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Dear Dr. Bajaj.

Review Editor

Journal of Visual Experiments

I would like to sincerely thank you for your willingness to extend the deadline for the revision and resubmission of our manuscript. We found the comments and feedback from you and the reviewers very relevant and highly helpful and we used them as guidance to improve the presentation of our manuscript. In summary, we made our best efforts to address the concerns and recommendation made by the reviewers by: 1) making all the suggested changes in the manuscript, 2) adding a new video, 3) revising figure 2, and 4) adding a new figure that includes a graph comparing microglia enumeration with the method described in our paper and conventional retinal flat mount preparation. Also, we made our sincere efforts to acknowledge and cite the most relevant publications in the field in the revised manuscript. Thus, we believe that our revised manuscript has effectively addressed the key concerns and recommendations stated by the reviewers. We agree with the first reviewer's comment that similar methods for intact whole retina imaging have been published over the last few years, however, we strongly believe that our method addresses some of the key shortcomings in the previously described methods and has merit for publication since currently a video paper describing intact whole eye preparation and imaging is lacking. In this regard, our publishing our methodology along with video would address this gap in the current literature.

Please find in the rebuttal letter our point-by-point response to the comments and recommendations expressed by the editorial team as well as the reviewers. After revaluating our manuscript, please let me know if additional clarifications or changes are needed to make our revised manuscript suitable for publication in the Journal of Visual Experiments.

Sincerely,

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TITLE:

Microsurgical Dissection and Tissue Clearing for High Resolution Intact Whole Retina and Vitreous Imaging

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KEYWORDS:

retina, whole retina imaging, retinal vasculature, collagen IV, innate fluorophore, endogenous fluorophore, GFP, light sheet microscopy, tissue optical clearing

SUMMARY:

Presented here is a protocol for intact whole retina imaging in which the outer opaque/pigmented layers of the eyeball are surgically removed, and optical clearing is applied to render retina transparent enabling the visualization of the peripheral retina and hyaloid vasculature in intact retina using light sheet fluorescent microscopy.

ABSTRACT:

Neuronal and vascular structures of the retina in physiologic and pathologic conditions can be better visualized and characterized by using intact whole retina imaging techniques compared to conventional retinal flat mount preparations and sections. However, immunofluorescent imaging of intact whole retina imaging is hindered by the opaque coatings of the eyeball, i.e., sclera, choroid, and retinal pigment epithelium (RPE) and the light scattering properties of retinal layers that prevent full thickness high resolution optical imaging for retina. Chemical bleaching of the pigmented layers and tissue clearing protocols have been described to address these obstacles; however, currently described methods are not suitable for imaging endogenous fluorescent

molecules such as green fluorescent protein (GFP) in intact whole retina. Other approaches bypassed this limitation by surgical removal of pigmented layers and the anterior segment of the eyeball allowing intact eye imaging, though the peripheral retina and hyaloid structures were disrupted. Presented here is an intact whole retina and vitreous immunofluorescent imaging protocol that combines surgical dissection of the sclera/choroid/retina pigment epithelium (RPE) layers with a modified tissue clearing method and light sheet fluorescent microscopy (LSFM). The new approach offers an unprecedented view of the unperturbed vascular and neuronal elements of the retina as well as the vitreous and hyaloid vascular system in pathologic conditions.

INTRODUCTION:

The interaction between the retinal neuronal and vascular elements in healthy and disease states is traditionally explored by immunofluorescent studies on physical sections of paraffin- or cryo-fixed retina tissue or retina flat preparations[1]. However, tissue sectioning disrupts retina neuronal and vascular continuity, and although three-dimensional reconstruction of the adjacent retina sections is suggested as a possible solution, it is still subject to errors and artifacts. Retina flat mount preparations also markedly disturb the integrity of retinal vascular and neuronal elements and the geographic connection between adjacent retinal areas[2]. Alternatively, intact whole retina imaging has recently been introduced to visualize the three-dimensional projections of retinal neuronal and vascular components in their natural anatomic position[2–5].

