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Mechanical vessel injury in zebrafish embryos

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Abstract:	Zebrafish (Danio rerio) embryos have proven to be a powerful model for studying a variety of developmental and disease processes. External development and optical transparency make these embryos especially amenable to microscopy, and numerous transgenic lines that label specific cell types with fluorescent proteins are available, making the zebrafish embryo an ideal system for visualizing the interaction of vascular, hematopoietic, and other cell types during injury and repair in vivo. Forward and reverse genetics in zebrafish is well developed, and pharmacological manipulation is possible. We describe a mechanical vascular injury model using micromanipulation techniques that exploits several of these features to study responses to vascular injury including hemostasis and blood vessel repair. Using a combination of video and timelapse microscopy, we demonstrate that this method of vascular injury results in measurable and reproducible responses during hemostasis and wound repair. This method provides a system for studying vascular injury and repair in detail in a whole animal model.
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Dear Editors,

Please consider the attached manuscript "Mechanical vessel injury in zebrafish embryos," for publication in your journal with an accompanying video. This manuscript details how to perform a mechanical injury on zebrafish embryos resulting in vascular breach, thrombus formation, and subsequent vascular repair. We provide evidence that these processes follow a reproducible pattern and can be measured, and demonstrate that mechanical vessel injury provides a novel platform for studying and visualizing vessel injury and repair in a whole animal model. We believe this technique is well suited to a video journal format as the micromanipulation involved in generating the injury is most easily replicated if the procedure can be visualized. This manuscript should be of interest for researchers studying injury, wound healing, and cellular processes associated with hemostasis and repair.

TITLE:
Mechanical vessel injury in zebrafish embryos

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KEYWORDS:
Zebrafish, hemostasis, vascular injury, wound healing, inflammation, microscopy

SHORT ABSTRACT:
This article describes a method for creating a mechanical vessel injury in zebrafish embryos. This injury model provides a platform for studying hemostasis, injury-related inflammation, and wound healing in an organism ideally suited for real-time microscopy.

LONG ABSTRACT:
Zebrafish (*Danio rerio*) embryos have proven to be a powerful model for studying a variety of developmental and disease processes. External development and optical transparency make these embryos especially amenable to microscopy, and numerous transgenic lines that label specific cell types with fluorescent proteins are available, making the zebrafish embryo an ideal system for visualizing the interaction of vascular, hematopoietic, and other cell types during injury and repair *in vivo*. Forward and reverse genetics in zebrafish are well developed, and pharmacological manipulation is possible. We describe a mechanical vascular injury model using micromanipulation techniques that exploits several of these features to study responses to vascular injury including hemostasis and blood vessel repair. Using a combination of video and timelapse microscopy, we demonstrate that this method of vascular injury results in

measurable and reproducible responses during hemostasis and wound repair. This method provides a system for studying vascular injury and repair in detail in a whole animal model.

INTRODUCTION:

Zebrafish have been used extensively to study a variety of topics in vascular biology, including vascular development, angiogenesis, and hematopoietic development and pathology[1-3]. Embryos develop a functional circulation as well as leukocytes and other components of the innate immune system by 1 day post fertilization (dpf) [1,4,5]. The conservation of the inflammatory and leukocyte response to injury has made the zebrafish embryo an informative model for such diverse inflammatory processes as tuberculous infection, enterocolitis, and tissue regeneration[6-9]. Zebrafish embryos have been used to study injury-related inflammation particularly in the context of epithelial wounding and the neutrophil response[10,11]. Injury to the embryo results in a highly conserved cellular response from cells at the injury site and the innate immune cells recruited to respond to the injury and regulate its resolution[11,12]. Other injury models have used focused laser pulses to spatially localize injury to specific cell types including neurons, muscle cells, and cardiomyocytes[13-15].

