figure 6: Visualization of muscle GFP expression in somites. Cross of *HGn39b* and *Ubi-zebrabow* were imaged on a confocal microscope for 55 h from about 30 somite stage. Muscle in green, all other cells in red. Scale bar 500 µm

Movie S1: Movie of an embryo of a cross of *Tg(kdr1: EGFP)mitfa*$^{b692/b692}$ and *Ubi-zebrabow* imaged from about 30 somite stage for 55 h on a confocal microscope using a 10x objective.

Movie S2: Movie of an embryo of a cross of *Tg(mnx:GFP)mitfa*$^{b692/b629}$ and *Ubi-zebrabow* imaged from about 30 somite stage for 55 h on a confocal microscope using a 10x objective.

Movie S3: Movie of an embryo of a cross of *Tg(kdr:enl.memRFP)mitfa*$^{b692/b692}$ and *Tg(mnx:GFP)mitfa*$^{b692/b692}$ imaged from about 2 dpf for 55 h on a confocal microscope using a 20x objective.

Movie S4: **Movie of an embryo of a cross of *HGn39b* and *Ubi-zebrabow* imaged from about 30 somite stage for 55 h on a confocal microscope using a 10x objective.**

DISCUSSION

A mounting method for extended time-lapse confocal microscopy of whole zebrafish embryos is described here. The most critical step for the mounting method is to identify the optimal concentration of agarose that will allow for unrestricted zebrafish embryo growth, and at the same time keep the embryos in a completely fixed position for confocal imaging. Because the optimal concentration of agarose is very narrow, this value is very sensitive to the errors in measurement of the weight of agarose and the volume of E3 during preparation of the solution. The optimal concentration may also depend on temperature and stage of the embryo. Thus, the optimal concentration will need to be re-defined for each new batch of agarose solution through repetition of tests of different concentrations.

Another critical step is in the mounting method is the addition of the second layer of agarose. The second layer holds the cover glass in place. It must be added carefully to the dish a little at a time so that it does not cause the cover glass to move. The second layer also serves as a permeable barrier for E3. Without E3, the embryos will dry out during the imaging. Without the second layer of agarose, the cover glass and the embryo will start floating.

A limitation of the proposed mounting method is that while it works well for inverted microscopes, it does not work for upright microscopes. Several attempts were made to perform time-lapse imaging using an upright microscope, by filling the glass bottom dish with E3, sealing it with parafilm, and turning it upside down. However, often this resulted in that the mounting collapsed halfway through the imaging. This might have been caused by increased heat in the sample after the long exposure to laser light, which causes the solidified agarose layer to melt.

By the end of the time-lapse microscopy the embryos started to show pericardial edema. Varying between different experiments edema was observed between 35 and 50 hours of imaging. Whether this was caused by embryo immobilization, or an effect of anesthetics, is currently not know. Tricaine is known to suppress the contraction of skeletal and cardiac muscles. Consequently, tricaine affects heart rate in adult fish[14] and embryos[15]. Other studies have also reported that tricaine treatment causes pericardial edema in zebrafish embryos[2, 16]. In this article, a concentration of Tricaine (0.16-0.20%) was used that is commonly used in

zebrafish research; still, the pericardial edema occurred by the end of our imaging period. The pericardial edema constituted a main restriction for enabling imaging of the embryos for even longer periods of time. Potential ways to decrease this cardiac toxicity are combining two different anesthetics, such as tricaine with eugenol, or using α-bungarotoxin mRNA injection for anesthetics[16]; beneficial effects of combinatorial or alternative anesthetizing compounds need to be further investigated for extended time-lapse imaging of zebrafish development.

In conclusion, the described mounting method is fast, easy, cost-effective and works on any inverted microscope. Regular glass bottom dishes and low melting agarose can be used, and no special molds, equipment or instrumentation are required. The layered mounting method allows for embryo growth while at the same time keeping the embryos in a fixed position. By using extended time-lapse imaging of whole organism new knowledge of tissue development can be obtained.

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DISCLOSURES

The authors have nothing to disclose.

