Rebuttal for Manuscript Submission to JoVE

**Title:** Visualization of a novel superior ocular sulcus during Danio rerio embryogenesis

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**Manuscript number:** JoVE59259

Dear Reviewers,

We thank the reviewers for their comments and constructive criticisms on the manuscript. All comments were considered carefully, and we have revised the manuscript to address the concerns.

Please note that, unless otherwise noted, the editorial comments from the editor and the minor comments from the reviewers related to spelling, grammar, word choice, and experimental details have been addressed in the revised Word document itself. Please see the annotated changes within the document.

Reviewer #1

Major Concerns:  
*1) The biggest concern with the paper is that the visualization of the superior ocular fissure is being done in the context of failure or late closure of this structure. Especially for the live visualization by stereomicroscopy and DIC (with or without the transgene), it would seem to be more appropriate to describe the process starting at the inception (20 hpf) of the superior fissure until its closure (28 hpf). In addition to detecting mutants which have delayed closure, encompassing the "normal" closure would yield more information regarding the processes involved (in other words are they similar to inferior fissure closure). Although the authors state that the experimental methods can be shifted to any time frame, it does not make a lot of sense as to why they are focusing on delayed closure.*

We have altered the manuscript to be multi-purpose. We have taken additional images of the open sulcus at 22 hpf using DIC and stereo microscopy. This allows us to provide a more complete methodology: also serving the needs of research laboratories interested in detecting the presence of the fissure, aligning its position with gene expression or other cells, and determining molecular treatments that prevent opening of the sulcus. We trust that this addition to the manuscript will make this a more valuable resource for the community.

*2) The protocol states to use PTU to inhibit pigmentation. For inferior fissure closure defects/colobomas, it can be easier to visualize them with pigmentation as the defect is more obvious against the darkly pigmented RPE. Is this not the case for the superior fissure closure, and if that's the case then is the superior fissure not all the way through the optic cup (in other words how deep is the fissure within the optic cup?)*

This is not the case for the superior sulcus. This is due to some pigmentation that appears along the dorsal boundary of the dorsal retina and grows inwards towards the lens. When viewed under the dissecting microscope, the dark pigmentation gives the impression of a superior sulcus closure delay even if the sulcus is closed as it forms a groove-like pattern. The manuscript has now been revised to emphasize and explain this.

Reviewer #2

Major Concerns:  
*1. The title and abstract frame this manuscript as presenting methods to visualize the novel superior ocular fissure. Yet the protocols only address the visualization of an aberrant structure in a mutant embryo (specifically gdf6a mutants). No wild type superior ocular fissure is shown throughout the manuscript for comparison, even when the mutant is shown at a timepoint when control embryos should have a superior ocular fissure (22 hpf; Figure 4). If the goal is strictly to visualize the abnormal structure, that should be reflected particularly in the title.*

Please see rebuttal for Reviewer #1, Major Concern 1.

*2. Throughout the manuscript, clarification is needed with respect to imaging methods and conditions. For example, what are the images in Figures 3 and 4? Were these acquired on compound microscope or via confocal microscopy? What objectives were used? Were these single confocal slices or projections? What image processing was carried out?*

This has been noted and revised within the manuscript.

Reviewer #3

Major Concerns:  
*1) Since this manuscript aims to provide detailed instructions for performing specific procedures, I think the authors should provide all the information and not refer to other papers. Specifically, the embryo mounting procedure mentioned in section 1.2.6 should be described in the text and not referred to the paper by Distel and Koster.*

This has been noted and revised within the manuscript.

*2) Image resolution is not good, at least on the PDF available for the review process. If this is also the resolution available for the published manuscript it is not high enough.*

This has been noted and revised within the manuscript.

Reviewer #4

Major Concerns:  
*Superior Ocular Sulcus (SOS) is not as Superior Ocular Fissure. A sulcus is a groove whereas a fissure implies a break or crack. I know the difference is subtle, but based on what we know of optic cup morphogenesis and the imaging of the SOS to date, sulcus is a better term and should be used to be consistent*

While the authors feel that the structure shares characteristics of both a sulcus and a fissure, the concern is duly noted, and this ambiguity is now addressed within introduction of the manuscript. For consistency with prior publications, we have shifted the nomenclature to sulcus.