Zebrafish embryos have been used as a model to study hemostasis and thrombosis in conditions of pharmacological and genetic manipulation, using both mechanical and laser-induced thrombus formation[16-19]. Components of the coagulation cascade appear to be well-conserved and transgenics have allowed for detailed studies of thrombocyte and fibrin deposition at the site of coagulation[17,20,21]. The procedure presented in this paper complements these methods by providing a system for studying mechanical vessel injury resulting in vessel breach, thrombus formation and resolution, and vessel repair.

PROTOCOL:

Procedures using zebrafish were approved by UCSF's Institutional Animal Care and Use Committee.

1. Preparation of tools

1.1. Insert minutia pin into a pin holder and clamp the pin.

1.2. Using fine tip forceps, carefully bend the tip of the pin to create a slight hook.

1.3. For manipulation and stabilization of the embryo during injury, bend the end of a 28 guage ½ inch needle mounted on an insulin syringe using needle-nose pliers.

2. Preparation of zebrafish embryos for injury

2.1. Set up zebrafish breeding pairs and collect eggs in egg water (60ug/mL aquarium salts) as shown previously[22].

2.2. Add 0.003% N-Phenylthiourea (PTU) to the egg water when the embryos are approximately 8 hours post fertilization (hpf) to prevent melanization.

2.3. Dechorionate two day post fertilization (dpf) embryos prior to the experiment using fine tip forceps. Note: Embryos can be injured anytime after circulation begins. The data presented here is for 2 dpf embryos, but the technique has been successfully applied in embryos up to 5 dpf.

2.4. Anesthetize the embryos with 0.02% buffered 3-aminobenzoic acid (Tricaine) approximately 10 min prior to manipulations.

3. Mechanical vessel injury of embryos

3.1. Transfer anesthetized embryos to a depression slide on a dissecting stereomicroscope using a transfer pipet.

3.2. Using the short flat side of the syringe needle to manipulate the embryo with the dominant hand, position the embryo on its side with the ventral surface facing away from the needle.

3.3. Position the minutia pin with the tip pointed directly against the ventral surface of the fish posterior to the urogenital opening. Position the minutia pin at a slight angle such that the curved tip is able to pierce through the periderm directly into the caudal vein (Figure 1).

3.4. Using the syringe needle to manipulate the embryo, pierce the caudal vein with the minutia pin by tapping the embryo into the pin to slightly hook the pin into the vein.

3.5. Using the syringe needle, pull the embryo away from the minutia pin to create a small tear in the vessel. Note: A successful injury will result in immediate bleeding from the vein.

4. Analysis of hemostasis

4.1. Choose only embryos with visibly circulating blood cells for this procedure.

4.2. Prepare to begin the timer as soon as the minutia pin is pulled from the vessel.

4.3. Start the timer as soon as blood loss can be visualized from the wound. When blood loss from the wound ceases, stop the timer and record total time as Bleeding Time. If coagulation is inhibited, record the time to when there are no longer visibly circulating blood cells.

5. Analysis of wound healing

5.1. Transfer post-injury animals onto glass-bottom imaging dishes for microscopy.

5.2. Remove the majority of the egg water.

5.3. Cover the embryos in 0.3-1.2% low melting agarose dissolved in egg water, heated to 42 ºC and supplemented with 0.02% Tricaine.

5.4. Position embryos on their sides using forceps.

5.5. After the agarose cools, fill the dish with 0.02% Tricaine in egg water.

5.6. Acquire images using brightfield, epifluorescence, or confocal microscopy.

5.7. Remove embryo from the agarose using forceps and transfer back to egg water.

REPRESENTATIVE RESULTS:

Mechanical vessel injury was performed on 2 dpf embryos (Figure2A-C). Injury results in a rapid and reliable coagulation response as measured by time to cessation of bleeding (Figure 2D). To determine whether or not differences in the coagulation response could be measured, the anticoagulant hirudin was administered to the embryos by injection into the Duct of Cuvier immediately prior to wounding (5-10 nL of 1 unit per uL hirudin dissolved in water)(for demonstration of injections into the Duct of Cuvier, see previous JoVE article[23])[24]. The administration of hirudin prior to injury resulted in significantly increased bleeding times versus vehicle control (Figure 2D).