In intact whole retina imaging, fluorescent signals from the vascular and neuronal elements of adjacent retina areas (tiles) of an intact whole retina are captured using a light sheet microscope; these tiles are then "stitched" together to reconstruct a three dimensional view of the entire whole retina[2–6]. Intact whole retina imaging provides an unprecedented view of the retina for studying the pathogenesis of retinal vascular, degenerative, and inflammatory diseases[2–6]. For example, Prahst et al. revealed a previously "un-appreciated" knotted morphology to pathological vascular tufts, abnormal cell motility and altered filopodia dynamics in an oxygen-induced retinopathy (OIR) model using live imaging of an intact whole retina[2]. Similarly, Henning et al., Singh et al., and Chang et al. demonstrated the complex three-dimensional retinal vascular network in intact whole retinas[3,4,6]. Vigouroux et al. used an intact whole eye imaging method to show the organization of the retina and visual projections in perinatal period[5]. In order to be able to create such unparalleled three-dimensional views of the retina, intact whole retina imaging protocols have overcome two major limitations: 1) the presence of opaque and pigmented coatings of the eyeball (sclera, choroid, and RPE) and 2) the limited penetration of the light through full retina thickness caused by the light scattering properties of the retinal nuclear and plexiform layers. Henning et al. and Vigouroux et al. applied H_2O_2 bleaching of choroid/RPE pigments so as to be able to image an intact retina[3,5]. However, bleaching is not suitable for animal strains with endogenous fluorophores such as green fluorescent protein (GFP) or after in-vivo immunofluorescent stainings[3,5,7]. In addition, Henning et al.'s method of H_2O_2 treatment was carried out in aqueous conditions which may generate microbubbles that result in retinal detachment. Moreover, the H_2O_2 treatment was performed at 55 ˚C, a condition that further deteriorates tissue antibody affinity. Furthermore, bleaching may introduce heavy autofluorescence originating from oxidized melanin[8]. Other depigmentation protocols for eye sections using potassium permanganate and oxalic acid were able to remove RPE pigments in

embryonic sections but this depigmentation method also has been shown to reduce the efficacy of immunolabeling[9,10]. As an alternative to bleaching, Prahst et al., Singh et al., and Chang et al. removed sclera and choroid and cornea to render a whole retina reachable to microscope light[2,4,6]. However, removing cornea, lens, and peripheral retina may distort and disrupt peripheral retina and hyaloid vessels making these methods unsuitable for studying peripheral retina and hyaloid vasculature.

All currently available intact whole eye imaging protocols include the use of a tissue optical clearing step to overcome the light scattering properties of retinal layers[2–5]. Tissue optical clearing renders retina transparent to microscope light by equalizing the refractive index of a given tissue, here retina, across all of its cellular and intercellular elements to minimize light scattering and absorption[11]. Choroid and RPE should be removed or bleached before tissue optical clearing is applied to the retina as the pigmented coatings of the eyeball (choroid and RPE) cannot be sufficiently cleared[6,12–18].

The participation and contributions of vitreous and hyaloid vascular system in pathologic conditions such as retinopathy of prematurity (ROP), persistent fetal vasculature (PFV), Norrie Disease, and Stickler Disease is best studied when retina and hyaloid vessels are not disrupted in tissue preparation[19–23]. Existing methods for intact whole retina imaging either removes the anterior segment of the eye, which naturally disrupts the vitreous and its vasculature, or apply bleaching agents, which may remove endogenous fluorophores. Published methods for visualizing the vitreous body and vasculature in their intact, untouched condition are lacking. We describe here a whole retina and vitreous imaging method that consists of surgical dissection of pigmented and opaque coatings of the eyeball, a modified tissue optical clearing optimized for retina, and light sheet fluorescent microscopy. Sample preparation, tissue optical clearing, light sheet microscopy, and image processing steps are detailed below.

PROTOCOL:

All experiments were approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee (IACUC). Animal use and care were in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for use of animals in ophthalmic and vision research. All the materials required to carry out this procedure are listed in the **Table of Materials**. Wear powder-free gloves while performing each step. For steps 6 and 7, also refer to the official microscope operating manual.

1. Preparation of the animals

1.1. Euthanize the experimental mice in accordance with applicable Institutional Animal Care and Use Committee-approved protocol (anesthesia with a combination of Ketamine 60 mg/kg and Dexmedetomidine 0.5 mg/kg followed by cervical dislocation was used here). Immediately proceed to stabilizing the animal on a platform for dissection and heart perfusion.

