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TITLE:

Measurement of Strial Blood Flow in Mouse Cochlea Utilizing an Open Vessel-window and Intravital Fluorescence Microscopy

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KEYWORDS:

Mouse, cochlear blood flow, intravital fluorescence microscopy, fluorescent labeled blood cells, open vessel-window, blood flow velocity, blood volume

SUMMARY:

An open vessel-window approach using fluorescent tracers provides sufficient resolution for cochlear blood flow (CoBF) measurement. The method facilitates the study of structural and functional changes in CoBF in mouse under normal and pathological conditions.

ABSTRACT:

Transduction of sound is metabolically demanding, and the normal function of the microvasculature in the lateral wall is critical for maintaining endocochlear potential, ion transport, and fluid balance. Different forms of hearing disorders are reported to involve abnormal microcirculation in the cochlea. Investigation of how cochlear blood flow (CoBF) pathology affects hearing function is challenging due to the lack of feasible interrogation methods and the difficulty in accessing the inner ear. An open vessel-window in the lateral cochlear wall, combined with fluorescence intravital microscopy, has been used for studying CoBF changes in vivo, but mostly in guinea pig and only recently in the mouse. This paper and the associated video describe the open vessel-window method for visualizing blood flow in the

mouse cochlea. Details include 1) preparation of the fluorescent-labeled blood cell suspension from mice; 2) construction of an open vessel-window for intravital microscopy in an anesthetized mouse, and 3) measurement of blood flow velocity and volume using an offline recording of the imaging. The method is presented in video format to show how to use the open window approach in mouse to investigate structural and functional changes in the cochlear microcirculation under normal and pathological conditions.

INTRODUCTION:

Normal function of the microcirculation in the lateral cochlear wall (comprising the majority of the capillaries in the spiral ligament and stria vascularis) is critically important for maintaining hearing function[1]. Abnormal CoBF is implicated in the pathophysiology of many inner ear disorders including noise-induced hearing loss, ear hydrops, and presbycusis[2-9]. Visualization of intravital CoBF will enable a better understanding of the links between hearing function and cochlear vascular pathology.

Although the complexity and location of the cochlea within the temporal bone precludes direct visualization and measurement of CoBF, various methods have been developed for the assessment of CoBF including laser-doppler flowmetry (LDF)[10-12], magnetic resonance imaging (MRI)[13], fluorescence intravital microscopy (FIVM)[14], fluorescence microendoscopy (FME)[15], endoscopic laser speckle contrast imaging (LSCI)[16], and approaches based on the injection of labeled markers and radioactively tagged microspheres into the bloodstream (optical microangiography, OMAG)[17-20]. However, none of these methods has enabled absolute real-time tracking of changes in CoBF in vivo, with the exception of FIVM. FIVM, in combination with a vessel-window in the lateral cochlear wall, is an approach that has been used and validated in guinea pig under different experimental conditions by various laboratories[14,21,22]**.**

An FIVM method was successfully established for studying the structural and functional changes in the cochlear microcirculation in mouse using fluorescein isothiocyanate (FITC)-dextran as a contrast medium and a fluorescence dye—either DiO (3, 3'-dioctadecyloxacarbocyanine perchlorate, green) or DiI (1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate, red)–for prelabeling blood cells, visualizing vessels, and tracking blood flow velocity. In the present study, the protocol of this method has been described for imaging and quantifying changes in CoBF in mouse under normal and pathological conditions (such as after noise exposure). This technique gives the researcher the tools needed to investigate the underlying mechanisms of CoBF related to hearing dysfunction and pathology in the stria vascularis, especially when applied in conjunction with readily available transgenic mouse models.

PROTOCOL:

NOTE: All procedures involving the use of animals were reviewed and approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University (IACUC approval number: TR01_IP00000968).

1. Preparation of the fluorescent-labeled blood cells

1.1. Anesthetize the donor mice (male C57BL/6J mice aged ~6 weeks) with an intraperitoneal (i.p.) injection of ketamine/xylazine anesthetic solution (5 mL/kg, see the **Table of Materials**).

NOTE: This anesthesia protocol is very reliable and maintains systemic blood pressure.

1.2. Collect ~1 mL of blood in heparin (15 IU/mL blood) by cardiac puncture, and centrifuge at 3,000 × *g* for 3 min at 4 °C.

1.3. Remove the plasma, wash the blood cell pellet with 1 mL of phosphate-buffered saline (PBS), and centrifuge 3x at 3000 × *g* for 3 min at 4 °C.

1.4. Label the blood cells with 1 mL of 20 μM DiO or Dil in PBS, and incubate in the dark for 30 min at room temperature[23,24].

1.5. Centrifuge and wash the labeled blood cells with 1 mL of PBS, centrifuge 3x at 3000 × *g* for 3 min at 4 °C, and resuspend the cell pellet in 30% hematocrit with ~0.9 mL of PBS (final volume ~1.3 mL) before injection.

2. Surgery to create an open window[25]

2.1. Prepare the sterile surgical instruments and imaging platform, and place a heating pad beneath the drape (**Figure 1A**). Anesthetize the mice (male C57BL/6J mice aged ~6 weeks) as described in step 1.1, and check the depth of the anesthesia by monitoring the paw reflex and general muscle tone. Place the animal on the warm heating pad, and maintain the rectal temperature at 37 °C.

2.2. Place the animal tail in the monitor system for monitoring blood pressure and heartbeat (see the **Table of Materials**). Record the animal's systolic blood pressure, diastolic blood pressure, and mean blood pressure (MBP) in the anesthetized condition.

NOTE: No difference was seen in the animal's MBP in the anesthetized and un-anesthetized state (107 ± 11 mmHg vs. 97 ± 7 mmHg). The animal's heart rate was stable under anesthetization, although lower (still within normal range) than in the non-anesthetized condition (357 ± 12 bmp vs. 709 ± 3 bmp). A slightly increased heart beat is expected in animals placed under restraint[26].

