Journal of Visualized Experiments

Visualization of the superior ocular sulcus during Danio rerio embryogenesis

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59259R2
Full Title:	Visualization of the superior ocular sulcus during Danio rerio embryogenesis
Keywords:	Eye development, coloboma, superior fissure, superior ocular sulcus, choroid fissure, zebrafish, ocular anatomy, superior coloboma, atypical coloboma, BMP signaling, dorsoventral axial patterning, dorsal eye
Corresponding Author:	Andrew J Waskiewicz University of Alberta Edmonton, Alberta CANADA
Corresponding Author's Institution:	University of Alberta
Corresponding Author E-Mail:	aw@ualberta.ca
Order of Authors:	Kevin H Yoon
	Sonya A Widen
	Melissa M Wilson
	Jennifer C Hocking
	Andrew J Waskiewicz
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Edmonton, Alberta, Canada

Cover Letter

UNIVERSITY OF ALBERTA

Department of Biological Sciences
Women & Children's Health Research Institute
Neuroscience and Mental Health Institute

Andrew J. Waskiewicz, Ph.D.
https://hocking.biology.ualberta.ca/labs/waskiewicz/
CW405 Biological Sciences Building
Edmonton, Alberta, Canada T6G 2E9
Tel: 780.492.4403
Fax: 780.492.9234
aw@ualberta.ca

Oct 12, 2018

Editor
JOVE

Dear Editor,

I would appreciate your consideration of our manuscript (*Visualization of a novel superior ocular fissure during Danio rerio embryogenesis*), for publication as a Research Article in *JOVE*. Our research presents a methodological follow-up to our recently published work in *PLOS Genetics* (Morphogenetic defects underlie Superior Coloboma, a newly identified closure disorder of the dorsal eye, Hocking et al. 2018).

Our previous study began with the identification of eight patients with superior coloboma, a congenital ocular anomaly characterized by gaps in tissues of the superior iris, retina or lens. The similarity to coloboma (a disorder caused by failure to close the inferior ocular fissure), suggested the hypothesis that superior coloboma represented a new embryonic fissure disorder. Yet, existing models of vertebrate eye development describe a continuous dorsal optic cup. Following up 2 reports from more than 20 years ago, we revisted morphogenesis of the dorsal eye, and identified an anatomical structure within the dorsal zebrafish, chick, mouse and newt eye, *the superior fissure*. We provided evidence that failure to close this fissure results in superior coloboma.

This manuscript describes three separate techniques for examining molecular genetic regulation of superior fissure closure. We suggest that analyses of superior fissure closure delay occur at 28 hpf and that investigators primarily utilize either stereomicroscope observation (protocol 1) or Laminin immunohistochemistry (protocol 2). To complement such work and visualize the cellular dynamics of fissure closure we present a method for injecting zebrafish with membrane labeled GFP (protocol 3).

The main purpose for the existence of this manuscript is to standardize protocols such that examinations of superior fissure closure will take place using similar methodologies in multiple laboratories. As this field is just beginning and we have recently identified new patients, we think this will serve as an essential tool to an emerging research area.

Yours sincerely,

Dr. Andrew J. Waskiewicz Ph.D.
Professor, Department of Biological Sciences

TITLE

Visualization of the superior ocular sulcus during *Danio rerio* embryogenesis

AUTHORS AND AFFILIATIONS

Kevin H Yoon[1], Sonya A Widen[1,2], Melissa M Wilson[1], Jennifer C Hocking[2,3,4,5], Andrew J Waskiewicz[1,2,6]

[1]Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada
[2]Women & Children's Health Research Institute, University of Alberta, Edmonton, Canada
[3]Division of Anatomy, Department of Surgery, University of Alberta, Edmonton, Canada
[4]Department of Cell Biology, University of Alberta, Edmonton, Canada
[5]Department of Medical Genetics, University of Alberta, Edmonton, Canada
[6]Neuroscience and Mental Health Research Institute, University of Alberta, Edmonton, Canada

Corresponding Author:
Andrew J Waskiewicz (aw@ualberta.ca)

Email addresses of Co-authors:
Kevin H Yoon (kyoon@ualberta.ca)
Sonya A Widen (widen@ualberta.ca)
Melissa M Wilson (mmwilson@ualberta.ca)
Jennifer C Hocking (jhocking@ualberta.ca)

KEYWORDS

Eye development, coloboma, superior fissure, superior ocular sulcus, choroid fissure, zebrafish, ocular anatomy, superior coloboma, atypical coloboma, BMP signaling, dorsoventral axial patterning, dorsal eye

SUMMARY

Here, we present a standardized series of protocols to observe the superior ocular sulcus, a recently-identified, evolutionarily-conserved structure in the vertebrate eye. Using zebrafish larvae, we demonstrate techniques necessary to identify factors that contribute to the formation and closure of the superior ocular sulcus.

ABSTRACT

Congenital ocular coloboma is a genetic disorder that is typically observed as a cleft in the inferior aspect of the eye resulting from incomplete choroid fissure closure. Recently, the identification of individuals with coloboma in the superior aspect of the iris, retina, and lens led to the discovery of a novel structure, referred to as the superior fissure or superior ocular sulcus (SOS), that is transiently present on the dorsal aspect of the optic cup during vertebrate eye development. Although this structure is conserved across mice, chick, fish, and newt, our current understanding of the SOS is limited. In order to elucidate factors that contribute to its formation and closure, it is imperative to be able to observe it and identify abnormalities, such as delay in the closure of the SOS. Here, we set out to create a standardized series of protocols that can be used to

efficiently visualize the SOS by combining widely available microscopy techniques with common molecular biology techniques such as immunofluorescent staining and mRNA overexpression. While this set of protocols focuses on the ability to observe SOS closure delay, it is adaptable to the experimenter's needs and can be easily modified. Overall, we hope to create an approachable method through which our understanding of the SOS can be advanced to expand the current knowledge of vertebrate eye development.

INTRODUCTION

The formation of the vertebrate eye is a highly conserved process in which carefully orchestrated intercellular signaling pathways establish tissue types and specify regional identity[1]. Perturbations to early eye morphogenesis result in profound defects to the architecture of the eye and are frequently blinding[2]. One such disease results from the failure to close the choroid ocular fissure in the ventral side of the optic cup[3]. This disorder, known as ocular coloboma, is estimated to occur in 1 out of 4-5000 live births and cause 3-11% of pediatric blindness, commonly manifesting as a keyhole-like structure that protrudes inferiorly from the pupil in the center of the eye[4-6]. The function of the choroid fissure is to provide an entry point for early vasculature growing into the optic cup, after which the sides of the fissure will fuse to enclose the vessels[7].