*The bias of this reviewer is against double publication of results. This is not a commentary specific to this particular study in JoVE per se, but it is something to consider. It is the view of this reviewer that the purpose of a journal such as JoVE is not simply to show the same data that were presented in another paper with a more detailed methods section, but to highlight a particular method that other scientists would like to do/replicate/extend. In this particular instance, I can appreciate that the precise timing of observing the SOS is something to note, but do the authors not have other images that weren't already published? Can the authors showcase something special about the SOS and the way it is studied relative to other parts of the retina, maybe outlining particular cells around the SOS to highlight morphological changes?*

We want to be absolutely clear that the results that we have included are not (and have not been) published elsewhere. They may originate from similar experiments as those published in *PLOS genetics*, but they are different embryos and/or from different days’ experiments. As to the merits of a methods paper, per se, we find it is essential. Many labs have said that they are unable to see the same structure that we have documented, so we want to ensure that the research community is relying on robust and reliable methodology. For the inferior fissure, this seems unnecessary, but we believe that the superior sulcus merits this special case and therefore the publication of a relevant methods paper.

*In general, it's difficult to know how specific to be with these protocols. It seems that the authors have also struggled with this question as in some cases, very detailed information is given whereas in other places, things (like how one would mount an embryo laterally) are assumed. Perhaps some of these things will be illustrated by the video.*

This has been noted and revised within the manuscript.

Reviewer #5

Major Concerns:  
None

For second round of editorial revisions:

*1. The editor has formatted the manuscript to match the journal's style. Please retain the same.*

Noted.

*2. Please address specific question marked in the manuscript:*

*Maybe the title can be changed to visualization of SOS closure to more clearly reflect the protocol.*

*Also, please explain what is novel about SOS?*

*Please have the title reflect the protocol described in the manuscript.*

Noted and changed. However, we cannot say “closure” as the manuscript is about the observation of the structure through its formation as well as closure.

*In the intro section, please describe why this mutant was chosen for the study. How is this linked to SOS to bring out more clarity.*

Noted and changed

*Include a note stating how would you visually identify these?*

Noted and changed

*Any citation for this?*

This is what we have observed in the lab, and there is no citation for this as we are the only lab that works on SOS.

*Need more clarity with respect to SOS development and closure. Maybe the developmental stages with respect to SOS can be described in the intro.*

This is provided in 1.2.3 and discussed throughout the manuscript.

*Need more clarity with respect to SOS development and closure. Maybe the developmental stages with respect to SOS can be described in the intro.*

Noted and changed.

*Please also explain how the embryo is imaged under the microscope. What is the difference between imaging under dissecting and compound microscope? What magnification? Etc?*

Noted and clarified.

*Please provide the step number.*

Noted and changed.

*In a Petridish?*

Noted and changed.

*What can be used? Please clarify.*

Noted and changed.

*Please check.*

Minutien pin is the correct term. It has been clarified within the document.

*How is this linked to SOS?*

Noted and changed.

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The figures not been taken or modified from any previous publications.

*Please provide scale bars for both the panels in the figure.*

Noted and changed.

*3. Once done please ensure that the highlight is no more than 2.75 pages including headings and spacings.*

Removed some highlighting to ensure 2.75 pages. Please see below.

**1. Protocol 1: Visualization of SOS using stereomicroscopy and DIC imaging**

1.1) Embryo collection

1.1.1) In a tank of dechlorinated water, prepare crosses of *gdf6a+/-* zebrafish in the evening by pairing a male zebrafish with a female zebrafish. Be sure to separate the male from the female by using a divider to ensure that the embryos are born within a small range of time.

1.1.2) The following morning, pull the divider and allow the zebrafish to breed for no longer than 30 min. Collect the embryos in Petri dishes with E3 media, described in The Zebrafish Book12, and place them in a 28.5°C incubator.

1.1.3) Remove any unfertilized eggs or dead embryos, which will appear white and opaque.

1.2) Preparation and live-imaging of zebrafish embryos

1.2.1) At 20 hpf, replace the E3 media with E3 media containing 0.004% 1-phenyl 2-thiourea (PTU) to prevent pigment production.

1.2.2) Ensure that all embryos are at the correct developmental stages at various points leading up to the time of observation.

1.2.3) Place the embryos under a dissecting microscope, and dechorionate the embryos by gently pulling apart the chorion using fine forceps. Visualize the SOS in the dorsal eye. The SOS may appear as an indentation at the dorsal margin of the eye, and a line should be visible across the dorsal eye. For normal SOS closure, observe the embryos at around 20-23 hpf. For examination of delayed SOS closure phenotypes, observe the embryos at 28 hpf or later.

1.2.4) Sort the embryos that show SOS closure delay from those that do not.