NOTE: Experimental animals are chosen based on the design of individual study.

1.2. Dissect the abdomen and thorax to expose the heart. Perform cardiac perfusion by transfusing the heart via a 27 G needle placed in the left ventricle and create a small (~1 mm) incision in the right atrium to allow egress of blood[24].

1.2.1. First, transfuse 30–50 mL of ice-cold phosphate balanced saline solution (PBS) and then, 30–50 mL of freshly prepared 4% paraformaldehyde (PFA).

1.2.2. To check for a successful PFA transfusion, check for visible muscle twitches throughout the body and tail. Proceed to the enucleation step.

2. Eyeball enucleation and fixation

2.1. Use a curved jeweler's forceps to gently push over the upper or lower eyelid to force the eyeball out of its socket. Use another set of jeweler's or similar forceps to puncture the conjunctiva from the side and hold the globe from the optic nerve side. Slowly lift the globe from its socket until it is severed from the optic nerve.

2.2. Transfer the globe to a tube containing freshly prepared ice-cold 4% PFA. Label the tube accordingly. Allow the globe to remain in 4% PFA in a 4 °C fridge for 12 h (overnight).

NOTE: Use a plastic transfer pipette with a cut tip to transfer the globe. Widen the opening of the cut tip with a second pipette tip to avoid damaging the sample with sharp edges.

3. Dissection of the sample (Figure 1 and Figure 2)

3.1. Under a stereomicroscope, locate the cornea-sclera junction (**Figure 1A**) and, use the sharp cutting tip of a 30 G needle to make a very superficial cut at the sclera approximately 0.5–1 mm behind the cornea-sclera junction (**Figure 1B**).

3.2. Advance one of the blades of a sharp tip dissecting scissors through the incision that was just made into the potential space between the sclera/choroid/RPE and the retina (**Figure 1C**). Advance the scissors and cut circumferentially until the sclera/choroid/RPE can be peeled off from the outer surface of the retina (**Figures 1D,E**).

NOTE: It is important to perform this step slowly and gently to avoid puncturing the retina. The first few cuts are particularly critical to avoid cutting through retina.

3.3. If needed, make radial relaxing cuts on the sclera/choroid/RPE to facilitate the process of circumferential cutting and the subsequent peeling of the optic nerve and sclera/choroid/RPE. Remove small patches of RPE (**Figure 1F**) using a size 1 painting brush soaked in PBS.

3.4. Transfer the whole intact eyeball to a tube containing PBS. Proceed immediately to the next step or preserve in 4 ˚C for immunolabeling.

NOTE: Marks may be placed on the eyeball after enucleation and then, after dissection to preserve the orientation of the eye if needed. The protocol may be paused here, and the samples may be preserved overnight in a 4 °C fridge before proceeding to the next steps.

4. Vascular staining

4.1. Permeabilize the tissue by immersing it in PBS containing 0.2% Tween-20 at room temperature for 20 min.

4.2. Wash the sample with PBS 3 times on a shaker for 10 min.

4.3. Incubate the sample with 5% normal goat serum (NGS) in PBS containing 0.25% Triton X-100 at room temperature for 1 h.

4.4. Incubate with the primary antibody at 4 ˚C overnight. Here, an anti-mouse Collagen IV antibody was used (final concentration was prepared in PBS containing 0.2% Tween-20).

4.5. Wash 3 times with PBS, for 5 min per wash.

4.6. Incubate the sample with fluorescent-labeled secondary antibodies. Here, an anti-rabbit Alexa Fluor 568 was used for 12 h at 4 ˚C (1:200 dilution in PBS containing 0.2% Tween-20).

4.7. Wash with PBS 3 times, for 1 h each, and then proceed with tissue clearing steps.

5. Optical clearing with 2,2'-thiodiethanol (TDE)

5.1. Prepare working TDE concentrations using stock TDE solution with PBS for a final concentration of 10%, 20%, 30%, 40%, 50%, and 60% volume to volume (v/v). Prepare at least 2 mL of solution for each eye sample to allow enough excess volume to penetrate the tissue.