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Figure 1

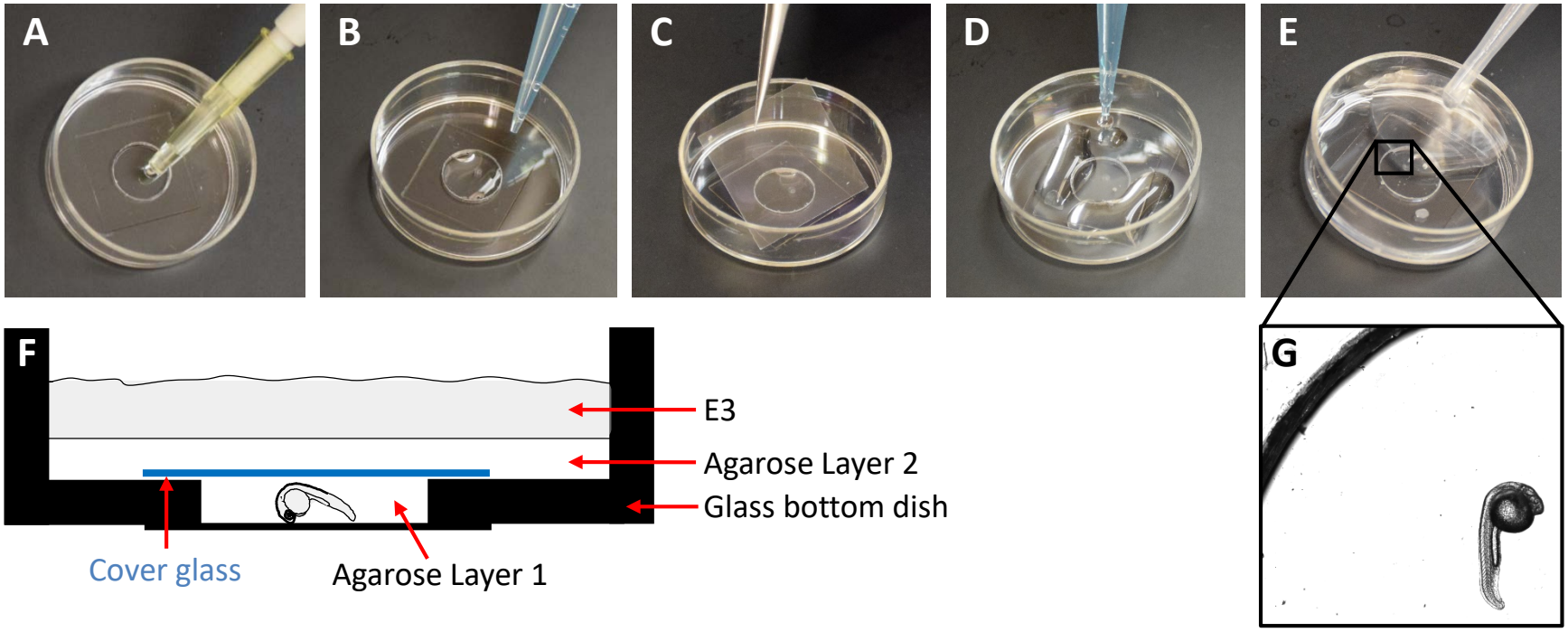

Figure 2