While ocular coloboma has been known since ancient times, we have recently identified a novel subset of coloboma patients with tissue loss affecting the superior/dorsal aspect of the eye. Recent work in our lab has led to the discovery of an ocular structure in the zebrafish dorsal eye, which we refer to as the superior ocular sulcus (SOS) or superior fissure[8]. It is important to note that the structure has characteristics of both a sulcus and a fissure. Similar to a sulcus, it is a continual tissue layer that spans from the nasal to the temporal retina. In addition, the closure of the structure is not mediated by a fusion of the two opposing basement membrane, and it appears to require a morphogenetic process by which the structure is populated by cells. However, similar to a fissure, it forms a structure that separates the nasal and temporal sides of the dorsal eye with the basement membrane. For consistency, we will refer to it as SOS in this text.

The SOS is evolutionarily conserved across vertebrates, being visible during eye morphogenesis in fish, chick, newt, and mouse[8]. In contrast to the choroid fissure, which is present from 20-60 hours post-fertilization (hpf) in zebrafish, the SOS is highly transient, being easily visible from 20-23 hpf and absent by 26 hpf[8]. Recent research in our lab has found that, similar to the choroid fissure, the SOS plays a role in vascular guidance during eye morphogenesis[8]. Although the factors that control the formation and closure of the SOS are not yet fully understood, our data did highlight roles for dorsal-ventral eye patterning genes[8].

Zebrafish is an excellent model organism to study the SOS. As a model system, it provides a number of advantages in studying eye development: it is a vertebrate model; each generation exhibits high fecundity (~200 embryos); its genome has been fully sequenced, which facilitates genetic manipulation; and approximately 70% of human genes have at least one zebrafish orthologue, making it an ideal genetics-based model of human disease[9,10]. Most importantly, its

development takes place externally to the mother, and its larvae are transparent, which allows for the visualization of the developing eye with relative ease[11].

In this set of protocols, we describe the techniques through which the SOS can be visualized in zebrafish larvae. The variety of visualization techniques used in this report will allow clear observation of the SOS during normal eye development, as well as the ability to detect SOS closure defects. Our example protocols will feature investigations of Gdf6, a BMP localized to the dorsal eye and known regulator of SOS closure. Further, these techniques can be combined with experimental manipulations to identify genetic factors or pharmacological agents that affect proper SOS formation and closure. In addition, we have included a protocol through which the fluorescent imaging of all cell membranes is possible, allowing the experimenter to observe morphological changes to the cells surrounding the SOS. Our goal is to establish a set of standardized protocols that can be used throughout the scientific community to offer new insights into this novel structure of the developing eye.

PROTOCOL

All methods described here have been approved by the University of Alberta Animal Care and Use Committee.

1. Protocol 1: Visualization of SOS using stereomicroscopy and differential interference contrast (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 Book[12], 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.

NOTE: Addition of PTU at a slightly later timepoint, such as 22-24 hpf, is unlikely to interfere with the experiment due to the early age of the embryos at the time of imaging. However, it is recommended to treat the embryos early to completely prevent pigmentation as there is a band of pigmentation that appears in the dorsal eye, which can interfere with the imaging of the SOS.

1.2.2) Ensure that all embryos are at the correct developmental stages at various points leading up to the time of observation. It is recommended that this is done at the stages at which somite number is clearly visible as outlined by Kimmel et al.[13]. Remove those that are developmentally immature.

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 center 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. This will ensure that the embryo is not at an oblique angle when being photographed.

1.2.6) Anesthetize embryos with 0.003% tricaine in E3 and place laterally 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. For more details, see Distel and Köster[14].

NOTE: If using an inverted microscope, the embryo can be placed against the glass of a glass-coverslip-bottom dish and imaged with a standard 20X objective lens.

1.2.8) Use a water immersion 20x objective lens to visualize the SOS with a compound microscope. Following visualization, gently pull the agarose from the embryos and fix in 4% paraformaldehyde (PFA) or allow to continue their development.

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

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

2.1.1) 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 made 4% PFA for 2 h on a room temperature (22-25 °C) shaker. Wash in 1x PBST for 5 min, four times.

NOTE: Following gastrulation, embryos may fix better after dechorionation.

2.1.2) Permeabilize embryos in 10 μg/mL proteinase K at room temperature for 5 min. Incubation time will depend on the developmental stage at which the embryos are fixed (see Thisse and Thisse[15]).

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 1xPBST 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 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 by diluting goat anti-rabbit Alexa Fluor 488 antibody in 1x PBST to a dilution of 1:1000.

NOTE: It is possible to adapt this step to suit the resources available to the experimenter by using a different secondary antibody.

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. Wait for the embryos to sink to the bottom of the tube.

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 forceps or other fine dissection tools to hold onto the posterior end to keep the head stationary. Use a fine minutien pin 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. Repeat this while moving posteriorly through the midbrain and into the hindbrain ventricle, essentially fileting the head down the midline. 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. Position four posts of vacuum grease at the corners (an appropriate distance apart for the coverslip being used) and cover with a glass coverslip, pushing down sequentially on each post until the coverslip makes contact with the samples. Pipette 70% glycerol at the edge of the coverslip so that the glycerol is pulled underneath, filling the space between the coverslip and the slide.

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

3.1) Synthesis of eGFP-CAAX mRNA

3.1.1) Linearize 1 μg of pCS2-eGFP-CAAX plasmid[16] with NotI in a reaction volume of 40 μL for 4 hours at 37 °C.

3.1.2) To stop the restriction digest reaction, add 10 μL RNase-free water, 2.5 μL 10% SDS and 2.0 μL 10 mg/mL Proteinase K.

3.1.3) Incubate 1 hour at 50 °C.

3.1.4) Add the following to the reaction (total volume 200 μL) and proceed to next step: 50 μL RNase-free water, 20 μL 3 M sodium acetate pH 5.2 and 75.5 μL RNase-free water

NOTE: RNase-free water is added in two separate occasions to prevent excessive dilution of the sodium acetate.

3.2) Purification of DNA through phenol/chloroform extraction and ethanol precipitation

3.2.1) Add 200 µL phenol:chloroform:isoamyl alcohol and vortex for 20 s. Separate the aqueous and organic phases through centrifugation at 18,000 x g for 5 min.