2.3. Open the left tympanic bulla via a lateral and ventral approach under a stereo microscope (see the **Table of Materials**), leaving the tympanic membrane and ossicles intact[21].

2.3.1. Make an incision along the midline of the animal's neck with its head immobilized and positioned to minimize movement (**Figure 1B**). Remove the left submandibular gland and posterior belly of the digastric muscle and cauterize.

2.3.2. Locate and expose the bony bulla by identifying the sternocleidomastoid muscle and facial nerve extending anterior toward the bulla.

2.3.3. Open the bony bulla with a 30 G needle, and carefully remove the surrounding bone with surgical tweezers to provide a clear view of the cochlea and stapedial artery, with its medial margin lying over the edge of the round window niche, and coursing anterior-superior towards the oval window (**Figure 1C,D**).

NOTE: Tracheotomy is optional and can be performed to keep the airway unobstructed.

2.4. Use a small knife blade (custom-milled #16 scalpel) to scrape the lateral wall bone at the apex-middle turn of the mouse cochlea, approximately 1.25 mm from the apex until a thin spot is cracked. Remove the bone chips with small wire hooks (**Figure 1E**).

2.5. Cover the vessel-window with a cut coverslip to preserve normal physiological conditions and provide an optical view for recording vessel images.

NOTE: All procedures are to be performed with caution. In addition to monitoring body temperature, the animal's vital signs, including blood pressure and heartbeat, should also be monitored throughout the surgery.

3. Imaging of CoBF under FIVM

3.1. Cut the leg skin to expose the right saphenous vein (**Figure 1F**).

3.2. Infuse 100 μL of the FITC-dextran solution (2000 kDa, 40 mg/mL in PBS) and 100 μL of a blood cell suspension (30% hematocrit) successively into the animal through the saphenous vein (**Figure 1G**) to enable visualization of the blood vessels and tracking of blood flow velocity.

3.3. Observe the blood flow in real time directly on a video monitor 5 min after the injection. Image the blood vessels using a fluorescence microscope equipped with a long working distance (W.D.) objective (W.D. 30.5 mm, 10x, 0.26 numerical aperture) and a lamp-housing containing a multiple band excitation filter and compatible emission filter (**Table of Materials**). Record the video using a high-resolution digital black and white charge-coupled device camera (**Table of Materials**) at 2 frames/s). Acquire more than 350 images per video to ensure successful analysis of the flow velocity.

NOTE: Blood vessels of both the spiral ligament and stria vascularis can be imaged by adjusting the optical focus (**Supplemental Video 1**).

4. Video analysis

4.1. Measure the vessel diameter using appropriate software (**Table of Materials**), and determine the distance between two fixed points across the vessel in the acquired images.

4.2. Calculate the blood flow velocity from captured video frames by tracing the movement of labeled blood cells in the spatial distance between image locations[27].

4.2.1. Open the video of blood flow in the software (Fiji [ImageJ] was used in this protocol), and set the scale of the images.

4.2.2. Track the selected DiO-stained blood cells using the tracking function. Use the distance the cells have moved and the interval of time between image frames in the video for auto-calculation of the flow velocity.

4.3. Calculate the volumetric flow (F) per the following equation: $F = V \times A$. (V: velocity; A: cross-sectional area of the vessel).

5. Noise Exposure

5.1. Place the animals in wire mesh cages. Expose them to broadband noise at 120 dB sound pressure level in a sound exposure booth for 3 h and for an additional 3 h the next day.

NOTE: This noise exposure regime, routinely used in this laboratory, produces permanent loss of cochlear sensitivity[28].

REPRESENTATIVE RESULTS:

After surgical exposure of the cochlear capillaries in the lateral wall (**Figure 1**), intravital high-resolution fluorescence microscopic observation of DiI-labeled blood cells in FITC-dextran-labeled vessels was feasible through an open vessel-window. **Figure 2A** is a representative image taken under FIVM that shows the capillaries of the mouse cochlear apex-middle turn lateral wall. The lumina of these vessels is made visible by the fluorescence of FITC-dextran mixed with plasma. Individually labeled blood cells distributed in the vascular network are also clearly visible in this image. Two distinct networks—capillaries of the spiral ligament and capillaries of the stria vascularis—are distinguished by location (under an upright microscope, the stria vascularis runs optically beneath the spiral ligament, and it contains more capillary loops and smaller vessels[25]). Both can be assessed for blood flow with adjustment of the optical focus. As shown in **Figure 2B,C**, the vessel density of the spiral ligament is sparser than that of the stria vascularis.

Vascular function in the noise-exposed mouse model was compared with vascular function in the control group. CoBF measurement was taken 2 weeks after noise exposure. **Figure 3A,B** are representative images showing the flow patterns in control and noise-exposed groups. A

disturbed pattern of blood flow was seen in the noise-exposed group (**Figure 3B**). Anomalies included reduced vessel diameter (**Figure 3C**) and increased variation in vessel diameter (**Figure 3D**). As illustrated in **Figure 4A,B**, the blood flow velocities in the control and noise-exposed groups were calculated by tracking the routes of the DiO-labeled blood cells **(Supplemental video 2)**. The results show that blood velocity and volume in the noise-exposed group were significantly lower than in the control group (**Figure 4C,D**). These data indicate that loud sound notably affects blood circulation and causes reduced and disturbed blood flow.

FIGURE AND TABLE LEGENDS:

Figure 1: Preparation of an open vessel-window for IVM imaging in mouse. (**A**) Preparation of instruments and tools for the surgery. (**B**) The left bulla was exposed via a lateral and ventral approach. (**C**) The cochlea was exposed after removing the bulla. (**D**) Magnified image of the cochlea, from the circle in (**C**). (**E**) An open vessel window was created at the apex-middle turn of the cochlear lateral wall (box). (**F** and **G**) Intravenous infusion of FITC-dextran and labeled blood cells through the saphenous vein. Abbreviations: OW = oval window; RW = round window; IVM = intravital microscopy; FITC = fluorescein isothiocyanate.