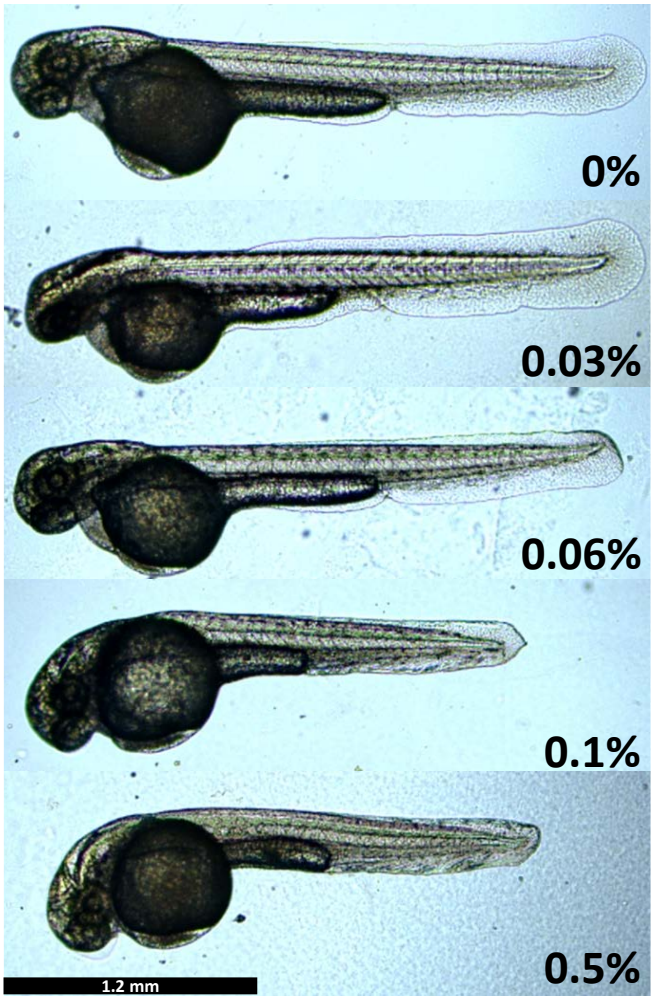

Figure 3

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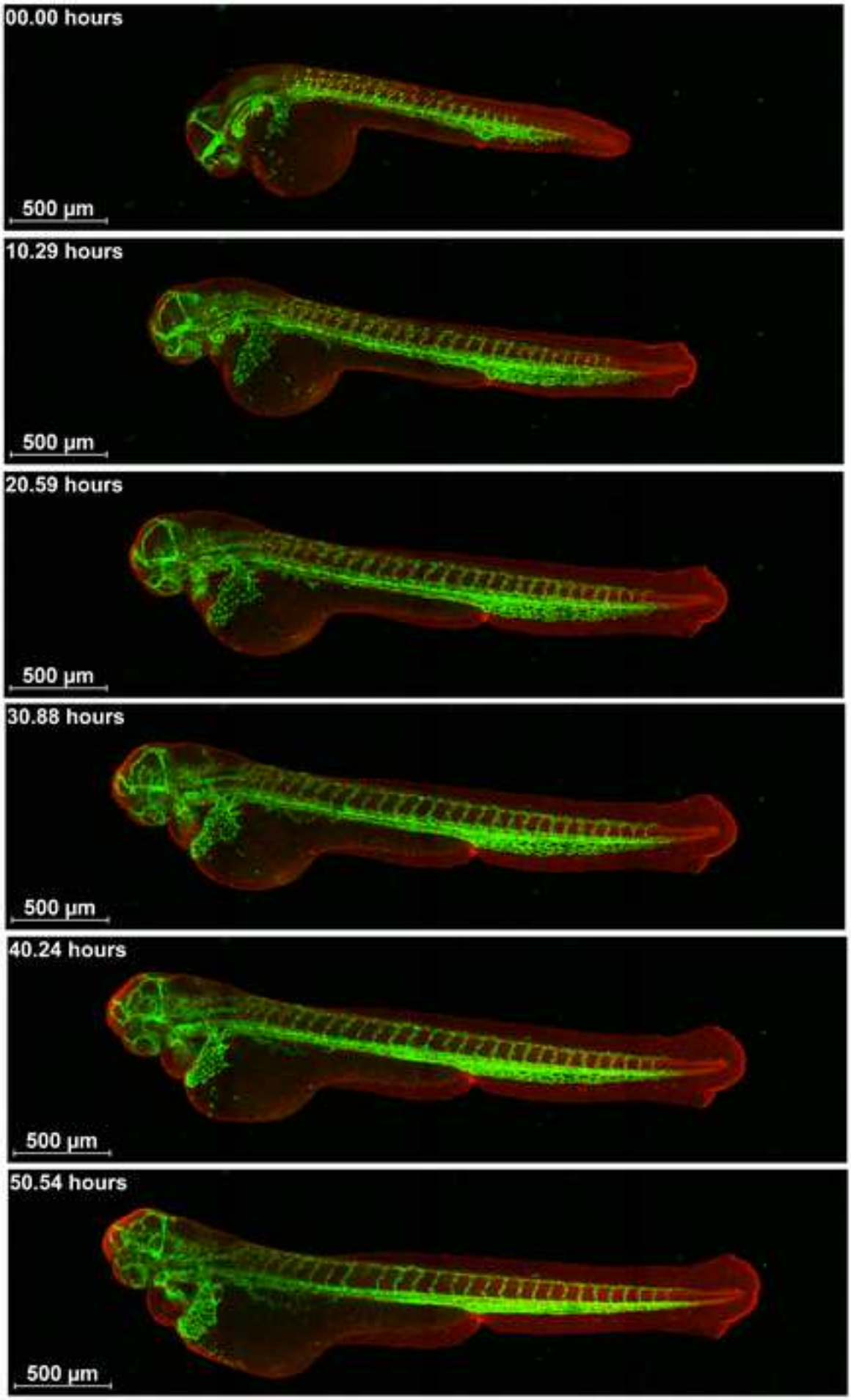