Evidence of vessel damage and coagulation can be seen immediately post-injury using transgenic lines for endothelial (*kdrl:egfp*) and red blood cell (*gata1a:dsred*) markers[25,26]. Images were acquired sequentially every 5 minutes for a 12 hour period using epifluorescence. Representative still images are shown throughout different stages of wound repair (Figure 3). Using a combination of differential interference contrast (DIC) and fluorescence microscopy, it is possible to measure distinct parameters of wound repair. In order to determine whether or not wound repair followed a reproducible pattern across experiments, the time to reestablished blood flow was measured in 4 groups of fish. Vessel injury resulted in a reliable stereotypical response of 253 ± 16 minutes to the reestablishment of blood flow through the wounded vessel (n = 4-5 fish per experiment, average ± SEM).

FIGURE LEGENDS:

Figure 1: Diagram of 2 dpf embryo showing placement of minutia pin for performing mechanical injury of the caudal vein (CV). Vascular compartment is shaded in grey.

Figure 2: Bleeding times can be visually measured after mechanical injury. Stills from real-time video of zebrafish vessel injury on 2 dpf embryos. Images are shown at the time of injury (A), during active blood loss from the wound (B), and after cessation of blood loss (C). All times indicated are in seconds. Embryos are oriented laterally with anterior at top and ventral surface facing to the left. Scale bar 100 µm. Administration of

the anticoagulant hirudin led to significantly increased bleeding times versus vehicle control (D) ($p<0.0001$, Student's t-test).

Figure 3: Visualizing mechanical injury and repair using transgenic markers. Stills from timelapse DIC and fluorescence microscopy after vessel injury using markers for vascular endothelium (*kdrl:egfp*) and red blood cells (*gata1a:dsred*) in 2 dpf embryos. Images showed a gap in vessels and local red blood cell accumulation (t = 25), partial repair with re-established blood flow (t = 210), and apparently complete restoration of normal vessel structure (t = 615). Time is indicated in minutes. Embryos are oriented laterally with anterior at top and ventral surface facing to the left. * indicates the position of the dorsal aorta. The injury (arrowhead) disrupted the caudal vein and part of the caudal plexus. Scale bar 25 µm.

DISCUSSION:

Zebrafish have been used successfully as a model for different types of wounds including laser injury[13-15], laser-induced thrombosis[16], and epithelial wounding[10]. We report a method of mechanical wounding that is simple to execute and produces a controlled injury in an *in vivo* model that is highly amenable to real-time microscopy. Injury results in a rapid and measurable hemostatic response and a reproducible wound repair program that can be monitored using video and timelapse microscopy.

Their simple and stereotyped vascular anatomy, which permits reproducible injury at a defined and microscopically accessible site, and the presence of most vascular and hematopoietic cell types make zebrafish embryos particularly useful for studying responses to injury. However, zebrafish embryos do not have functional lymphocytes during the first weeks of development[5,6], making this system most appropriate for studying the contribution of innate immunity in inflammation and repair. At present, a wide variety of transgenic zebrafish exist with markers for cells and proteins that participate in thrombus formation, coagulation, inflammation, and wound repair, including lines that label thrombocytes, fibrinogen, erythrocytes, leukocytes and vascular endothelium[17,21,25-31]. These and other tools should make it possible to follow processes involved in hemostasis and repair in significant detail.