Figure 2: Representative images of cochlear capillaries in the lateral wall. (**A**) DiI-labeled blood cells (red, arrow) in strial vessels labeled with FITC-dextran (green). (**B**) DiO-labeled blood cells (green) in spiral ligament vessels labeled with FITC-dextran (arrows, green). Note the vessels are sparse. (**C**) DiO-labeled blood cells (green) in strial vessels labeled with FITC-dextran (green). Note the vessels are denser. Scale bars: 50 µm and 100 µm. Abbreviations: DiI = 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate; DiO = 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate; FITC = fluorescein isothiocyanate.

Figure 3: Change in vessel diameter in the stria two weeks after noise exposure. (**A** and **B**) Representative images of the blood circulation after labeling vessels with FITC-dextran (green) and blood cells with DiO (green) in control and noise-exposed (NE) groups with high-resolution IVM. (**C**) Mean vessel diameter calculated for control and NE groups. Compared to the control group, the vessel diameter was reduced in the noise-exposed group. (n = 18, t (34) = 2.880, $^{**}p$ = 0.007, Student's t-test, mean ± standard deviation [SD]). (**D**) Variance of vessel diameter in control and NE groups. The vessel diameter varied much more in the NE group than in the control group. (n = 6, t (10) = 6.630, $^{****}p < 0.0001$, Student's t-test, mean ± SD). Scale bars: 100 µm. Abbreviations: DiO = 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate; FITC = fluorescein isothiocyanate; IVM = intravital microscopy.

Figure 4: Blood flow changes in the stria two weeks after noise exposure. (**A** and **B**) Representative images show the tracking routes (red, green, and blue lines) of DiO-labeled blood cells (green) for blood flow velocity measurement in control and noise-exposed groups. (**C** and **D**) Blood flow velocity (µm/s) and volumetric flow rate ($\mu m^3/s$) were respectively calculated for control and noise-exposed groups. Blood flow rate and volumetric flow rate in the noise-exposed group were lower than in the control group (n = 54, $t_{velocity}$ (106) = 19.705, $^{****}p_{velocity} < 0.0001$;

t_{volume} (106) = 15.342, ****p_{volume} < 0.0001, Student's *t*-test, mean ± standard deviation). Scale bars: 100 µm. Abbreviations: DiO = 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate; FITC = fluorescein isothiocyanate; NE 2W = two-week noise exposure.

Supplemental video 1: Blood vessels of both the spiral ligament and stria vascularis.

Supplemental video 2: Tracking the routes of DiO-labeled blood cells for calculation of the blood flow velocities in the control and noise-exposed groups. Abbreviations: DiO = 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate.

DISCUSSION:

The protocol described here is a feasible approach for imaging and investigating CoBF in the lateral wall (particularly in the stria vascularis) in mouse models using an open vessel-window combined with FIVM. The method provides sufficient resolution for determining the blood flow velocity and blood flow volume using fluorescently labeled blood cells as a tracer (**Figure 3** and **Figure 4**). This approach can be used for several different applications such as the assessment of vascular permeability and pericyte contractility and for tracking bone marrow cell migration[25]. The open vessel-window approach, involving the removal of cochlear lateral wall bone, opens different avenues for research. Acute changes in CoBF in response to trauma, infection, noise, foreign bodies, ototoxic drugs, or potentially any agent affecting the lateral wall can be monitored in real-time[25, 29].

Overall, this method is robust and can be used for investigating structural and functional changes in CoBF under normal and pathological conditions such as those arising from inflammation, noise, and aging. Importantly, a constant and normal endocochlear potential (EP) can be maintained after the surgery, indicating that the procedure is harmless to cochlear function when performed carefully enough[25]. However, the successful establishment of the open vessel-window does require a high degree of surgical skill. Care must be exercised in removing the bone of the cochlear lateral wall so as to prevent loss of perilymphatic fluid and micro-injury to the outer layer of the cochlear spiral ligament. These may adversely affect cochlear homeostasis and compromise the imaging.

An open vessel-window, in conjunction with an FIVM system, enables real-time visualization of blood flow in the cochlear lateral wall and measurement of blood flow velocity in the recorded regions. The IVM system has several advantages over other methods. For example, the animal can be conveniently positioned and manipulated as needed. There is flexibility to adjust the contrast of fluorescence labeled plasma and blood cells to optimize the visualization of vascular architecture and highlight relevant structures. The molecular weight of FITC-conjugated dextran can be selected to optimize the evaluation of vascular permeability. The use of intravenous infusion for substance delivery minimizes the administered volume needed to visualize the plasma. However, the solution should be slowly and cautiously injected to avoid oncotic imbalance in the plasma.

The labeled blood cells serve as flow indicators for the calculation of the blood flow velocity or other parameters of the microcirculation. The choice of whether to use DiI ($\lambda_{excitation}$ [λ_{ex}] = 550 nm; $\lambda_{emission}$ [λ_{em}] = 564 nm) or DiO (λ_{ex} = 484 nm; λ_{em} = 501 nm) to label the blood cells, in contrast to the FITC-dextran used to label the plasma, is determined by the experimental goals. In general, DiI provides better contrast for visualization of the vessel lumen and individual blood cells, whereas DiO is preferred when simultaneous imaging is used for visualization of blood cells and lumen in the same acquisition channel. The latter may be needed to obtain the shorter acquisition time needed to optimize the accurate measurement of flow velocity. Tracking is best accomplished with a small number of labeled cells. Blood cell fluorescence will mask the vessel lumen fluorescence if all blood cells in the vessel are labeled[21]. In addition, care should be exercised to limit the amount of solution administered, so as to minimize dilution- and viscosity-related blood flow changes in the cochlea[30].