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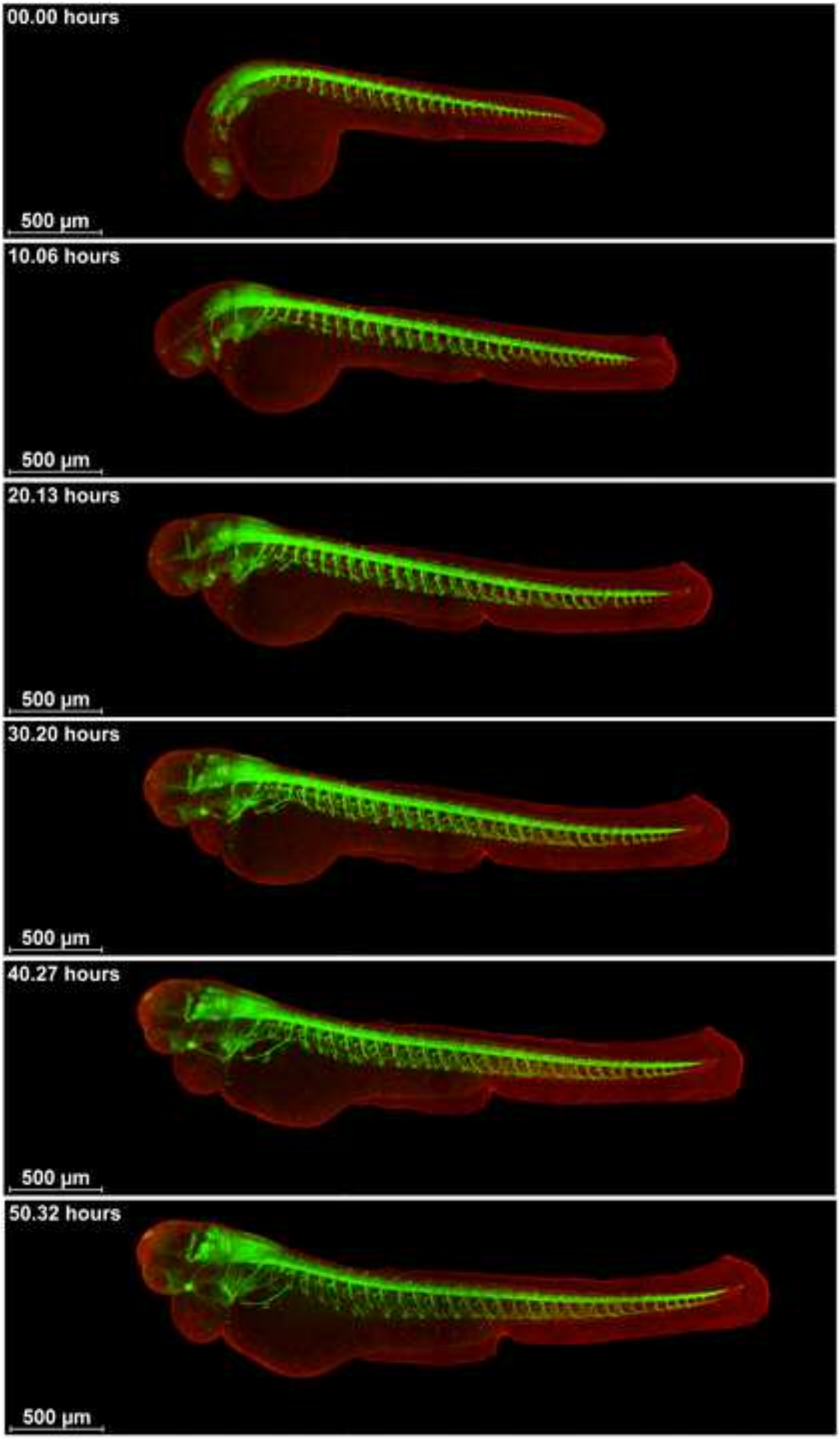