3.2.2) Transfer the upper aqueous layer to a new microcentrifuge tube, making sure to avoid the transfer of the bottom organic layer. Add an equal volume of chloroform to the new tube.

NOTE: Addition of chloroform is optional, but it is recommended to ensure complete removal of phenol from the sample.

3.2.3) Vortex for 20 s. Separate the aqueous and organic phases through centrifugation at 18,000 x g for 5 min.

3.2.4) As before, transfer the upper aqueous layer to a new microcentrifuge tube, making sure to avoid the transfer of the bottom organic layer.

3.2.5) Add 1/10 volume of 3 M sodium acetate pH 5.2.

3.2.6) Precipitate DNA by adding 3 volumes of 100% RNase-free ethanol and chill at -20 °C for 15 min. Centrifuge at 18,000 x g for 20 min at 4 °C. A pellet should be visible. Decant the supernatant.

3.2.7) Wash the pellet with 100 µL of cold 70% ethanol/RNase-free water. After gently mixing to break the pellet loose, centrifuge at 18,000 x g for 15 min at 4 °C. A pellet should be visible. Decant the supernatant.

3.2.8) Air-dry the pellet for 5 min and resuspend the DNA in 7 µL water.

NOTE: The pellet may need to be dried for longer than 5 min depending on the airflow available.

3.3) Transcription and purification of eGFP-CAAX mRNA

3.3.1) In an RNase-free manner, prepare an *in vitro* transcription reaction with a commercially available Sp6 RNA polymerase kit, using about 1 µg of purified linearized plasmid DNA obtained in Step 3.2. Incubate for 2 h at 37 °C.

NOTE: Sp6 RNA polymerase must be used for the production of capped mRNA.

3.3.2) Add 1 µL of DNase (2 U/µL; RNase free) and incubate for 30 min at 37 °C.

3.3.3) Purify the mRNA with any commercially available RNA purification kit. Aliquot the mRNA to avoid repeated freeze-thaw, and store at -80 °C.

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 the 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. Wash the embryos in 1x PBST for 5 min, four times, and dechorionate, if not previously done. Dissect the eyes and mount them on slides as described in Protocol 2.3.

REPRESENTATIVE RESULTS

The zebrafish SOS appears at 20 hpf in the presumptive dorsal retina[8]. By 23 hpf the SOS transitions from its initial narrow architecture to a wide indentation and by 26 hpf it is no longer visible[8]. Therefore, to examine the SOS during normal zebrafish eye development, the embryos must be observed between 20-23 hpf. During this period, the SOS is observable through the dissecting microscope and via DIC imaging as a thin line in the dorsal eye that separates the nasal and temporal halves of the developing retina (**Figure 1**). In addition, a subtle indentation may be visible in the dorsal boundary of the eye (**Figure 1**). Following immunofluorescent staining of laminin, the thin line can be confirmed to be the basement membrane (**Figure 1**).

To examine molecular pathways resulting in delayed SOS closure, we chose to observe the embryos at 28 hpf as this is a timepoint that is sufficiently removed from the time of normal SOS closure and is, therefore, a reliable marker of SOS closure delay due to experimental manipulations. Through direct visualization of 28 hpf zebrafish under the dissecting microscope, it is possible to evaluate SOS closure delay due to experimental manipulation. When SOS closure is delayed, its prolonged presence can be seen as a pronounced cleft in the dorsal side of the eye under the dissecting microscope (**Figure 2**). When observed under the compound microscope using DIC or Nomarski optics, this feature is even more prominent, and the nasal and temporal sides of the eye are separated by the SOS, which is clearly visible as a line in the dorsal eye (**Figure 3**).

The SOS is lined with basal lamina components, including laminin. Therefore, immunofluorescent staining provides a complementary method of evaluating SOS closure in fixed embryos. When imaging the embryonic eye from a lateral view, the basal lamina demarcates the outside margin of the eye, both ocular fissures, and the border between the lens and the retina (**Figure 4**). The SOS is oriented directly opposite to the choroid fissure in the dorsal aspect of the eye. Whole embryos can be mounted laterally, with somewhat better optical clarity achieved if eyes are previously microdissected. By 28 hpf, in wildtype zebrafish, laminin staining demonstrates clearly that the SOS is completely closed, which makes this the ideal stage for monitoring delays in fissure closure.

Injection of eGFP-CAAX mRNA allows visualization of the cell membranes of a live or fixed embryo (**Figure 5**). Successful one-cell stage injection is sufficient to produce embryos with complete cell membrane fluorescence. In the lateral view, all cellular boundaries should be marked by GFP fluorescence, and as such, cell morphology is also clearly observed. This allows the visualization of the morphological changes to the cells that lead to SOS closure.

FIGURE LEGENDS:

Figure 1: Observation of SOS during normal zebrafish eye development. Zebrafish embryos were collected and imaged at 22 hpf. **A-B.** Lateral view of 22 hpf embryos live-imaged with the dissecting microscope (A) and via DIC imaging (B), respectively. The SOS is marked by a red asterisk. **C.** Laminin immunofluorescent staining of a 22 hpf embryo. Embryos were fixed in 4% PFA and obtained for whole-mount immunofluorescent staining of laminin. The embryos were fileted and mounted in 70% glycerol/PBS. Single slice images were obtained through confocal imaging with a software. The SOS is marked by a white asterisk. All figures were annotated and assembled using Adobe Illustrator software. Scale bars represent 50 μm.

Figure 2: Dissecting microscope images of SOS closure delay in zebrafish larvae. Wildtype and *gdf6a*$^{-/-}$ embryos were collected and live-imaged at 28 hpf. **A.** Lateral view of a wildtype embryo with a closed SOS. **B.** Lateral view of a *gdf6a*$^{-/-}$ embryo with an SOS closure delay (asterisk). A sharp depression is observable in the dorsal aspect of the eye due to the failure of the SOS to close appropriately. Scale bars represent 50 μm.

Figure 3: Representative DIC images of the SOS in the zebrafish embryonic eye. Wildtype and *gdf6a*$^{-/-}$ embryos were collected, anesthetized, and placed laterally in 1% Ultrapure low-melting point agarose in E3 on a 35 mm Petri dish. The dish was filled with E3, and a compound microscope with a 20x water-dipping objective was used for DIC imaging. **A.** Lateral view of a wildtype embryo with a closed SOS. **B.** Lateral view of a *gdf6a*$^{-/-}$ embryo with an SOS closure delay (asterisk). The SOS is observable as a thin line in the dorsal aspect of the eye. Scale bars represent 50 μm.

