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.