In past decades, several relatively non-invasive methods were established to assess CoBF without removing the cochlear bony wall, including LDF, MRI, endoscopic LSCI, and injection of microspheres into the blood plasma. However, none of these approaches is useful for evaluating the absolute flow rate in individual vessels. For example, although endoscopic FME is versatile and small enough for relatively non-invasive imaging of CoBF, it can only be used to image limited regions near the round window. It is not suitable for imaging blood flow in the stria vascularis. This is a severe limitation as the stria vascularis plays such a crucial role in maintaining the EP, ion transport, and endolymphatic fluid balance, all essential for hearing sensitivity. Although OMAG is useful for visualization of blood flow in the apical area of the intact cochlea, the resolution is too poor for imaging individual capillaries in the stria vascularis of a mouse model[19].

Two-photon microscopy can be used to visualize the three-dimensional anatomy of the cochlear vasculature through an intact cochlear lateral wall. Moreover, it can be used to evaluate vessel diameter, red blood cell velocity, shear stress, and related hemodynamic parameters in the living cochlea. However, the limitation with two-photon microscopy is that the acquisition time is too long for real-time imaging[31]. This paper demonstrates how capillaries in the cochlear lateral wall (and in the stria vascularis) of a mouse model can be visualized with fluorophore labeling in an open vessel-window preparation with an FIVM system. Mouse models are widely used and preferred as a mammalian model for the investigation of human health and disease.

The mouse was among the first mammalian species in which molecular tools were developed for genetic modification, and the mouse model remains the most robust. Although surgery in a mouse model is challenging (due to the small size of the mouse cochlea), this method provides for successful investigation of CoBF and associated biological processes in the mouse cochlear lateral wall in vivo and in real time. Recently developed transgenic mouse models add further to the armamentarium available for using an open vessel-window and FIVM approach in a mouse model for better understanding CoBF associated cochlear homeostasis.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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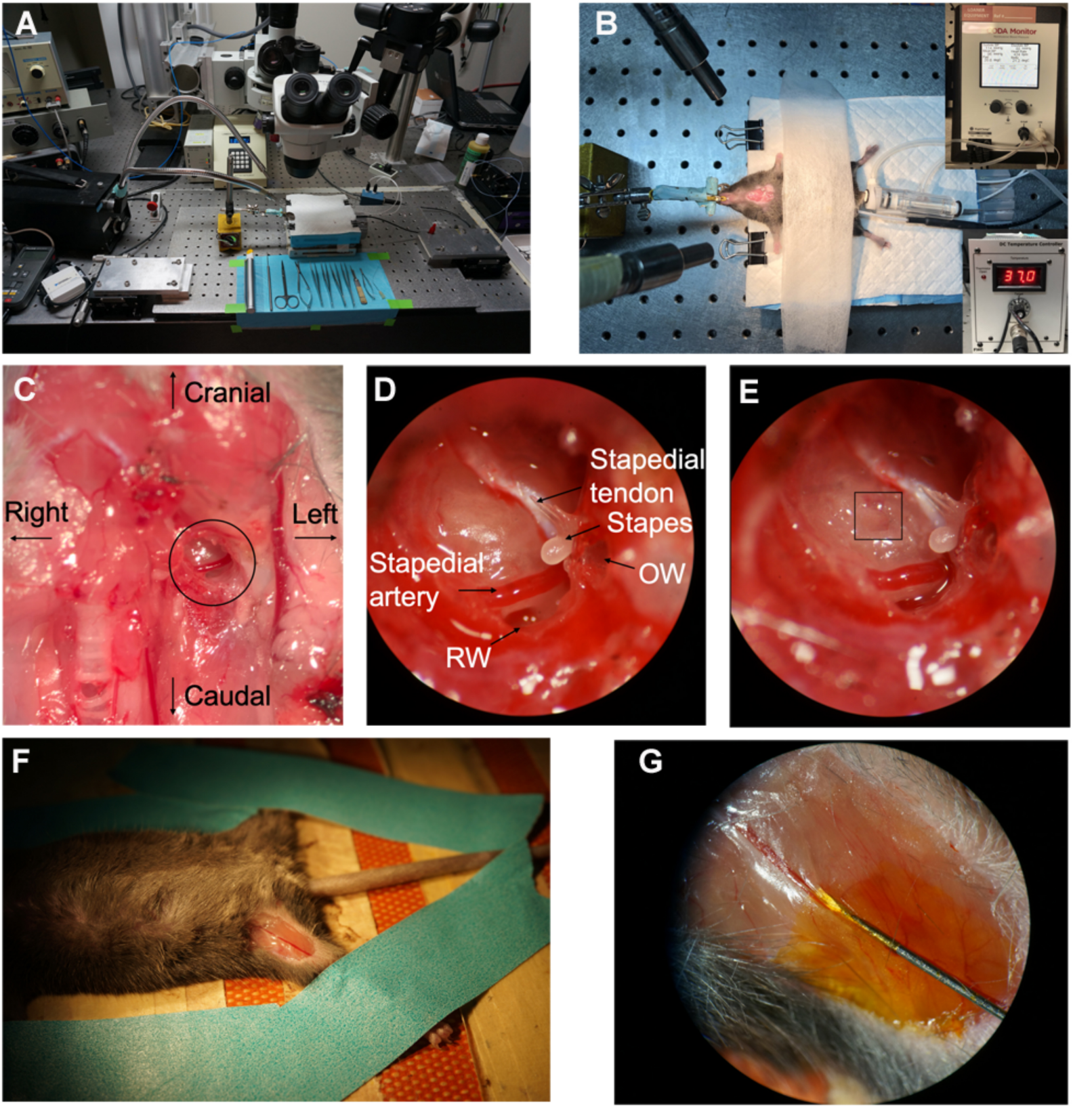