Figure 4: Representative images of laminin immunofluorescent staining in embryonic zebrafish eye. Wildtype and *gdf6a*$^{-/-}$ embryos were collected and fixed in 4% PFA at 28 hpf. The basal lamina was immunostained, and the embryos were fileted and mounted in 70% glycerol/PBS for confocal imaging. Single slice images were obtained using ZEN software. **A.** Lateral view of a wildtype embryo with a closed SOS. **B.** Lateral view of a *gdf6a*$^{-/-}$ embryo with an SOS closure delay (asterisk). The basal lamina is shown outlining the eye in green, with the SOS visible in the dorsal part of the eye in *gdf6a*$^{-/-}$ embryos. Scale bars represent 50 μm.

Figure 5: Imaging of the zebrafish embryonic eye following eGFP-caax mRNA injection. Wildtype embryos were injected with 300 pg of eGFP-caax mRNA at 1-cell stage. At 22 hpf and 28 hpf, respectively, the embryos were anesthetized and mounted laterally in 1% Ultrapure low-melting point agarose in E3 on a 35 mm Petri dish. A confocal microscope with a 20x water-dipping objective was used for imaging, and single slice images were obtained using a software. **A.** Lateral view of a *gdf6a*$^{-/-}$ embryo at 22 hpf with a visible open SOS (asterisk). **B.** Enlarged

panels of a *gdf6a*$^{-/-}$ embryo at 22 hpf. **C.** Lateral view of a *gdf6a*$^{-/-}$ embryo at 28 hpf with a SOS closure delay. **D.** Enlarged panels of a *gdf6a*$^{-/-}$ embryo at 28 hpf. Scale bars represent 50 μm and 10 μm in Panels A and C, and B and D, respectively.

DISCUSSION:

Here, we present a standardized series of protocols to observe the SOS in the developing zebrafish embryo. To determine closure delay phenotypes, our protocols have focused on the ability to distinguish the separation of two discrete lobes of the dorsal-nasal and dorsal-temporal sides of the eye, similar to techniques used to visualize choroid fissure closure delay phenotypes in the ventral eye.

These visualization techniques can be used in conjunction with a variety of genetic manipulation techniques to study the effects of inhibiting or inducing expression of certain genes to study their roles in the closure of the SOS. We have chosen to demonstrate these protocols using *gdf6a*$^{-/-}$ embryos as we have previously shown that its loss can affect proper closure of the SOS, but the protocols can be used to study the effects of manipulating the expression of any gene as required. It is recommended that any morphological changes to the dorsal eye are studied preliminarily with observations using the dissecting microscope. The other techniques should be used once an initial link is established definitively, as they are more time-consuming and lower throughput.

While the protocols can be easily modified to suit the needs of the experimenter, there are several aspects that must be followed carefully. Because of the transient nature of this structure, it is imperative to ensure that all observed embryos are of the same developmental stage. For our work, we find it important to allow only a small window of breeding time and to periodically sort the embryos throughout early development. The most important step of equalizing stages is at 20 hpf, when you can still accurately count somites (24)[17], and we find this much more reliable than the staging hallmarks that delineate time at 28 hpf. In addition, pigmentation must be inhibited or removed to ensure successful visualization of the structure. We have observed the pigmentation in the eye begins to start around 22 hpf, and there is a pattern of pigmentation in the dorsal eye that can interfere with proper visualization of the SOS. Therefore, it is highly recommended to treat the embryos with PTU prior to pigmentation to ensure successful visualization. Additionally, dissection of the embryonic eye prior to slide mounting without causing damage requires some practice. It is also imperative to laterally mount the eyes as parallel as possible to the slide. It is recommended that the experimenter practices these techniques with extra embryos prior to the experiment.

With the exception of the immunofluorescent staining of the basal lamina, all of the protocols described here can be completed using live embryos. This allows continual visualization of the SOS throughout early embryogenesis, allowing the experimenter to conduct time-lapse studies of the morphological changes involved in the closure of the SOS. In the past, we have used retina-specific transgenes, such as *Tg(rx3:eGFP)*, which marks the neural retina during early development. Although it lacks the ability to visualize cell membranes, the use of *Tg(rx3:eGFP)* has the advantage of not requiring microinjections and has been our primary method of visualizing gross morphological changes to SOS architecture in real-time. That protocol has not

been included here, as similar methods have been discussed previously in this journal[18,19]. However, investigation of cell biological basis of SOS formation and closure will require membrane fluorescent proteins. Specifically, the injection of eGFP-CAAX mRNA allows visualization of the cell membranes around the SOS as seen in Figure 5, which allows us to study the dynamics of cell shape changes in the dorsal eye that are required for proper SOS closure. While eGFP-CAAX can be useful for performing live-imaging of SOS closure, it is made difficult by the presence of the enveloping layer in zebrafish. In addition, care must be taken when analyzing results from mRNA injections because it can result in mosaicism, making it difficult to directly compare embryos based on quantification of eGFP expression strength. This could be ameliorated through the use of transgenic zebrafish lines that fluorescently label cell membranes specifically in the developing retina, such as *Tg(vsx2.2:GFP-caax)*.

One of the challenges of our protocols lies with any treatment that is not fully penetrant. We have previously noted that SOS delays can be seen in about 10% of control embryos at 28 hpf[8], and this underlying presence of embryos with an SOS within any given experimental group could make it difficult to observe subtle effects of experimental manipulation. This could be addressed by blinding the experimenter to reduce experimenter bias and by increasing the number of embryos used within each experimental group to increase the power of the experiment. In addition, the stage of analysis could be shifted to 29-30 hpf.

With this set of protocols, we seek to standardize the way through which SOS closure delays are visualized. The techniques described above have been shown to be reliable in detecting and visualizing SOS closure delays in a variety of experimental settings and are adaptable to the experimenter's needs. While we have used techniques such as scanning electron microscopy or time-lapse imaging of transgenic embryos to visualize the SOS in greater detail, our aim here is to create a standardized set of protocols that are amenable to high-throughput experimental designs to visualize a large number of embryos in a single day with emphasis on the ability to score closure delay phenotypes. In addition to its use with *gdf6a*$^{-/-}$ embryos, we have been able to observe SOS closure delay phenotypes using these visualization techniques alongside pharmacological treatments, morpholino injections, and RNA overexpression studies. As the role of the SOS in eye development is elucidated further through various means, we hope that this standardized set of protocols provide the scientific community a common language through which this novel structure is studied.