1.2.5) To photograph these embryos using a dissecting microscope, prepare a Petri dish containing 1% agarose in E3. Lightly prick the centre of the agarose to create a shallow hole in which the yolk of the embryo can sit when the embryo is placed on the agarose.

1.2.7) To image the embryos using a compound or confocal microscope, transfer the embryo into 35 mm Petri dish containing a small bolus of non-gelled 1% low-melting point agarose in E3 (w/v). Quickly position the embryo laterally using a fine fishing line or an eyelash and wait for the agarose to cool. Once the agarose is firm, pour enough E3 into the dish to cover the agarose.

1.2.8) Use a water immersion 20X objective lens to visualize the SOS with a compound microscope.

**2. Protocol 2: Whole-mount immunofluorescent staining of laminin**

2.1) Whole-mount immunofluorescent staining of laminin: Day 1

* + 1. Dechorionate embryos as described in Step 1.2.3, if not already done. Fix embryos in a microcentrifuge tube at the desired timepoint in freshly made4% PFA for 2 h on a room temperature (22-25°C) shaker. Wash in 1X PBST for 5 min, four times.

2.1.2) Permeabilize embryos in 10 μg/mL proteinase K at room temperature for 5 min.

2.1.3) Wash in 1X PBST for 5 min, four times.

2.1.4) Block embryos in a solution of 5% goat serum and 2 mg/mL bovine serum albumin (BSA) in 1X PBST for 1-2 h on a room temperature shaker.

2.1.5) Prepare primary antibody solution by diluting rabbit anti-laminin antibody in block solution at a 1:200 dilution.

2.1.6) Incubate the embryos in anti-laminin primary antibody (L9393, Millipore Sigma) overnight on a 4°C shaker.

2.2) Whole-mount immunofluorescent staining of laminin: Day 2

2.2.1) Wash in 1X PBST for 15 min, five times.

2.2.2) Prepare secondary antibody solution (ab150077, Abcam) by diluting goat anti-rabbit Alexa Fluor 488 antibody in 1X PBST to a dilution of 1:1000.

2.2.3) Incubate the embryos in secondary antibody overnight on a 4 °C shaker. Shield from light as much as possible from this step onwards.

2.2.4) Wash in 1X PBST for 15 min, four times. The embryos can be stored at 4 °C for up to a week, if necessary.

2.3) Dissection and mounting of embryonic eyes

2.3.1) If desired, place the embryos in a small Petri dish and deyolk the embryos in 1X PBST. Do this by gently disrupting the yolk with fine forceps and removing the yolk cells through mild scraping of the yolk sac.

2.3.2) Prepare the following concentrations of PBS-glycerol series solutions in microcentrifuge tubes: 30%, 50%, and 70% glycerol in PBS. Transfer embryos into 30% glycerol/PBS, making sure to place the embryos on top of the solution and transferring as little of the previous solution as possible.

2.3.3) When embryos have sunk to the bottom, transfer them to 50% glycerol/PBS. Repeat and transfer to 70% glycerol/PBS.

2.3.4) Once the embryos have sunk in 70% glycerol/PBS, move them to a small plastic dish for dissections.

2.3.5) Sever the embryo posterior to the hindbrain, and use the posterior tissue for genotyping, if necessary.

2.3.6) Move the head to a glass slide, transferring as little glycerol as possible. Use a fine minutien pin (26002-10, Fine Science Tools) or other fine dissection tools to gently insert into the forebrain ventricle from the anterior and push downward to separate the right and left halves of the head from each other. This minimizes manual manipulation of the eye and surrounding tissue, thereby leaving the SOS undamaged.

2.3.7) Mount each side of the head midline down, eye up.

2.3.8) Image samples within a day, or seal around the coverslip with nail polish and image samples only after the nail polish has dried. Store in the dark at 4°C.

**3. Protocol 3: Visualization of SOS using eGFP-CAAX mRNA**

3.4) Injection and visualization

3.4.1) Obtain embryos as outlined in Protocol 1.1.

3.4.2) Using a microinjection apparatus, inject 300 pg of eGFP-CAAX mRNA at the 1-cell stage.

3.4.3) Screen for embryos with bright expression of eGFP in the eyes using a fluorescence stereoscope.

3.4.4) Image the embryos as described in Protocol 1.2.

3.4.5) Alternatively, dechorionate and fix the embryos at the desired timepoint in 4% PFA for 4 hours at room temperature or overnight at 4°C. Dissect the eyes and mount them on slides as described in Protocol 2.3.