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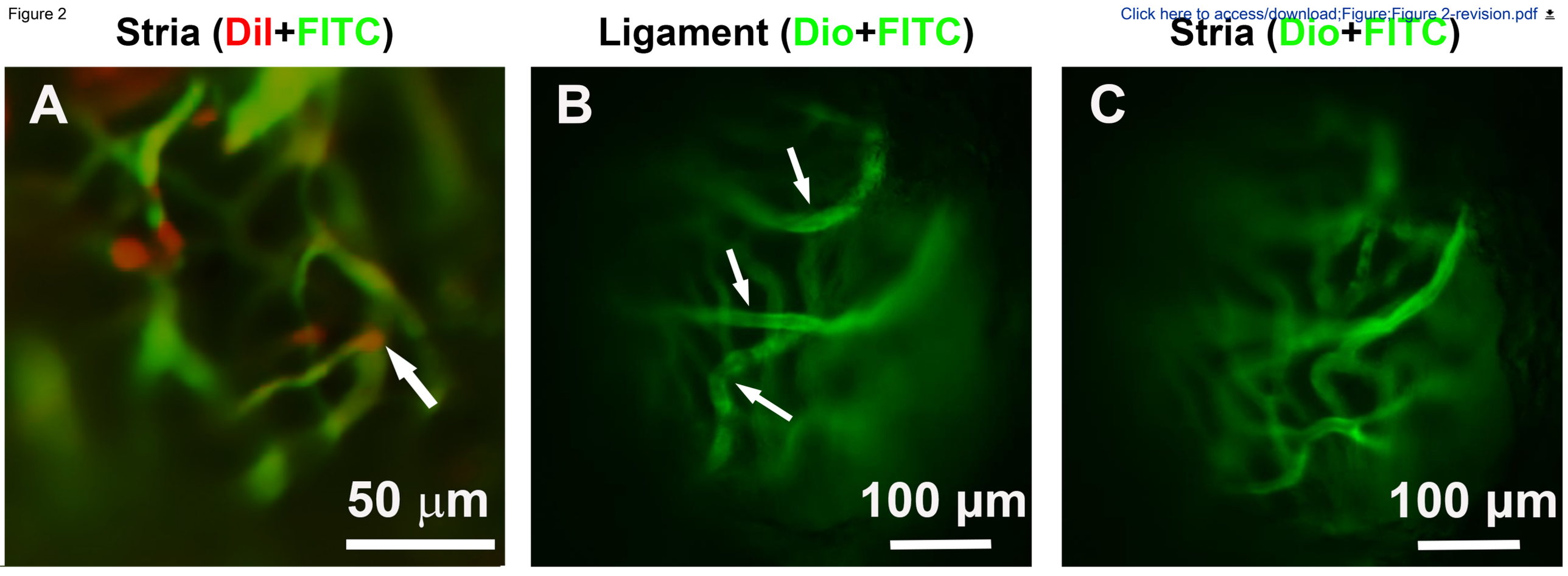

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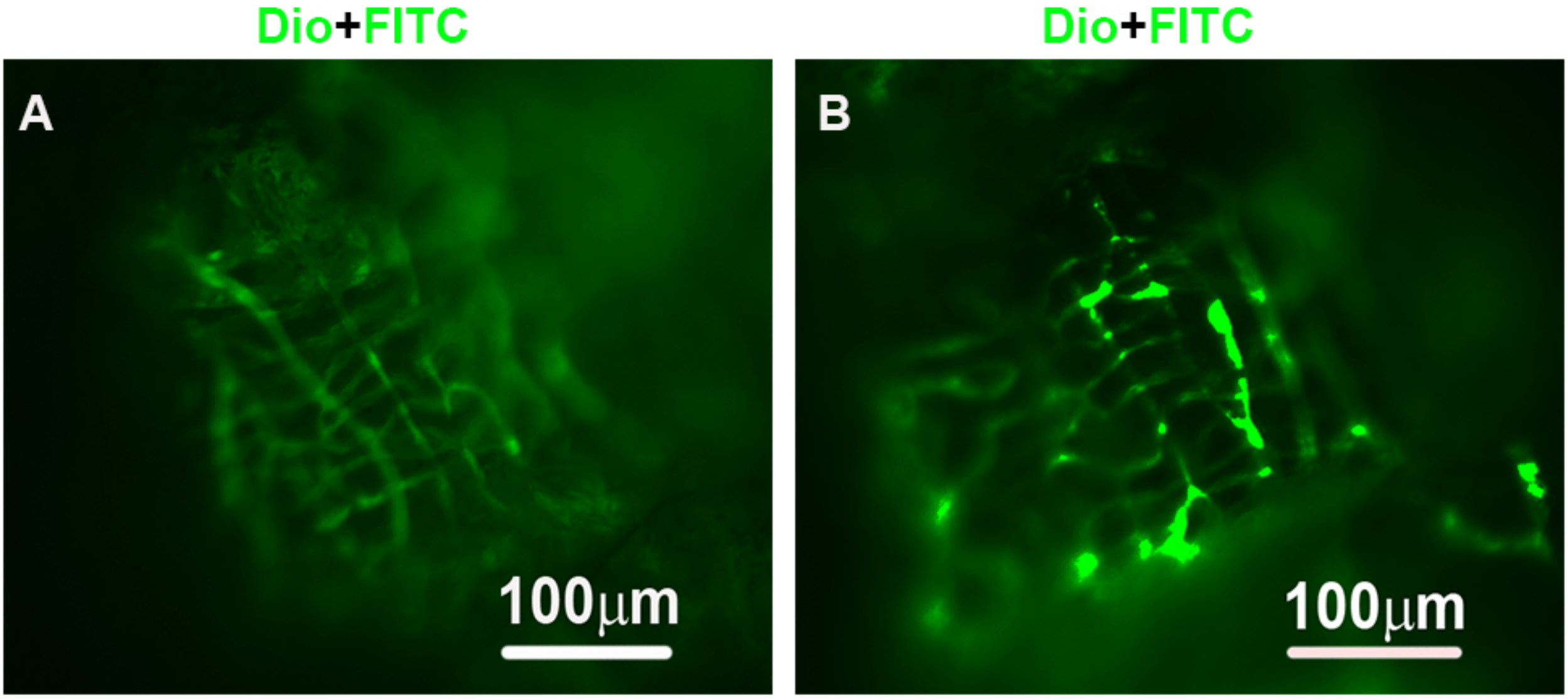