5.2. Incubate the samples in a 6 or 12 well plate well at increasing concentration of TDE. Start by immersing the intact whole eyeballs in 10% TDE solution for 2–4 h on a shaker at room temperature. Successively, transfer the sample to a higher TDE concentration for 2–4 h in each TDE concentration (**Figure 2C**).

NOTE: Retina starts to clear at concentrations of 40%–50%, but maximum clearing occurs after incubation in a 60% solution. Retina becomes less transparent at concentrations of 70% and higher (**Figure 2D**).

5.3. Stop the clearing process overnight, if needed, at any of the successive clearing exchange steps.

6. Whole eye imaging using a light sheet microscopy

6.1. Mount the intact whole eye samples considering the configuration of the light sheet microscope platform being used. Follow the microscope and acquisition software instructions to set up acquisition parameters including light sheet alignment and the illumination and detection of optical paths.

NOTE: The samples used in this experiment were glued from the cornea side to the tip of a hypodermic needle on an insulin syringe (**Figure 2E**). The sample was then suspended inside the microscope chamber.

6.2. Fill the microscope chamber with 60% TDE as clearing solution.

6.3. Immerse the sample within the light sheet microscope chamber in 60% TDE solution (the final clearing concentration).

6.4. Image the cleared eye by means of a variety of commercial or custom-built confocal and light sheet microscopes. In this protocol, a dual-side illumination light sheet microscope is used.

6.5. Use low resolution and low magnification imaging (5x, NA 0.16) to image cellular morphology and cellular process tracing especially when combined with tiling. Use high resolution and magnification imaging (20x, NA 1.0) to image both cellular morphology and large sub-cellular organelles such as nuclei and mitochondrial clusters.

7. Post-acquisition image processing

NOTE: Post-acquisition processing depends on the type of file and software compatible with the imaged files.

7.1. Apply deblurring or deconvolution to further augment the raw images prior to stitching the imaged tiles. A Weiner filter can be applied to deblur the images. Alternatively, images can be iteratively deconvolved after denoising with the Richardson-Lucy deconvolution and a theoretical or experimentally measured PSF using modelling tools such as the ImageJ PSF generator plugin[25].

7.2. Perform the stitching of pre-processed z-stacks and an affine and non-rigid volume transformations followed by multi-view volume registration and fusion using a variety of commercial or public-domain software packages (ImageJ – BigStitcher plugin)[26].

REPRESENTATIVE RESULTS:

A zero-angle projection of peripapillary vascular network and microglia is shown in **Figure 3A**. Also, intact whole retina microglia distribution in a CX3CR1^{-GFP} mouse is presented in **Figure 3B**. A major advantage of the method presented here, is its ability to image innate fluorophores. **Figure 3C,D** show microglia in representative Z projections (green channel) from samples prepared with the current method of intact whole eye imaging (**Figure 3C**) and flat mount preparations (**Figure 3D**). Microglia were quantified and compared in randomly selected regions

of interest from intact whole retina and flat mount preparations. No statistically significant difference was noted between the flat mount and intact whole retina imaging in terms of microglia numbers (**Figure 3E**). All representative images in **Figure 3** were captured from CX3CR1^{-GFP} mouse retina.

GFP-tagged microglia (green channel) and the vascular network (red channel) in a CX3CR1^{-GFP} mouse retina that was imaged using the current intact whole retina imaging protocol is further described in **Supplemental Video 1**. Endogenous and in vivo staining fluorophores would have been bleached if the pigment bleaching methods used by Henning et al.[3] and Vigouroux et al.[5] were used to remove choroid and RPE pigmentation. **Supplemental Video 2** shows a view of the hyaloid vessels and retrolental vascular plexus in their natural state. It should also be noted that removing the anterior segment of the eye as described by Prahst et al.[2] and Chang et al.[4] would have disturbed the hyaloid vessels.

FIGURE AND TABLE LEGENDS:

Figure 1: Dissection of sclera/choroid/retinal pigment epithelium. (**A-F**) Removal of outer opaque and pigmented layers allows high resolution optical imaging of retina and vitreous cavity without interference from surrounding pigmented structures.