ACKNOWLEDGMENTS:
This work was supported by Canadian Institutes of Health Research (CIHR), Natural Sciences and Engineering Research Council (NSERC), Alberta Innovates Technology Futures, and Women and Children's Health Research Institute (WCHRI).

DISCLOSURES:
The authors have no conflicting interests to declare.

REFERENCES:

1. Chow, R.L., Lang, R.A. Early eye development in vertebrates. *Annual Review of Cell and Developmental Biology.* **17**, doi:10.1146/annurev.cellbio.17.1.255 (2001).
2. Slavotinek, A.M. Eye development genes and known syndromes. *Molecular Genetics and Metabolism*. **104** (448-456), (2011).
3. Gregory-Evans, C.Y., Williams, M.J., Halford, S., Gregory-Evans, K. Ocular coloboma: a reassessment in the age of molecular neuroscience. *Journal of Medical Genetics.* **41** (12), doi: 10.1136/jmg.2004.025494 (2004).
4. Onwochei, B.C., Simon, J.W., Bateman, J.B., Couture, K.C., Mir, E. Ocular colobomata. *Survey of Ophthalmolgy*. **45**, 175–194 (2000).
5. Williamson, K.A., FitzPatrick, D.R. The genetic architecture of microphthalmia, anophthalmia and coloboma. *European Journal of Medical Genetics*. **57**, 369–380 (2014).
6. Chang, L., Blain, D., Bertuzzi, S., Brooks, B.P. Uveal coloboma: clinical and basic science update. *Current Opinion in Ophthalmology*. **17**, 447–470 (2006).
7. Kaufman, R., et al. Development and origins of Zebrafish ocular vasculature. *BMC Developmental Biology*. **15** (18), doi:10.1186/s12861-015-0066-9 (2015).
8. Hocking, J.C., et al. Morphogenetic defects underlie Superior Coloboma, a newly identified closure disorder of the dorsal eye. *PLOS Genetics.* **14** (3), doi:10.1371/journal.pgen.1007246 (2018).
9. Lawson, N.D., Wolfe, S.A. Forward and reverse genetic approaches for the analysis of vertebrate development in the zebrafish. *Developmental Cell.* **21** (1), doi:10.1016/j.devcel.2011.06.007 (2011).
10. Howe, K., et al. The zebrafish reference genome sequence and its relationship to the human genome. *Nature*. **496** (7446), doi: 10.1038/nature12111 (2013).
11. Bilotta, J., Saszik, S. The zebrafish as a model visual system. *International Journal of Developmental Neuroscience*. **19**, 621-629 (2001).
12. Westerfield, M. The Zebrafish Book; A guide for the laboratory use of zebrafish (Danio rerio). 5th edition, University of Oregon Press. Eugene, Oregon. (2007).
13. Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F. Stages of embryonic development of the zebrafish. *Developmental Dynamics.* **203**, 253–310 (1995).
14. Distel, M., Köster, R.W. In vivo time-lapse imaging of zebrafish embryonic development. Cold Spring Harbor Protocols. doi: 10.1101/pdb.prot4816 (2007).
15. Thisse, C., Thisse, B. High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nature Protocols*. **3**, 59-69 (2008).
16. Kwan, K.M., Otsuna, H., Kidokoro, H., Carney, K.R., Saijoh, Y., Chien C. A complex choreography of cell movements shapes the vertebrate eye. *Development*. **139**: 359-372, doi: 10.1242/dev.071407 (2012).
17. Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullman, B., Schilling, T.F. Stages of Embryonic Development of the Zebrafish. *Developmental Dynamics*. **203**: 253-310 (1995).
18. Gfrerer, L., Dougherty, M., Liao, E. C. Visualization of Craniofacial Development in the sox10: kaede Transgenic Zebrafish Line Using Time-lapse Confocal Microscopy. *J. Vis. Exp.* (79), e50525, doi:10.3791/50525 (2013).
19. Percival, S. M., Parant, J. M. Observing Mitotic Division and Dynamics in a Live Zebrafish Embryo. *J. Vis. Exp.* (113), e54218, doi:10.3791/54218 (2016).