Mechanical injury complements laser injury for the study of hemostasis in zebrafish. While laser-induced injury has been used for years to trigger thrombus formation in zebrafish embryos and mouse models, the mechanisms by which laser injury triggers coagulation and thrombocyte/platelet activation are not fully known[16,32]. Mechanical injury provides a physiologically relevant method for inducing coagulation by vascular breach and, presumably, tissue-factor-dependent initiation of coagulation cascade. The finding that hirudin treatment significantly increased bleeding times after injury suggests that this model is thrombin-dependent. Mechanical injury additionally complements laser injury by providing sufficient disruption of a blood vessel to provide an opportunity to follow vessel repair. Previous studies have successfully used mechanical injury by scalpel incision and needle puncture to show differences in bleeding times in conditions of pharmacological and genetic manipulation [19,33]. The minutia pin injury used in the current model may complement other injury models by providing a more reproducible injury due to the small and defined size of the wound it produces and by providing an opportunity to better study vessel recanalization and repair.

Epithelial wounding in the zebrafish has proven to be a powerful model for studying inflammation and wound repair[10]. The ability to introduce a vascular injury provides an opportunity to assess repair of more complex wounds in settings where fibrin provides a provisional matrix, thrombi and debris are cleared, and vessels regenerate. As these processes participate in normal tissue repair and in acute and chronic inflammation and vascular pathology, this method should help to model aspects of human disease in a system where cellular behaviors can be monitored in real time in a whole animal model.

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DISCLOSURES:
The authors declare that they have no competing financial interests.

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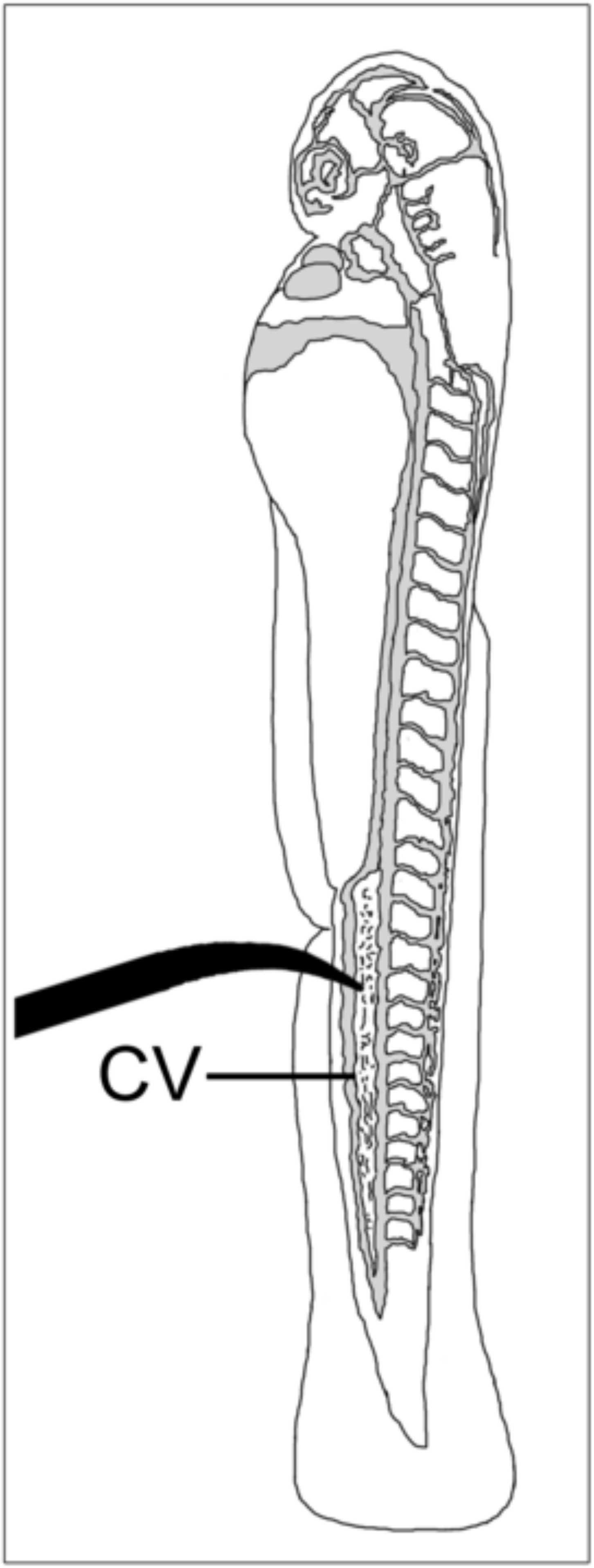