Figure 5

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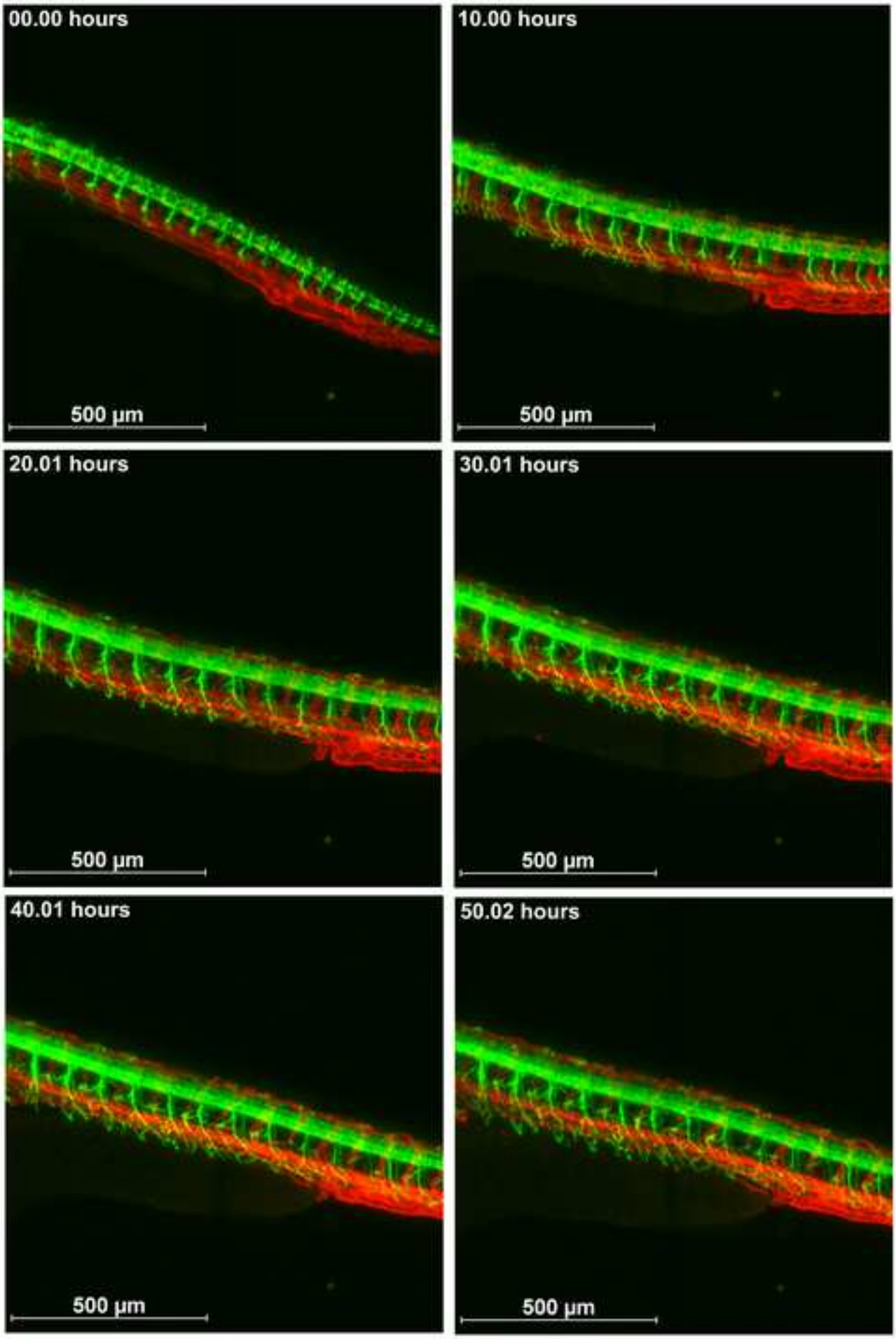

Figure 6

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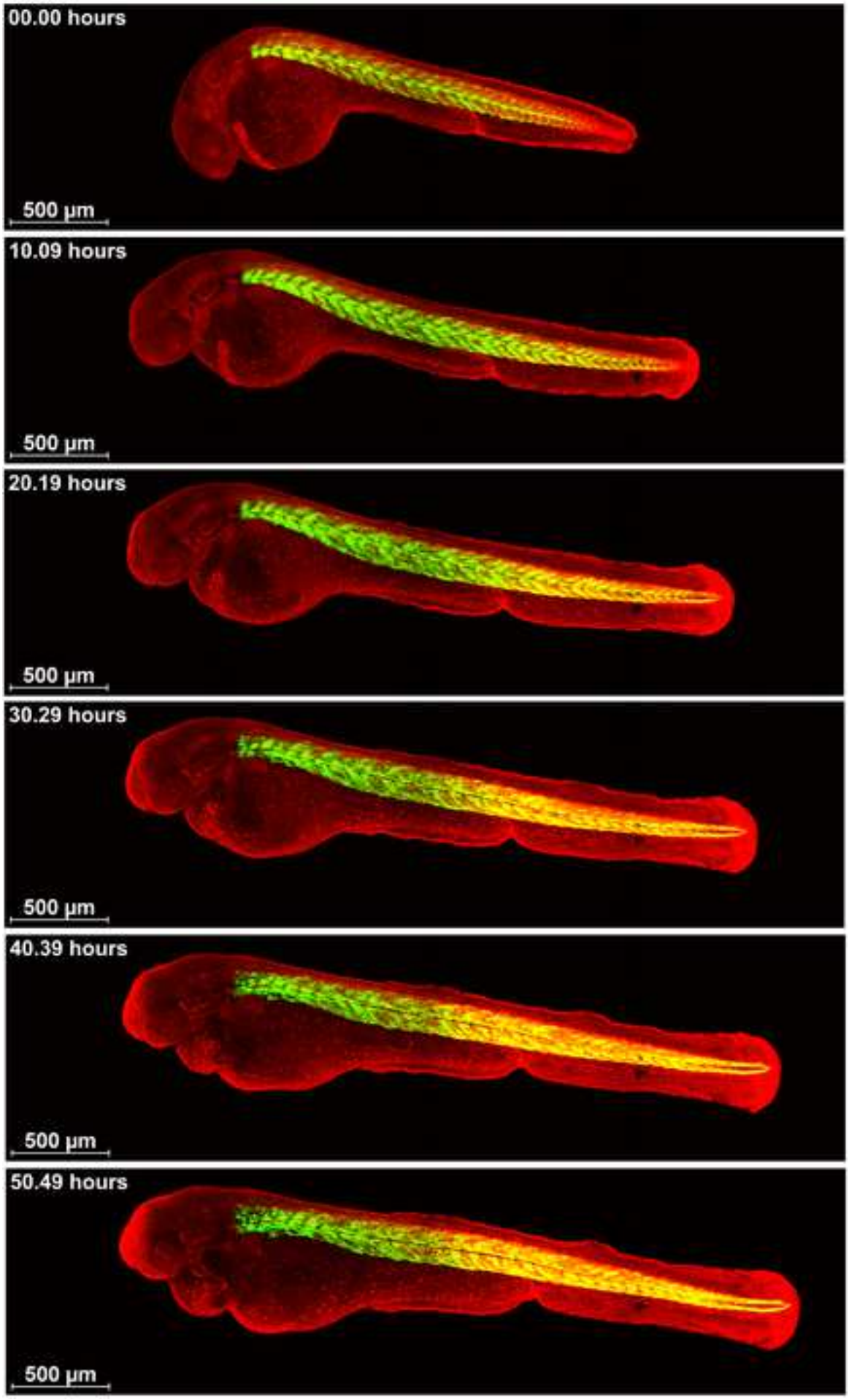