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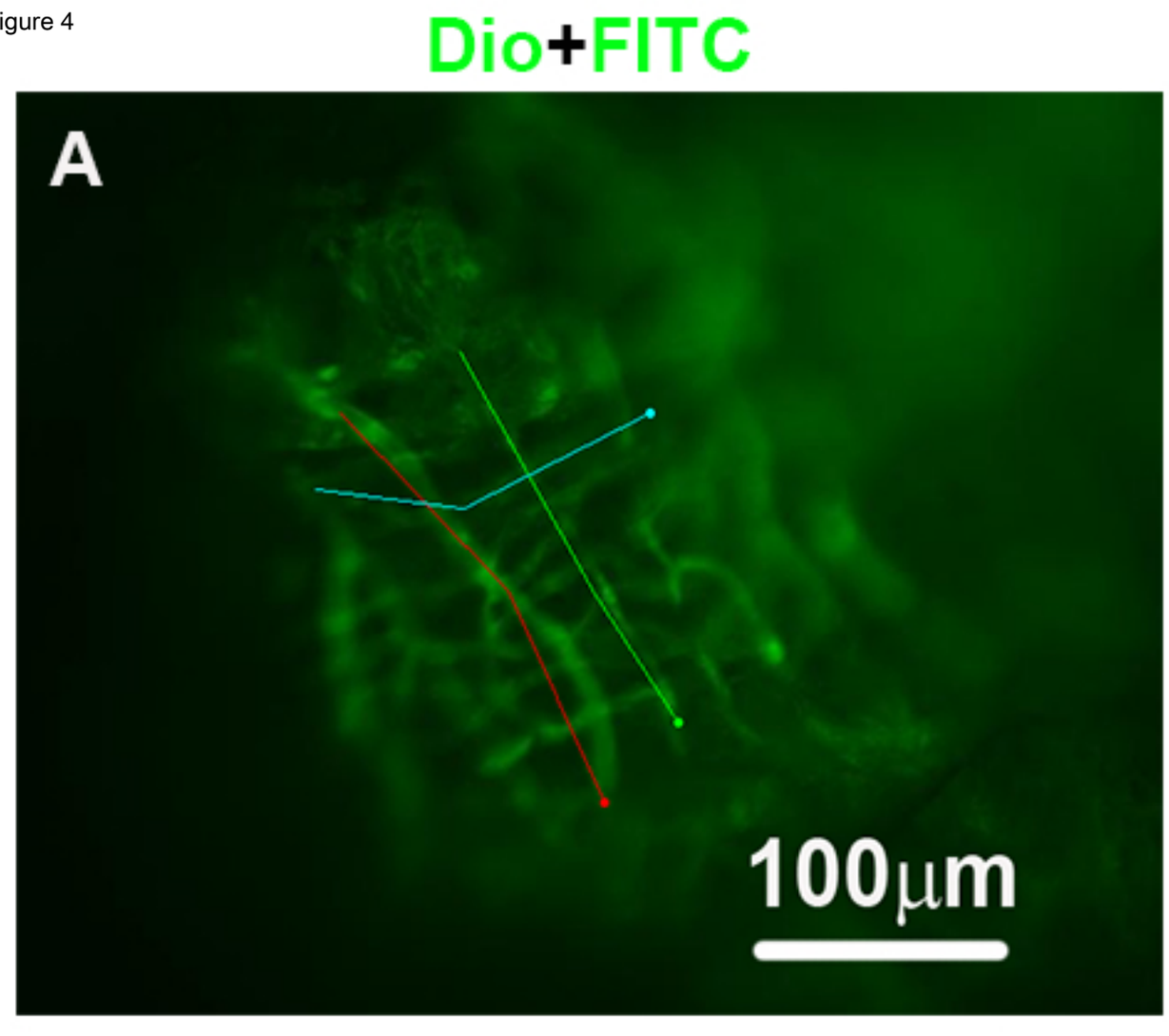