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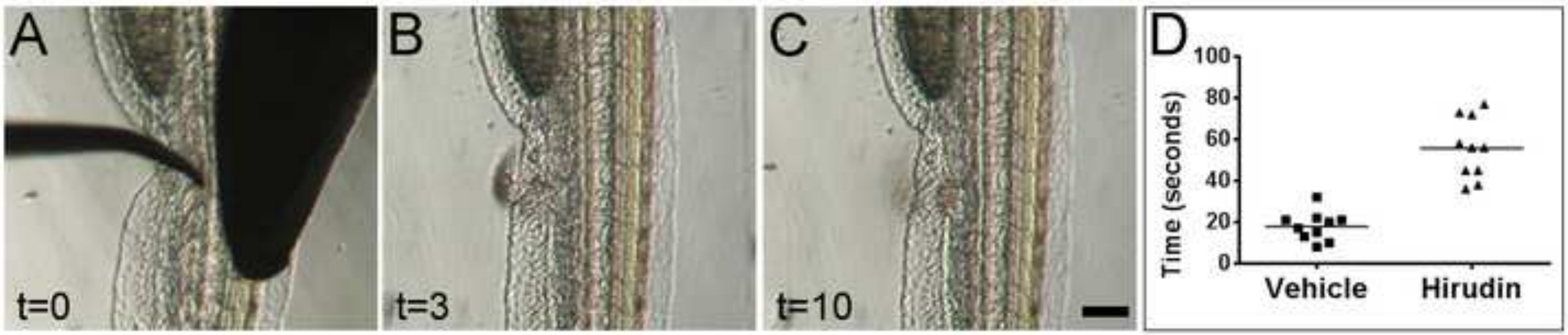