Video 1

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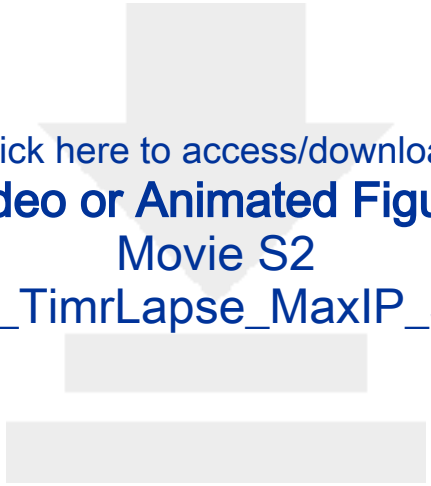
Video or Animated Figure

Movie S1 Vasculature_TimeLpase_MaxIP_55Hours.mp4

Video 2



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Video or Animated Figure

Movie S2

MotorNeuron_TimrLapse_MaxIP_55Hours.mp4

Video 3

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Video or Animated Figure

Movie S3 MNXxKDR_TimeLapse_MaxIP_55Hours.mp4

Video 4

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Video or Animated Figure

Movie S4 Somites_TimeLapse_MaxIP_55Hours.mp4

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Low melting agarose	Sigma-Aldrich, MO	A9414	Store dissolved solution at 4°C
35 mm glass bottom dishes with No. 0 coverslip and 10 mm diameter of glass bottom	MatTek Corporation, MA	P35GCOL-0-10-C	
Tricaine (MS-222)	Sigma-Aldrich, MO	E10521	Store dissolved solution at 4°C
N-phenylthiourea (PTU)	Sigma-Aldrich, MO	P7629	Store dissolved solution at -20°C
Micro cover glass 22x22 mm	VWR	48366 067	
Leica DMi8 fluorescence microscope	Leica	NA	
LAS X software	Leica	NA	Microscope software
DMC4500 digital microscope camera	Leica	NA	
Nikon A1S confocal microscope	Nikon Instruments Inc.	NA	
Nikon NIS AR Version 4.40	Nikon Instruments Inc.	NA	Microscope software

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Title of Article:	LAYERED MOUNTING METHOD FOR EXTENDED TIME-LAPSE CONFOCAL MICROSCOPY OF WHOLE ZEBRAFISH EMBRYOS
Author(s):	Sanat Upadhyay, Leoncio Vergar, Pranjali Sha, Jan-Åke Gustafsson, Ioannis Kakadiaris, Maria Bondesson

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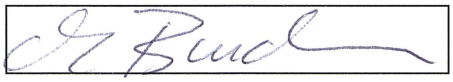
Signature:	
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Replies to Reviewers' comments

We would like to sincerely thank the Reviewers for very helpful comments. All reviewers criticized Figure 1, and thus this figure has been completely re-made with photographs. Please, see our detailed responses below to each comment (in bold). Referral to line numbers are according to the version with track changes.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
Manuscript Number: JoVE60321 Full Title: LAYERED MOUNTING METHOD FOR EXTENDED TIME-LAPSE CONFOCAL MICROSCOPY OF WHOLE ZEBRAFISH EMBRYOS
The major issue with this paper is the ambiguity of the description of preparation of the sample dish (line 147-163) and the diagram of it in figure 1. It appears that the fish and layer 1 of agarose is stuck to the bottom of the petri dish, if so what is supporting them? This needs to be redrawn to better reflect the verbal description and/or additional views of the setup may be needed. It is suggested to add "ranging from 0.025 to 0.04% agarose as optimized below" for layer 1.