Figure 2: Sample preparation for intact whole eye imaging. (**A**) After sampling, the eyeball was covered with opaque sclera, choroid, and RPE. (**B**) Opaque sclera/choroid/RPE were dissected out but the retina tissue was still relatively optically opaque for microscopic imaging. (**C**) Successive immersion in increasing concentrations of TDE rendered the sample clear. After the sample was clear (**D**), it was mounted onto the light sheet microscope platform for high resolution imaging. (**E**) For the light sheet microscope system that was used in this experiment, the intact whole retina was mounted to the tip of a hypodermic needle on a syringe using cyanoacrylate glue. The sample was then placed inside the imaging chamber that was filled with the final concentration of the clearing agent.

Figure 3: Representative results for intact whole retina imaging. (**A**) Zero angle projection of the peripapillary vascular network and microglia in an 8-month old female CX3CR1^{-GFP} mouse intact whole retina. (**B**) Flat projection of the green channel showing microglia distribution in an intact whole retina from an 8-month old female CX3CR1^{-GFP} mouse. Peripheral retina was distorted in this flat projection of the cup shaped intact retina. (**C-D**). Representative 250 µm x 250 µm square flat projection from an intact whole retina scans (**C**) and flat mount preparation (**D**), both from 8-months old female CX3CR1^{-GFP} mice (scale bar 50 µm). (**E**) Retinal microglia enumeration in flat mount preparations versus intact whole retina imaging using light sheet fluorescent microscope: Three regions of interest (ROI) were randomly selected from the mid-peripheral retina for microglia quantification. Orthogonal projections of the entire retina thickness in green channel was prepared for each ROI. Microglia numbers were counted within ImageJ. A two tailed Student's t-test did not show statistically significant difference in the number of microglia in flat mount and intact whole eye preparations.

Supplemental video 1: Intact whole retina imaging of a CX3CR1^{-GFP} mouse. Retinal vessels were

visualized in their entirety. Imaging was performed without choroid/RPE pigment bleaching. In this GFP-tagged mouse (and similar strains with innate fluorophores), choroid/RPE bleaching would have removed the GFP-tagged microglia signals. In this video, the three-dimensional depiction of retinal microglia distribution highlights this method's strength in detecting microglia distribution and activation.

Supplemental video 2: Hyaloid vasculature and retrolental vascular plexus in a 4-day old mouse. Anterior segment of the eyeball, including the cornea, iris, and lens, was not removed, leaving the vitreous cavity and its vasculature untouched.

DISCUSSION:

Retina and vitreous development and pathologies are best studied with intact whole retina imaging techniques in which the retina is not cut for sections or for flat mount preparations. Existing intact whole eye imaging methods either incorporate pigment bleaching, which removes innate fluorophores, or involve physical removal of the opaque coatings of the eyeball (RPE, choroid, and sclera) along with the anterior segment of the eye, which may disturb peripheral retina and vitreous body. Chang et al. and Prahst et al. removed the outer coatings of the eyeball as well as the cornea thereby possibly disrupting the vitreous body and hyaloid vasculature and peripheral retina[2,4]. As such, their method may not be suitable for studying cellular features of retina and vitreous development and pathologies involving hyaloid vasculatures. Alternatively, Henning et al. and Vigouroux et al. used pigment bleaching as a technique to render choroid and RPE transparent for microscopic imaging[3,5]. However, pigment bleaching removes GFP and may compromise the performance of other innate/endogenous fluorophores as well as the fluorophores used for in vivo staining as imaging probes[7]. In the method introduced here, no bleaching was used, and the sclera, choroid, and RPE were dissected out while the cornea, iris, and lens were left untouched allowing for the visualization of the anterior segment of the eye, peripheral retina and hyaloid vessels in their undisturbed state.

The existing methods described for visualization of hyaloid vessels in diseases such as persistent fetal vasculature (PFV) and retinopathy of prematurity (ROP) involve dissecting anterior segment of the eye that disturbs the vitreous body and inevitably hyaloid vasculature[22,23]. In contrast to these methods, the current protocol does not perturb hyaloid and retrolental vasculature thus allowing for the visualization of the intact hyaloid vasculature as shown in **Supplemental Video 2**.