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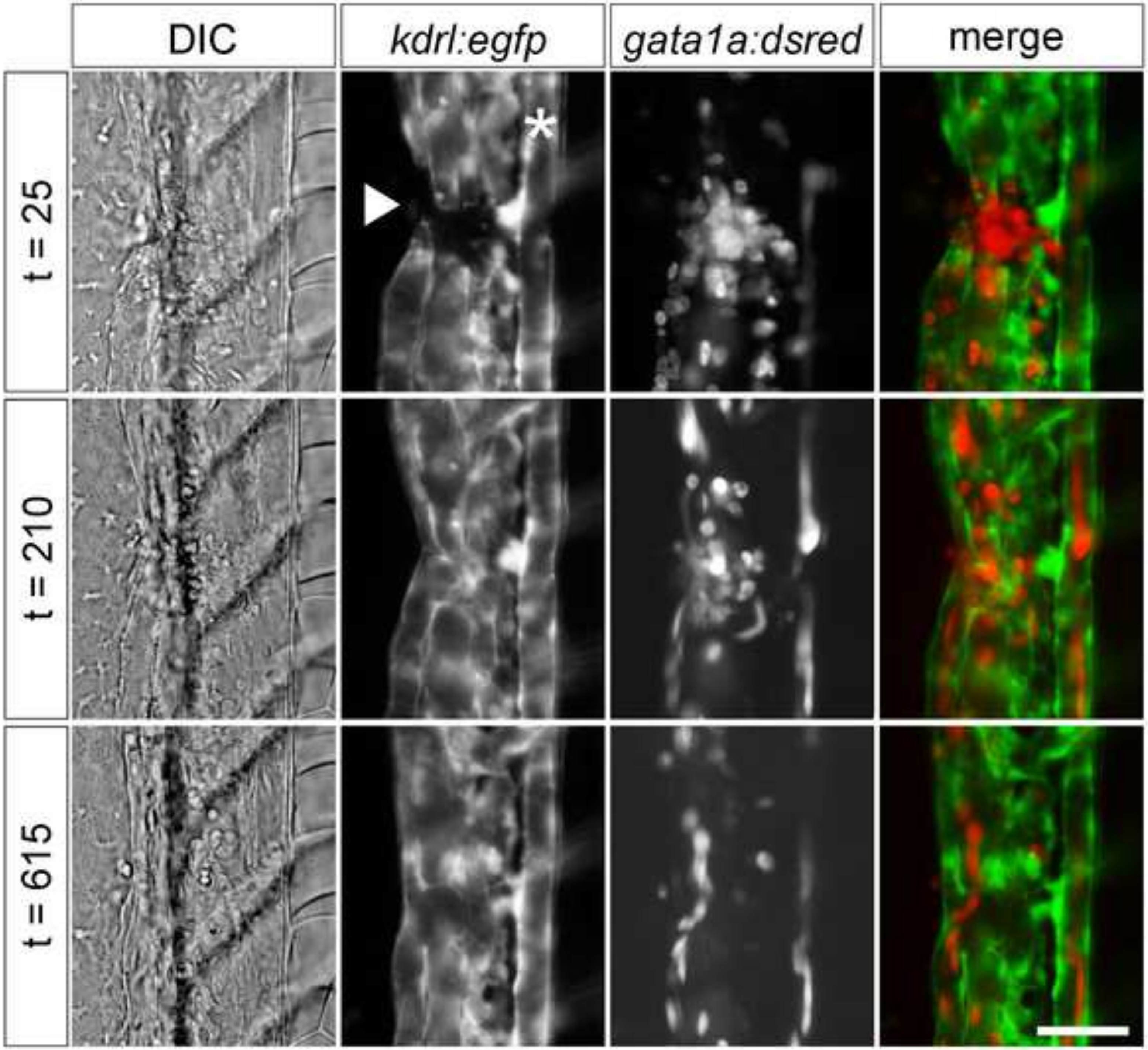

Excel Spreadsheet- Table of Materials/Equipment

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Name of Material/ Equipment	Company	Catalog Number
Minutia Pins	Fine Science Tools	26002-10
Pin Holder	Fine Science Tools	26016-12
Dumont #5 Fine Tip Forceps	Fine Science Tools	11254-20
Glass Depression Slide	Aquatic Eco-Systems	M30
Low Melting Agarose	Lonza	50081
N-Phenylthiourea (PTU)	Sigma Aldrich	P7629
3-aminobenzoic acid (Tricaine)	Sigma Aldrich	E10521
Hirudin	Sigma Aldrich	H7016
Glass bottom imaging dishes	Mattek	P35G-1.5-14-C
Dissecting microscope	Olympus	SZH10
Fluorescence microscope	Zeiss	Axio Observer
Aquarium salts	Instant Ocean	
Insulin syringe with 28G1/2 needle	Becton Dickinson	329461

Comments/Description

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Response to reviewers

Reviewer #1:
The authors present a description of mechanical vascular injury in zebrafish embryos and larvae for the purpose of studying hemostasis and wound healing. They demonstrate reproducible results, and this should be useful information for the scientific community.

Major points:
1) "Note: Embryos can be injured anytime after circulation begins." Up to what ages have been tested? Are there any differences in the results (e.g. at 24, 48, 72 or 96 hours)?

We have added a sentence into the methods saying that the technique has been applied to animals up to 5 dpf, but we have not done extensive characterization of these later stages.

2) The following questions/issues are likely to be more clear with the video, but are not clear in the text:
a. What does "tapping the embryo into the pin" mean?
b. "Using the tuberculin syringe, pull the embryo away from the minutia pin…" In the pictures it appears that the syringe needle is being used to provide pressure to support the embryo while inserting the pin. It is not clear how one can use it to pull the embryo.

This will hopefully become apparent in the video.

c. "Choose only embryos with robust circulation for this procedure." Please be more specific.