We agree with the reviewer and have completely re-made Figure 1 and re-written the text to better describe the procedure. Please, see section 4.

Line 157: please clarify "place a dechorionated embryo laterally": What is really the intent of the word laterally

We intended to say that the embryo should be mounted laying with one of the lateral sides down. This has been clarified in the text (section 4.3).

Line 181: What is meant by "finer grid"?

We meant dilutions of the agarose in finer steps. This has been clarified (section 5.2).

Line 273: It would be helpful to add the maximum length of time, the system could be used without causing issues with pericardial edema.

Pericardial edema was seen after different times in different experiments, from after around 35 to 50 hours of imaging. This information has been added to the text (please, see lines 416-417).

Reviewer #2:
Manuscript Summary:
Upadhyay et al. describe a method for mounting larval zebrafish for few-day live imaging. The paper is well-motivated: immobilizing zebrafish while enabling normal development is challenging, and sharing methods may be useful to a wide range of users. The data are clear. Overall, this is a worthwhile paper. I suggest various revisions, but these should be quite

straightforward.

Major Concerns:
I struggled to understand the geometry of what the authors are trying to describe, and I can't make any sense of Figure 1.

Figure 1 has been remade and we hope the new version is more clear.

Regarding: "add the first agarose solution to the small well created by the cover slip in the bottom of the dish" -- it first needs to be stated that this method makes use of glass-bottom dishes, and that the coverslip-based bottom creates a shallow well in the dish.

Yes, we agree. This has been added to the text. Please, see section 4.1.

In Figure 1: How is "Agarose Layer 1" magically suspended below the chamber bottom (presumably the thick black line)?

The layer 1 agarose is suspended before the cover glass is added. Please, see the new figure 1.

"Cover the small well with a glass cover slip." What small well? The whole glass chamber bottom?

Please, see the new figure 1.

A better diagram, and a photo, are necessary. I realize there will be a video, but the figures should be capable of standing on their own.

Yes, we agree with the reviewer. Please see the new figure 1.

What is the distance between the two coverslips?

It is approximately 1.2 mm. This information has been added to section 4.2.

It's also unclear what the point is of the second agar layer -- is it simply to hold the first layer and coverslip in place? Do its properties matter? The authors should elaborate on this -- what happens if the upper agar concentration is raised or lowered?

The function of the second layer is to hold the coverslip in place and to serve as a permeable barrier for the E3. Without E3, the embryos will dry out during the imaging. Without the second layer of agarose, the coverslip will start floating and layer 1 becomes diluted. This information has been added to lines 401-405.

Combining the last two points, I think the authors' method is basically keeping the larvae in a weak gel together with physical constraint provided by the short spacing between coverslips. This is fine -- it's a good idea -- but it should be stated and discussed.

Yes, this is correct. Please, see section 4.5.

Minor Concerns:
The authors' discussion of existing mounting methods is very good. The critiques of FEP tubing are inaccurate, however. The authors write: "However, only one embryo at a time can be visualized, and SPIM microscopes are not yet available for every lab." (1) One can stack embryos in FEP tubing. This is illustrated in Figure 4 of "High-speed panoramic light-sheet microscopy reveals global endodermal cell dynamics" by Schmid et al. (2) The lack of availability of SPIM microscopes is irrelevant to the *mouting* method, as one could of course use FEP tubing for confocal, widefield, or other microscopies. A better critique of FEP tubing is that its performance is very sensitive to the type of FEP tubing used, though I don't know if this criticism is documented. Also, it would be hard to make an array of many specimens, as the authors' method is capable of.

We thank the reviewer for this comment and agree with it. We have added the study by Schmid et al. and removed the discussion of availability of SPIMs. Please, see lines 87-90.

Reviewer #3:
Manuscript Summary:
The authors describe a multilayered methodology to hold live zebrafish embryos in a fixed position for video and time-lapse microscopy. This method successfully holds embryos in a fixed position for up to 55 hr despite the extensive growth that occurs during that period and is superior to other methods. The technique will be of broad interest in the zebrafish community (and possibly even for those who work on Xenopus).