Figure 1

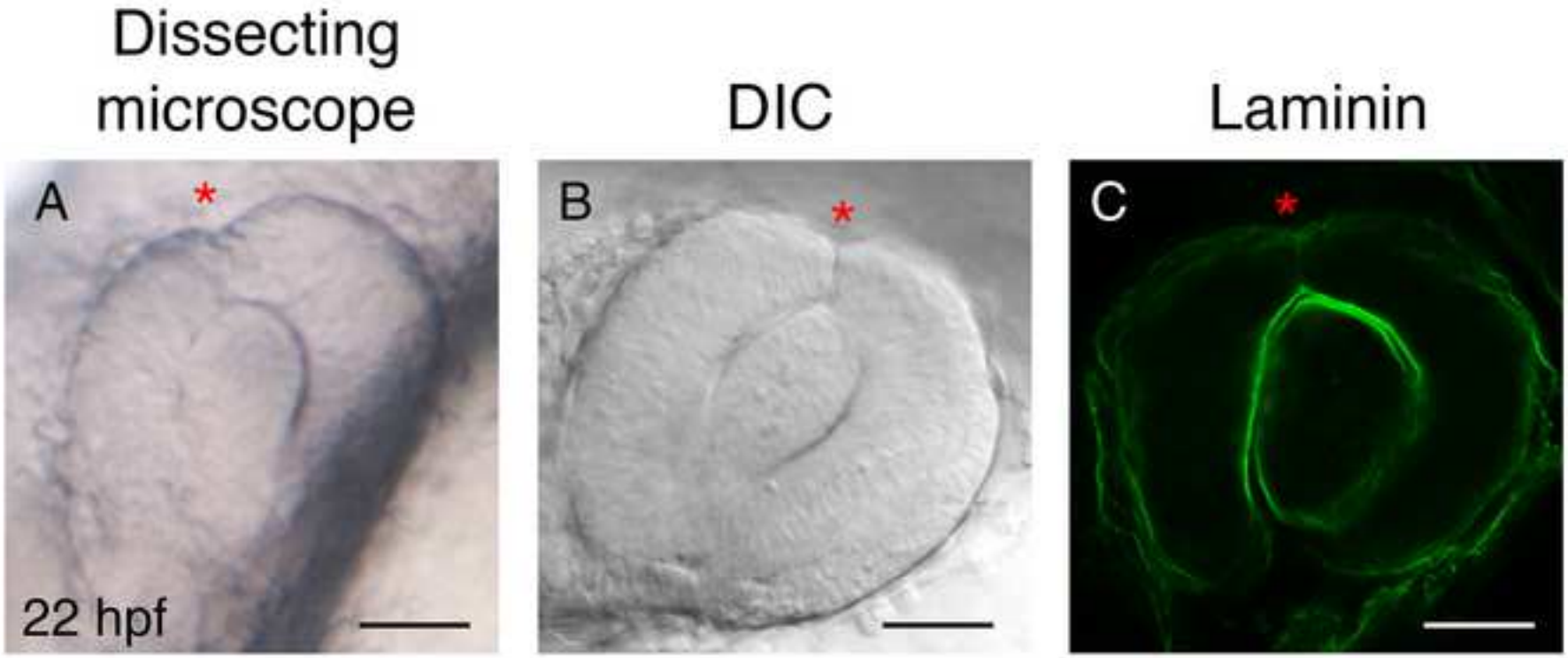


Figure 2

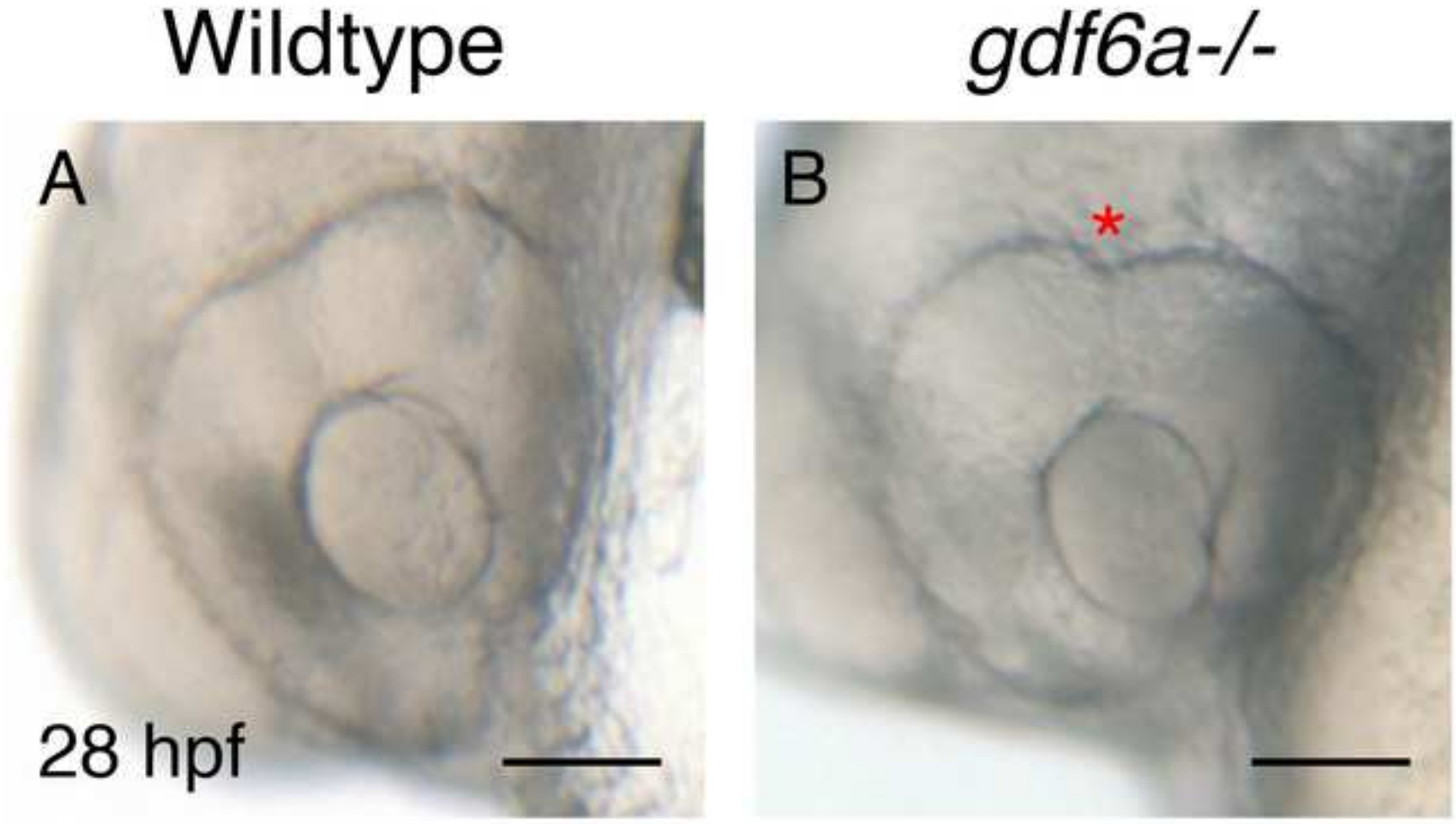


Figure 3