All the existing methods for intact whole eye imaging, including the method outlined above, involve tissue optical clearing, a process that matches a tissue's refractive index with the surrounding medium to minimize light scattering and absorption[11,27]. Three major tissue clearing approaches include hydrophobic, hydrophilic and hydrogel-based methods depending on the agent used to clear the tissue[13,27–31]. A variety of these tissue clearing techniques have been successfully used in the previously described intact whole eye imaging methods, but they have all been time consuming, taking 3 to 7 days[4,5]. The protocol detailed here uses a modification of the previously-described 2,2'-thiodiethanol (TDE) exchange method to suit retina tissue[32]. In the current protocol, the intact whole retina/vitreous was immersed in a successively increasing

concentration of TDE to gradually equalize the tissue's refractive index and to render it clear in less than two days. TDE is a glycol derivative hydrophilic clearing agent miscible with water in any ratio that makes it possible to create a gradient of concentration to allow penetration of high refractive index solution into the tissue. TDE does not quench the fluorescence of various fluorophores unlike certain other clearing methods such as CLARITY and CUBIC[33]. In addition, TDE does not have the potential to damage equipment, as is the case for organic solvent-based clearing agents such as benzyl benzoate/benzyl alcohol (BABB). It also prevents the growth of contaminating organisms that is observed with the fructose based SeeDB clearing method[33].

Light Sheet Fluorescent Microscopy is an efficient tool with a high temporal and spatial resolution for imaging thick tissues[2–6,34]. In LSFM, a thin sheet of light is directed through the sample to excite fluorophores only in a thin imaging plane that in turn enables lower phototoxicity and photobleaching, faster imaging, and higher contrast due to minimal out of focus excitation. Imaging of the dissected and optically cleared whole murine eyes as described here may be performed utilizing one of the many commercially available LSFM or confocal microscopes. In the current method, LSFM offers a fast and detailed view of retinal neurons and vessels and enables a quantitative analysis of microglia density.

The current method is limited in showing the photoreceptor-RPE interaction because RPE is being removed during dissection. Such interaction may be better studied with methods that uses bleaching and skip the dissection[3,5]. Tissue shrinkage and possible distortion are inherent limitations of all the tissue fixation and clearing methods including the method described here. These limitations should be acknowledged when interpreting results obtained with the current and other similar methods. Two critical steps that contribute to the success of this method include good surgical dissection of the ocular coatings without damaging the retina in combination with the application of TDE as optical clearing agent. Dissecting sclera/choroid/RPE layers in a small rodent eye can be technically challenging and cutting through peripheral retina or the anterior segment of the eye should be avoided. Mastering this technique requires the same set of skills utilized in retina flat mount preparation and, in our experience, essential dexterity can be acquired after performing the dissection steps described above on three to five eyes. The incubation period for each clearing step may be modified if the method needs to be applied on larger animal (such as rabbit) eyes. Whether hyaloid vessel stain better with a modified immunolabeling technique by injecting the primary and secondary antibodies into the vitreous cavity needs to be explored in future.

In summary, the method described here is based on further development of previously described techniques for high resolution whole retina imaging enabling imaging of the molecular and structural targets including innate and in-vivo staining fluorophores in an intact whole retina and vitreous. This method addresses several of the limitations of existing intact whole retina preparation methods while offering a new approach for an unprecedented visualization of the hyaloid vasculature in intact retina.

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DISCLOSURES:

No relevant commercial conflict of interest.

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29. Zaman, R. T. et al. Changes in morphology and optical properties of sclera and choroidal layers due to hyperosmotic agent. *Journal of Biomedical Optics.* **16**, 077008 (2011).
30. Chung, K., Deisseroth, K. CLARITY for mapping the nervous system. *Nature Methods.* **10**, 508–513 (2013).
31. Renier, N. et al. IDISCO: A simple, rapid method to immunolabel large tissue samples for volume imaging. *Cell.* **159**, 896–910 (2014).
32. Costantini, I. et al. A versatile clearing agent for multi-modal brain imaging. *Scientific Reports.* **5**, 9808 (2015).
33. Aoyagi, Y., Kawakami, R., Osanai, H., Hibi, T., Nemoto, T. A rapid optical clearing protocol using 2,2'-thiodiethanol for microscopic observation of fixed mouse brain. *PLoS One.* **10**, e0116280 (2015).
34. Icha, J. et al. Using Light Sheet Fluorescence Microscopy to Image Zebrafish Eye Development. *Journal of Visualized Experiments* (110), e53966 (2016).