Major Concerns:
I have two concerns. First, the method section on how to perform the two layered mounting technique is unclear. Figure 1 shows the chamber holding the zebrafish embryos underneath the glass dish, but this does not make much sense. The description of the mounting in 4.1, 4.2 and 4.3 is confusing. "Add the first agarose solution to the small well created by the cover-slip in the bottom of the dish". I think means, put a coverslip in the bottom of the dish, then add the first agarose solution to create the well. If not, then I have no idea how this well gets created in the first place.

We agree with the reviewer that this was not clearly described, and have modified the text (as described above) and improved figure 1.

My second concern is really more of a question. Does this method work for embryos at earlier stages? This utility of this method would be extended if it could be used to video embryos throughout the gastrulation stages.

We are currently trying to optimize this mounting method for imaging of early embryogenesis, and we have successfully imaged embryos from the shield stage (about 6 hpf). One problem we are facing is that addition of tricaine to the very early embryo slows down the development, but without it the embryo starts to twitch.

Minor Concerns:
The movies all have a green tinge. It might be better to show them in black and white, if possible.

Because the movies were taken at dual wavelengths in double transgenic fish (GFP and RFP), we have kept the color in the movies.

Reviewer #4:
LAYERED MOUNTING METHOD FOR EXTENDED TIME-LAPSE CONFOCAL MICROSCOPY OF WHOLE ZEBRAFISH EMBRYOS
Sanat Upadhyay et al

In this manuscript, the authors present their useful optimization of the mounting of zebrafish embryos in low melting point agarose. The approach has generated some nice results, and many authors persist in mounting their specimens in a manner that seems certain to alter the very processes they wish to investigate. The manuscript uses a very nice system for showing that the mounting does not perturb development, as the labeled vasculature system is very revealing of issues. Thus, both the mounting and the diagnostics deployed argue strongly that the manuscript offers something useful and appropriate.

Major Concerns:
There are two things that need to be resolved.
1 - The images of the zebrafish are lovely and numerous, but the images of the actual mounting chamber are far less. The dishes are drawn as a conceptual object and is not an adequate rendering of the real geometries. Real photos and a more complete treatment of some things seem important to the authors, and less so to me. It the coverslip between the agarose layers really required? Is it worth the dramatically reduced amount of transport of oxygen to the fish and waste products away? The details and the variants considered both seem to deserve more weight than the authors give them.

We agree with the reviewer and have changed the text and figure 1 accordingly. The second layer of agarose holds the coverslip in place, but also allows for E3 (and we assume oxygen) to permeate to the embryo, preventing it from drying out. This information has been added to section 4 and the discussion lines 401-405.

2 - The journal does not use the uniqueness of a solution as the motivation for acceptance, making the incomplete discussion of alternatives and the claimed shortcomings annoying to this reviewer. Other tools of laminae of agarose in light sheet imaging other than the one cited are not mentioned. The layered approach of Shih from the 90's is not mentioned. The lovely paper describing molded agarose wells that support long term culture without perturbation from Megason (Methods in Mol Biol, 2009) that shows arraying and good development seems to deserve coverage. The Hirsinger JoVE paper from two years ago seems tagged on.

The introduction has been re-written to meet this criticism. Please, see lines 83-107. The excellent paper by Megason suggested by the reviewer has been added. I am afraid we were not able to identify the paper by Shih. The papers we found by Shih did not cover

mounting for long time imaging.

With the improvement of the balance, tone, and contents needed to resolve these two issues, I would strongly argue for the paper to appear in JoVE.

Replies to editorial comments

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For example: Leica DMi8 automated fluorescence 318 microscope, Nikon A1S Confocal 323 microscope, etc.

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Done

5. Please include an ethics statement before the numbered step of the protocol

Done

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

Done

7. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

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9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

Done

10. Please ensure you answer the “how” question, i.e., how is the step performed?
11. 3.3: How is this done?

Explanation has been added.

12. 6: Please convert this to numbered action steps in the imperative tense. Please include all the button clicks, the knob turns, etc.

Any inverted microscope with time-lapse functionality can be used, so I moved the description of how we did the imaging to the results section. The exact procedure for setting up a time-lapse will vary between microscopes. Please, let me know if this is OK or if you would like me to move it back to the protocol. We can add step-wise description for the Nikon confocal microscope that we used.

13. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of a .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

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All figures are original and not published previously.

16. Did you compare the results with other mounting methods as well?

No, but we describe them in the introduction.

17. What are the markers used for staining? Citation?

No staining was used. We use live transgenic fish that express tissue-specific fluorescence. The references to the fish lines are given. The advantage of using live transgenic fish is now highlighted in the results section.

18. Please include all the Figure Legends together at the end of the Representative Results and before the Discussion section in the manuscript text.

Done

19. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

The discussion has been revised accordingly.

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