Response to reviewers' critiques

Dear Section Editor,

Our sincere thanks to you and the two reviewers for the timely review of our study (JoVE61857). We very much appreciate the time and effort the editor and two reviewers put into improving the quality of our manuscript. Below, we address point by point the critiques and concerns raised by the editor and reviewers. All changes made in the revision are bolded and underlined for easy identification by the editor and reviewers.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We really appreciate this opportunity to further polish the manuscript. We have carefully gone over the whole manuscript and made revisions, including correction of the spelling and grammar issues.

2. Unfortunately, there are sections of the manuscript that show overlap with previously published work. Please revise the following lines: 28-30, 46-47, 57-61, 70-72, 168-170, 224-227, 230-232, 234-237, 239-246, 248-253, 258-260, 264-268, 271-277, 282-285

We apologize for the improper citation of published work. We have revised the descriptions in these sections in the Revision.

3. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

We thank the editor for the good suggestion. We concur with the editor's concern regarding how much detail is provided in the protocol. We have now added more detailed information in the protocol section of the Revision.

4. Please specify the age/gender/strain of the mouse used.

Detailed information on the animal used has been added to the protocol. See 1.1 & 2.1, Pages 2-3 (lines 80-103).

5. 1.3: How much PBS is used to wash?
6. 1.5: Centrifuge at what speed and for how long? Please specify all volumes used throughout.

We used 1 ml PBS to wash the blood cell pellet by centrifugation at 3000 x g for 3 minutes at 4°C. This information has been added to all relevant procedures. See 1.3 (lines 87-88) & 1.5 (lines 94-96), Page 3.

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

Thanks for the suggestion. We have mimized the use of personal pronouns in the Revision.

8. Please do not abbreviate journal titles in the references.

All abbreviated journal titles have been changed to their full titles in the references.

(P.S., we have added Jinhui Zhang, M.D., Ph.D to the Revision. We overlooked her contribution in doing the fine surgery; we have also replaced the previous panel B, figure 1 with a new image).

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This protocol would be useful in hearing research.The relation between cochlea blood flow and pathophysiology of hearing is so close. The method to calculate the blood flow in real time in vivo should be developed. This manuscript is definitely important. But it needs some modifications.

Thanks to Reviewer #1 for postive comments on the application of our technique. We agree the manuscript is improved with more detailed information. More detailed information and discussion are provided in the Revision. Errors identified by reviewers or noticed by ourselves have been corrected.

Major Concerns:
The blood flow peripherally is affected by many minor changes during the experiment(i.e. temperature, heart beat, noise, time, and anethesia). In the method, there are no concerns to control these factors except

of rectal temperature (even though there is no warm pad). If these factors are not controlled, the result of variations could not be from the disease.

Yes, this is an excellent point. We fully agree with the reviewer. All operations need to be conducted carefully and animal vital signs closely monitored to maintain the cochlear homeostasis for accurate data collection. In our experimental setting, we use a warm heating pad and monitor the animal's rectal temperature throughout the operation. We also monitor animal blood pressure and heart rate before and after anesthesia. This additional information has now been added to prototol section 2.3 on Page 3, line 107-113. This information is also emphasized the in experimental note in section note after 2.6 on page 3 and line 140-143.

The tracheotomy wound was seen in the Fig 1. Did you use IP anethesia? The figure is suspicious. This incision approach is anterior (ventral) midline approach, not posterior ventral (bulla approach).

Yes, we used IP anesthesia on the animal. Tracheotomy is optional, only done if necessary to keep the airway unobstructed during the surgery. A note to this effect has been added on Page 3, line 131. We did open the left tympanic bulla via a lateral and ventral approach, as described in step 2.4. The text describing the procedure is modified on Page 3, lines 115-129 in the Revision.

In Figure 2, there are so confusion for wording 'stria vescularis'. Is vessels in (A) same with stria vessels in (C)? Why does not the image show the red blood cells in (C)?

We apologize for the misspelling and incorrect legend in figure 2C. The difference between figure 2A and C is the difference in fluorescence dye used for blood cell labeling. FITC-dextran was used to label the plasma for the visualization of the blood vessels in both images. In figure 2A blood cells are labeled by Dil (red) to provide better contrast relative to the vessel lumen and individual blood cells. However, there isn't sufficient time to acquire an image in 2 channels (requiring filter change) for real-time tracking. Thus, Dio (green) is preferred for labeling the blood cells. A simultaneous image taken in the same channel is more accurate for flow velocity measurement, as shown in figure 2C. Of note, the amount of Dio administered for labeling blood cells needs to be well controlled. If not, the cell labeling could easily mask the FITC-dextran signal of the vessel lumen. This is explained in the discussion section on Page 6, lines 262-283 in the Revison. The legend has also been corrected in the revised figure.

The stria ligament does not seem to be separated from the stria vascularis, because the strial ligaments cover the stral vascularis in the lateral wall. If you want to see the stria vascularis, you need to re-focus the depth more with the lens. The imaging technique is lack of informations. DId The zeiss LSM 7MP use the laser? More information to take the picture would be added in the discussion.

Response to reviewers' critiques

We appreciate the reviewer's suggestion. Yes, the stria vascularis marks the upper portion of the spiral ligament containing numerous capillary loops and small blood vessels. On our upright FIVM system, the stria is optically under the spiral ligament. Thus we are able to distinguish and image both of them by simply adjusting the optical focus. We demonstrate this in Figures 2B and C. The 2 images are taken through the same open vessel window. Only adjustment of optical focus has been made. Arrows in Figure 2B point out capillaries of the spiral ligament -straighter, larger, and sparser than in the stria - while in Figure 2C we show the higher vessel density capillaries of the stria vascularis. This is noted in the protocol section on Page 4, lines 160-161 and explained in the results section on Page4, lines 195-197 of the Revison.