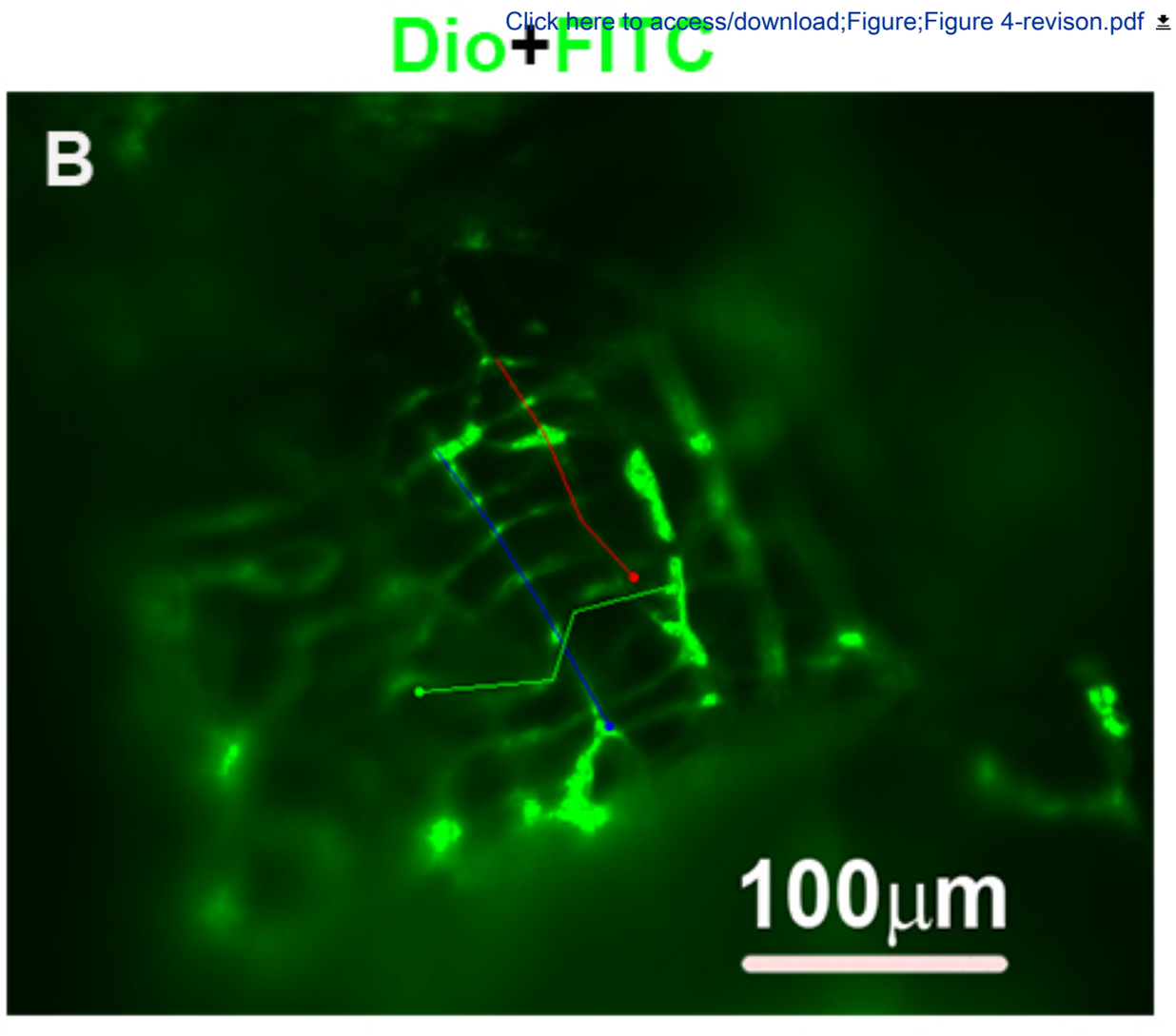

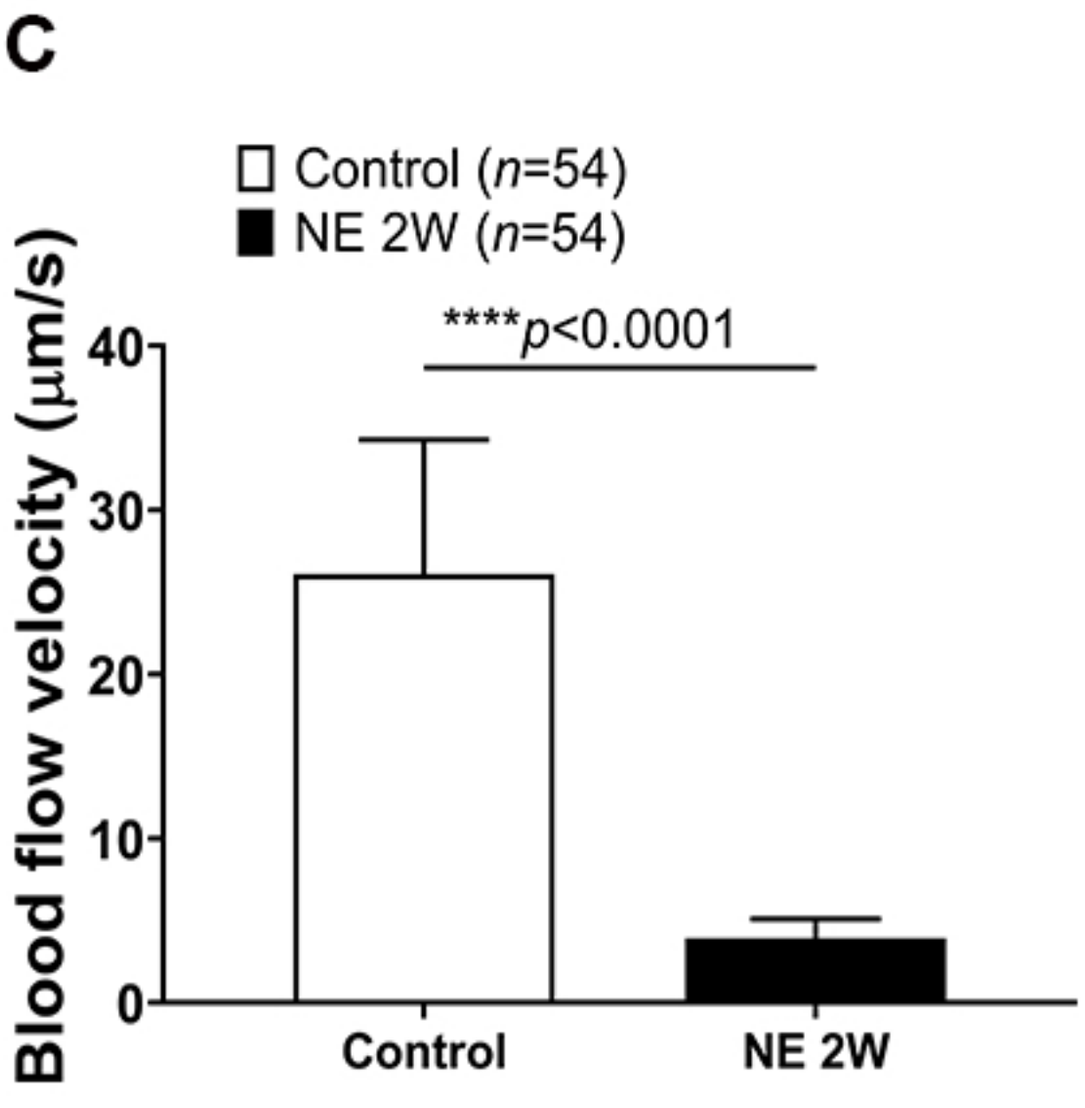

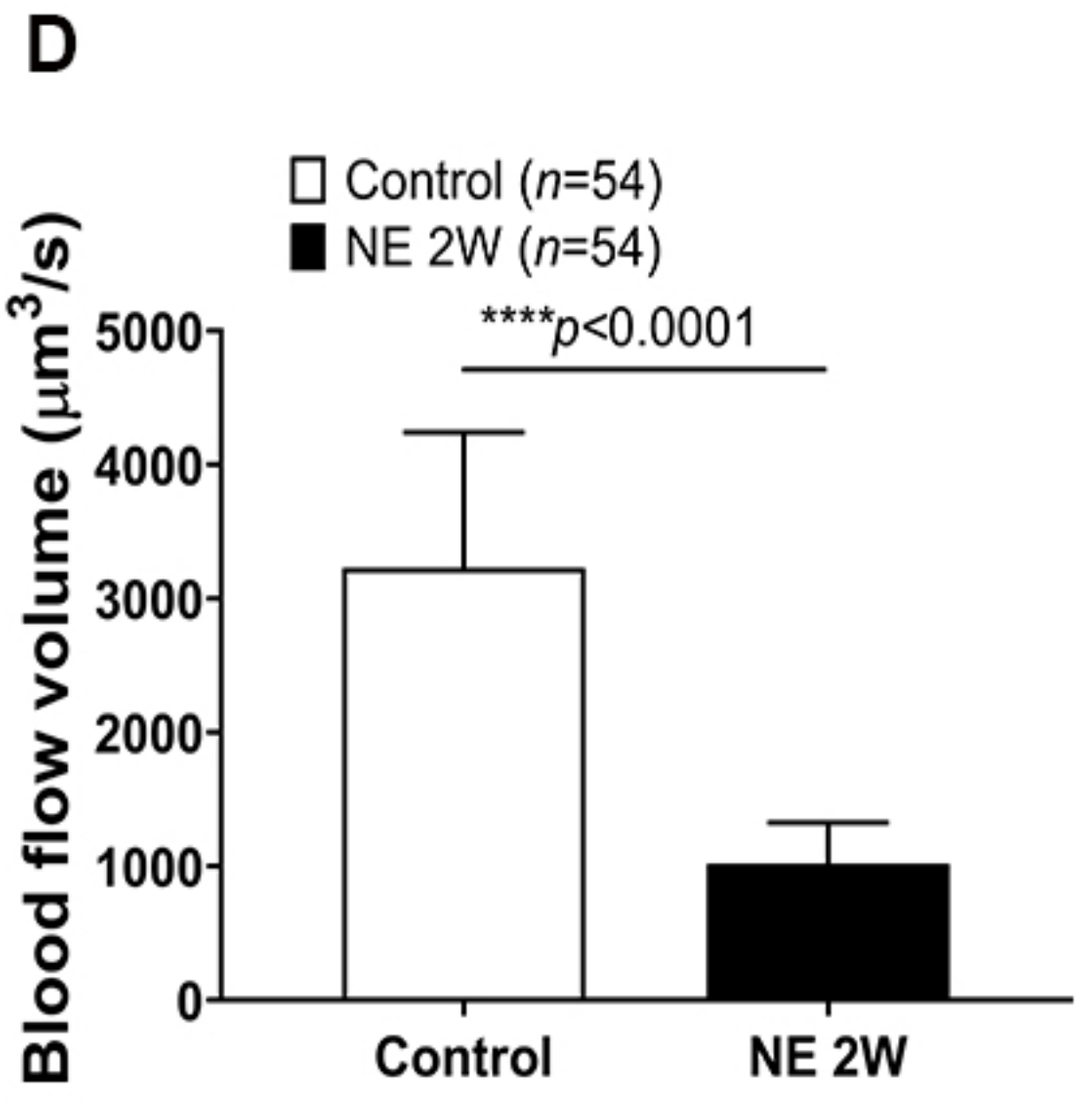

Name of Material/Equipment	Company	Catalog Number	Comments/Description
0.9% Sodium Chloride	Hospira	NDC 0409-1966-02	0.6 mL (for 1 mL)
1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate	Sigma Aldrich	468495	20 µM
3,3′-Dioctadecyloxacarbocyanine perchlorateDio (3,3′-Dioctadecyloxacarbocyanine perchlorate	Sigma Aldrich	D4292	20 µM
CODA Monitor system	Kent scientific		CODA Monitor, for monitoring blood pressure and heartbeat
Coverslip	Fisher Scientific	12-542A	
DC Temperature Controller	FHC	40-90-8D	
Fiji/ImageJ	NIH		diameter
FITC-dextran (2000 kDa)	Sigma Aldrich	FD2000s	40 mg/mL
Heparin Sodium Injection, USP MDV	Mylan	NDC 67457-374-12	5000 USP units/mL
Katathesia (100 mg/mL)	Henry Schein	NDC 11695-0702-1	0.2 mL (for 1 mL)
Microscope Objective	Mitutoyo	378-823-5	Model: M Plan Apo NIR 10x
ORCA-ER Camera	Hamamatsu		Model: C4742-80-12AG
PBS	Gibco	2085387	
Xyzaine (100 mg/ml, 5x diluted for use)	Lloyd	LPFL04821	0.2 mL (for 1 mL)
Zoom Stereo Microscope	Olympus		Model: SZ61, fluorescent microscope

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).

Supplemental video 1

Click here to access/download

Supplemental Coding Files

strial-spiral ligament-supplymental video 1 for revision.mp4

Supplemental video 2

Click here to access/download

Supplemental Coding Files

blood cell movie-supplymental video 2 for revision.mp4