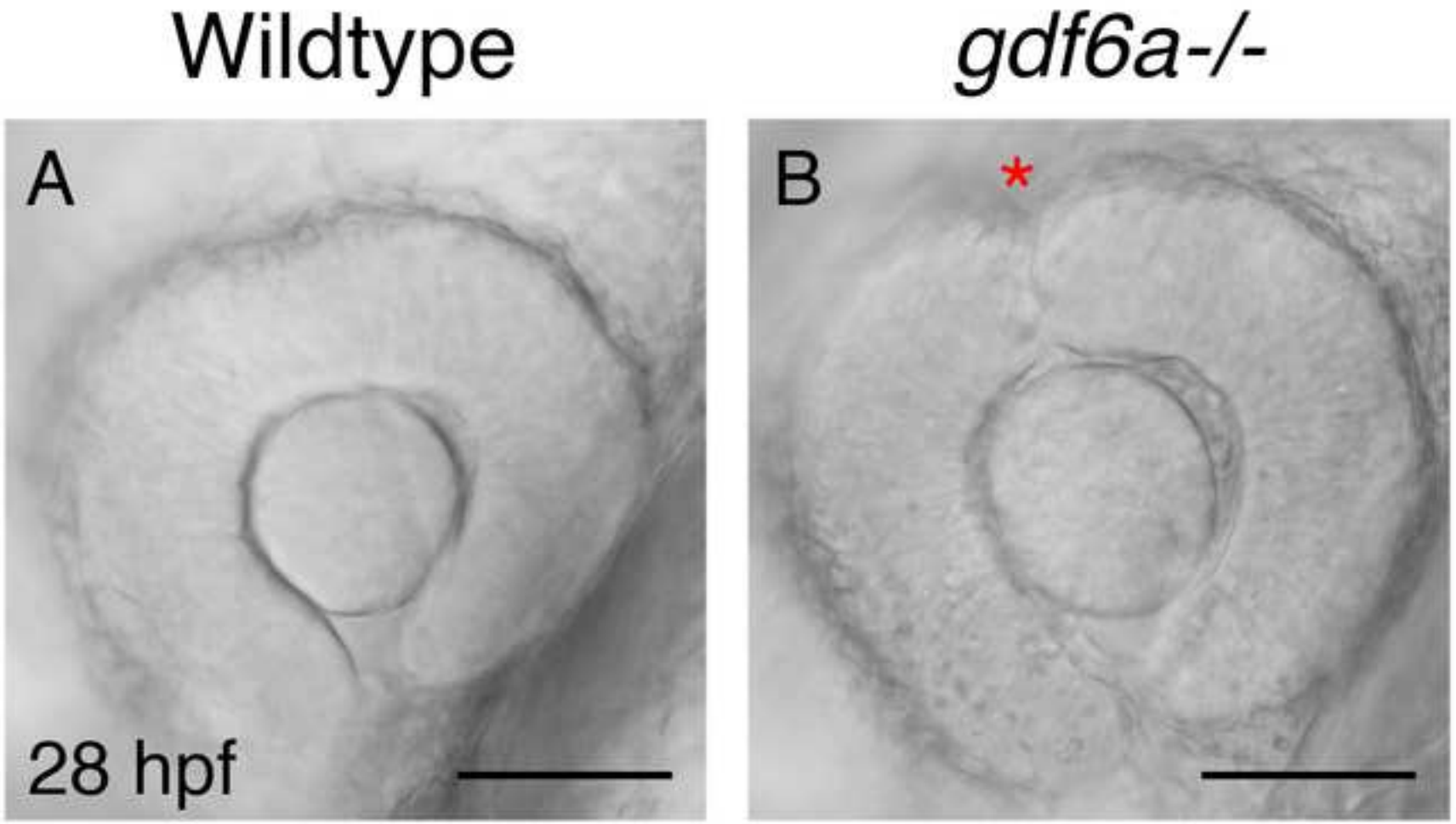


Figure 4

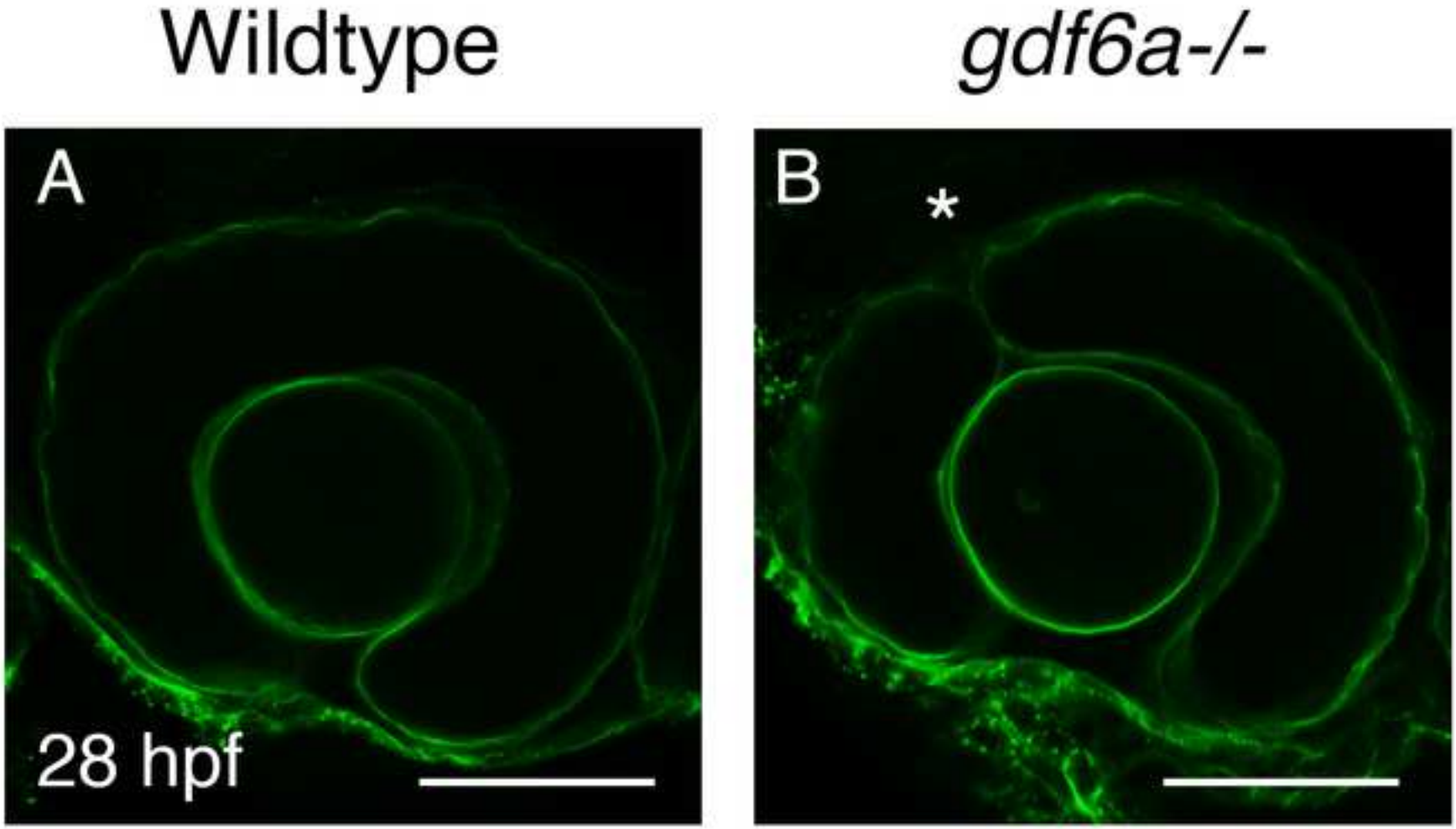
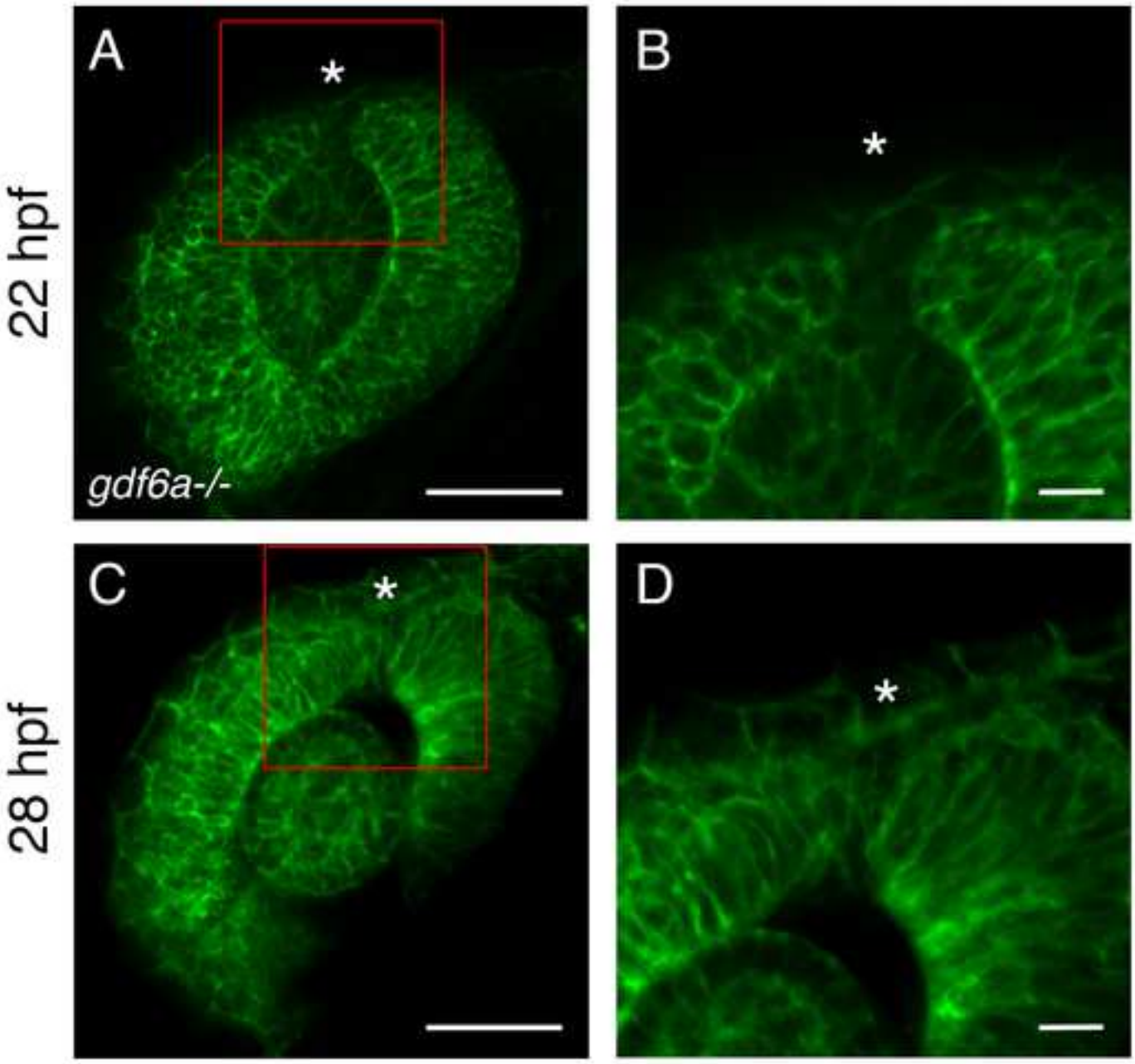