This has been changed to "Choose only embryos with visibly circulating blood cells for this procedure."

d. "record the time to exsanguination, or when there are no longer visibly circulating blood cells." This statement is not completely clear. Are these two different observations or the same observation stated in two different ways?

This has been changed to "record the time to when there are no longer visibly circulating blood cells"

e. It is difficult to appreciate the bleeding in Figs. 2B, C. An improvement would be to do this with the transgenic lines as in figure 3.

Because the red blood cells lyse almost immediately after entry into the fish water, this is unfortunately not possible. It might be possible to see the fluorescence briefly if the injury were performed under fluorescence and not brightfield, but I do not think I can perform the injury in those conditions.

3) There should be a description and demonstration of Duct of Cuvier injection unless there is another JoVE video that can be referenced for this.

I have included a reference to a JoVE video, and I have pointed out in the text that it is a JoVE article.

4) "…Duct of Cuvier immediately prior to wounding (5-10 nL of 1 unit per uL hirudin dissolved in water)(Figure 2A)." Fig. 2A seems to be the incorrect reference here.

This has been corrected.

5) It would be easier to appreciate Fig. 3 if the middle two columns were pseudo-colored in green and red respectively.

The human eye can pick up more contrast from monochrome than pseudo-colored images and thus having the images displayed in monochrome allows for more accurate representation of the data.

Minor points:
1) Detailed information should be provided for the syringe needle size.

This has been added.

2) "Using the tuberculin syringe to manipulate the embryo…" Technically it is not the syringe that is being used to manipulate the embryos, it is the needle attached at the end.

This has been changed throughout the methods.

3) "Time bleeding…" is not proper grammar.

This has been changed to "Start the timer as soon as blood loss can be visualized from the wound."

4) Define egg water.

The text has been changed to read "aquarium salts" rather than the brand name.

Reviewer #2:
Hilary and Coughlin are describing a method of mechanical injury of the zebrafish larvae to study blood clotting. The technical aspects and experimental details of the paper are very well presented. The authors portray the information in an elegant manner such that the experiment detailed in this paper would be easily reproducible in any laboratory. Thus, this mechanical injury should certainly be useful to those interested in creating wounds in zebrafish. Unfortunately, this method of combining mechanical injury with time lapse photography has previously been reported for use in zebrafish larvae, particularly for hemostasis studies. This reviewer found the following references by searching PubMed:

1. Jagadeeswaran and Liu (1997) A hemophilia model in zebrafish: analysis of hemostasis., Blood Cells, Molecules, and Diseases.

2. Bielczyk-Maczyńska, et al. (2014) A Loss of Function Screen of Identified Genome-Wide Association Study Loci Reveals New Genes Controlling Hematopoiesis., PLoS genetics.

In light of the above cited mechanical injury model, it is not appropriate to describe this method without referencing the existing mechanical injury model. However, this reviewer recognizes that such a detailed description may be useful to the hemostasis community and encourages the authors to discuss these previous publications and present the material in such a way that the differences of this method compared to those previously published becomes apparent.

We thank the reviewer. The last paragraph of the introduction has been changed to include the citations. We have included a discussion of the different published mechanical injuries in the last paragraph of the discussion.

Furthermore, the variation inherent to needle induced injury makes this mechanical method less attractive than laser injury methods.

We believe the laser and mechanical models are complementary. Mechanical injury is likely a more physiological trigger of tissue damage and hemorrhage than laser injury, and laser injury does not allow for the study of vessel recanalization. We now discuss this in the last paragraph of the revised manuscript.

If this method were to be standardized (e.g. by mechanization), then it would be more useful than previously described methods. In summary, as it stands, with the exception of the use of GFP lines and the associated fluorescence images, the methodological contributions of this paper have unfortunately already been published.

Editorial Comment: Novelty is not a requirement for publication of a method in JoVE. However we ask that you carefully consider the references and points presented by this reviewer and include additional information as appropriate in your revised manuscript.