We apologize for the rough and incorrect imaging information. We used an Olympus BXFM fluorescence microscope equipped with a long working distance objective (W.D. 30.5 mm, 10 x, 0.26 NA) for the imaging. The instrument lamp housing includes a multiple band excitation filter and a compatible emission filter, and the video was recorded on a Hamamastu ORCA-ER high-resolution digital B/W CCD Camera at 2 frames/sec. The information has been added to the protocol section on Page 4, lines 153-158.

The noise induced mouse should represent the hearing thresholds (deaf?). The severity of hearing would affect the vasculature of cochlea.

Yes, the noise exposure does cause loss of hearing sensitivity and it does correlate with vascular changes in the cochlea, as we previously reported. The noise exposure model we use (mouse exposed to broadband noise at 120 dB for 3 hours per day for 2 consecutive days) induces a significant shift of the hearing threshold and structurally damages blood vessels in the stria vascularis [1, 2].

Why are there different for population between Fig3 (C) and (D)? The variation seen in (C) is same between both groups, but in (D) more variation in noise group.

This is a good question. We apologize for the confused way in which the data are presented. For figure 3C we sampled more vessels in the open vessel window so as to compare the difference in average strial vessel diameter in the control and noise exposed group. In figure 3D we specifically compared the range in vessel diameter determined from the largest and smallest vessels in the control and noise exposed groups. The noise exposed group displayed a wider range of vessel diameter. To make this clearer, we have revised figure 3D.

Reviewer #2:

Manuscript Summary:

The authors describe thoroughly an interesting procedure for investigating the cochlear strial blood flow via intravital microscopy. The manuscript is interesting and only minor clarification are required prior to

publication.

Major Concerns:
None

We appreciate the valuable comments of reviewer #2 on our study. We now address the specific concerns raised by the reviewer.

Minor Concerns:
Step 1.3: Volume of PBS? Centrifuge parameters during washing?

1 ml PBS was used to wash the blood cell pellet by centrifugation at 3000 x g for 3 minutes at 4°C. We have added this information to step 1.3 & 1.5, on Page 2, lines 87-88 & lines 94-96.

Step 1.4: What is the volume in which cells are suspended? What is the volume of the added solution that contains the fluorophore?

Step 1.5: What is the final volume?

The fluorescence labeled blood cells were suspended in ~30% hematocrit with PBS, so the ratio of added PBS volume to pellet blood cell volume should be 7:3. The hematocrit of mouse blood is around 40%. We collected ~ 1 ml blood from the animal and added ~0.9 ml PBS. The final blood cell suspension volume is ~1.3 ml. This information has been added to step 1.5 on Page 2, lines 94-96. The volume of added solution containing the fluorophore is 1 ml, as stated in step 1.4 on Page 2, line 90.

Figure 1A: Does agree with the text.

We apologize for the confusing illustration. We modified the description of steps 2.1 - 2.3 on Page 3, lines 100-113.

Figure 1B: A zoom-in picture would be more clear.

We agree with the Reviewer's suggestion. We are sorry not to be able to provide a zoom-in image in the current situation. However, we do have a video recorded at higher magnification. Thus we modified the description of step 2.4.1 to better bmatch the figure on Page 3, lines 118-119.

Step 3.1: What is the Mol. Weight of the Dextran?

Response to reviewers' critiques

The molecular weight of the dextran we use is 2000 KDa. This information has been added to procedure 3.2 on Page 4, line 149.

Step 3.3: What is the objective's numerical aperture?

We use an objective with a numerical aperture of 0.26. This information has been added on Page 4, line 153-158.

Figure 2B,C: Why both fluorophores represented with green?

This is a good question. Although Dil (red) provides better contrast for vessel lumen and distinguishing individual blood cells, too much time is required to acquire an image in 2 channels (filter change is required) for real-time tracking. Dio (green) is preferred, as a single simultaneous image can be acquired, saving time, and giving a more accurate measurement of flow velocity. A careful titration of the amount of Dio used to label blood cells must be done, since a bright Dio signal could easily mask the FITC-Dextran signal of the vessel lumen. This is explained in the discussion section on Page 6, lines 273-283 in the Revison.

Line 254: λex / λem for DiI?

The company we purchase Dil from only provides its emission λ_{max} = 549 nm. We checked with another company. They specify the λex / λem for DiI is 550 nm / 564 nm , which is consistent with the filter we use for Dil. This information has been added to the details on Page 6, line 273-274.

Could the authors add a representative sequence showing the injected blood cell kinetics? Now only one figure is shown (Fig 2A).

Good suggestion. As explained above, we use Dio labeled blood cells and FITC-dextran labeled plasma, both green and acquired in the same channel, when measuring blood cell kinetics. A representative video has been added to the supplemental materials, as indicated in the Result section on Page 4, line 205-207.

Fig 3 and 4: Color-legends indicating what the are presented colors are missing.

Thanks to the reviewer for pointing this out. The missing color-legends have been added to the figures in the Revison.

Response to reviewers' critiques

References:

1. Shi, X. Cochlear pericyte responses to acoustic trauma and the involvement of hypoxia-inducible factor-1alpha and vascular endothelial growth factor. *Am J Pathol*. **174**(5), 1692-704, dio:10.2353/ajpath.2009.080739 (2009).

2. Hou, Z. *et al.* Acoustic Trauma Causes Cochlear Pericyte-to-Myofibroblast-Like Cell Transformation and Vascular Degeneration, and Transplantation of New Pericytes Prevents Vascular Atrophy. *Am J Pathol*. **190**(9), 1943-1959, dio:10.1016/j.ajpath.2020.05.019 (2020).