Figure 5



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1-phenyl 2-thiourea	Sigma Aldrich	P7629-10G	
100 mm Petri dish	Fisher Scientific	FB0875713	
35 mm Petri dish	Corning	CLS430588	
Agarose	BioShop Canada Inc.	AGA001.1	
Bovine serum albumin	Sigma Aldrich	A7906-100G	
DIC/Fluorescence microscope	Zeiss	AxioImager Z1	
Dissection microscope	Olympus	SZX12	
Dissection microscope camera	Qimaging	MicroPublisher 5.0 RTV	
Dow Corning High-vacuum grease	Fisher Scientific	14-635-5D	
Ethyl 3-aminobenzoate methanesulfonate salt (Tricaine)	Sigma Aldrich	A5040-25G	
Goat anti-rabbit Alexa Fluor 488	Abcam	ab150077	
Goat serum	Sigma Aldrich	G9023	
Image capture software	Zeiss	ZEN	
Incubator	VWR	Model 1545	
Microscope Cover Glass (22 mm x 22 mm)	Fisher Scientific	12-542B	
Microscope slide	Fisher Scientific	12-544-2	

Minutien pin	Fine Science Tools	26002-10
mMessage mMachine Sp6 Transcription Kit	Invitrogen	AM1340
NotI	New England Biolabs	R0189S
Paraformaldehyde (PFA)	Sigma Aldrich	P6148-500G
Phenol:Chloroform:Isoamyl Alcohol pH 6.7 +/- 0.2	Fisher Scientific	BP1752-100
Proteinase K	Sigma Aldrich	P4850
Rabbit anti-laminin antibody	Millipore Sigma	L9393
TURBO Dnase (2 U/µL)	Invitrogen	AM2238
Ultrapure low-melting point agarose	Invitrogen	16520-100
UltraPure Sodium Dodecyl Sulfate (SDS)	Invitrogen	15525017

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Visualization of a novel superior ocular fissure during Danio rerio embryogenesis
Author(s):	Yoon, Kevin H., Widen, Sonya A., Wilson, Melissa M., Hocking, Jennifer C., Waskiewicz, Andrew J.

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

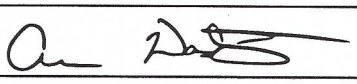
12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Andrew J. Waskiewicz
Department:	Department of Biological Sciences
Institution:	University of Alberta
Title:	Professor
Signature:	
Date:	October 12, 2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Rebuttal for Manuscript Submission to JoVE

Title: Visualization of a novel superior ocular sulcus during Danio rerio embryogenesis
Authors: Kevin H Yoon, Sonya A Widen, Melissa M Wilson, Jennifer C Hocking, and Andrew J Waskiewicz
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.

Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
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 Book[12], 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

2.1.1) 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 made 4% 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.