Figure2

Click here to access/download;Figure;Revised figure 2.tif

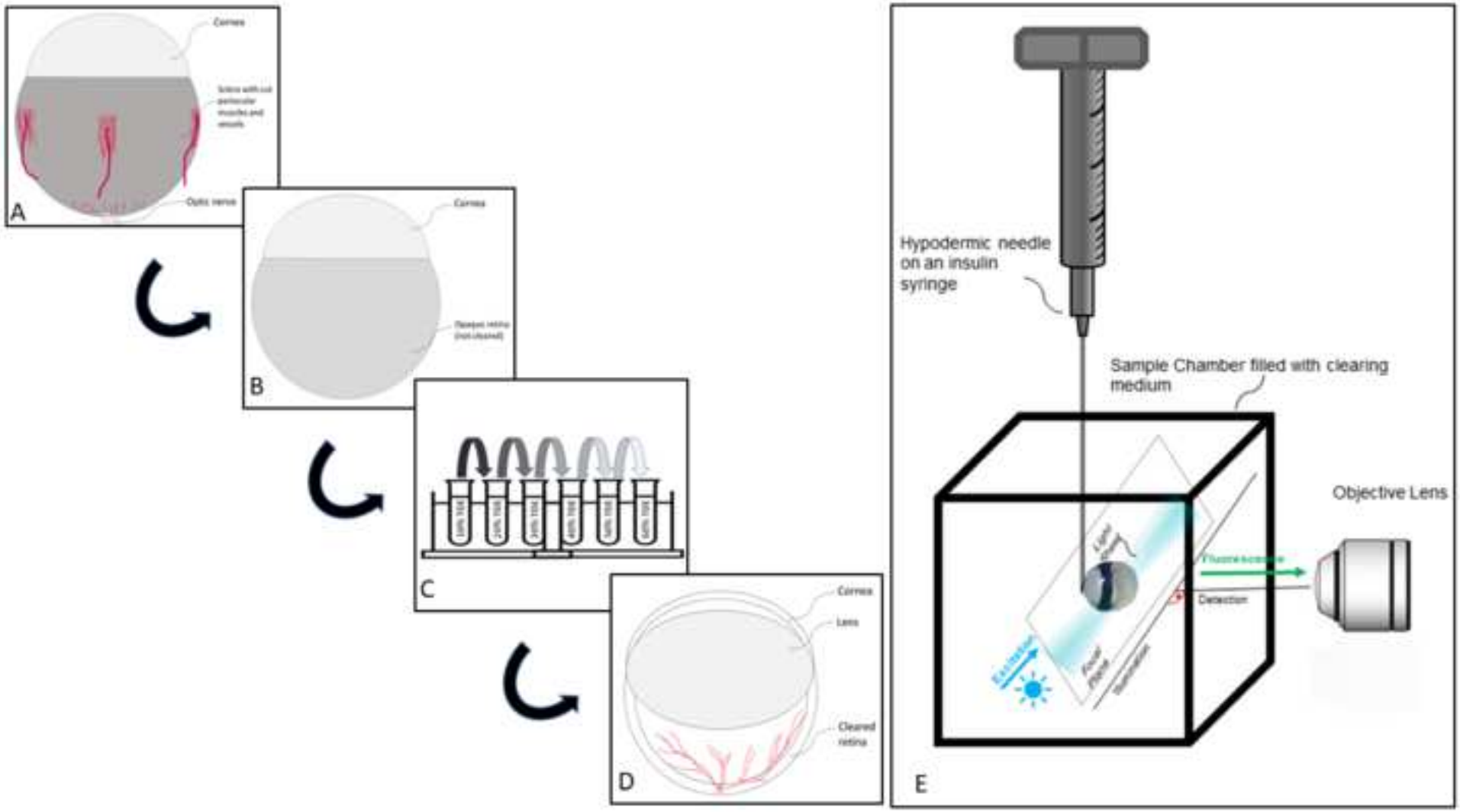


Figure3

Click here to access/download;Figure;Revised Figure 3.tif

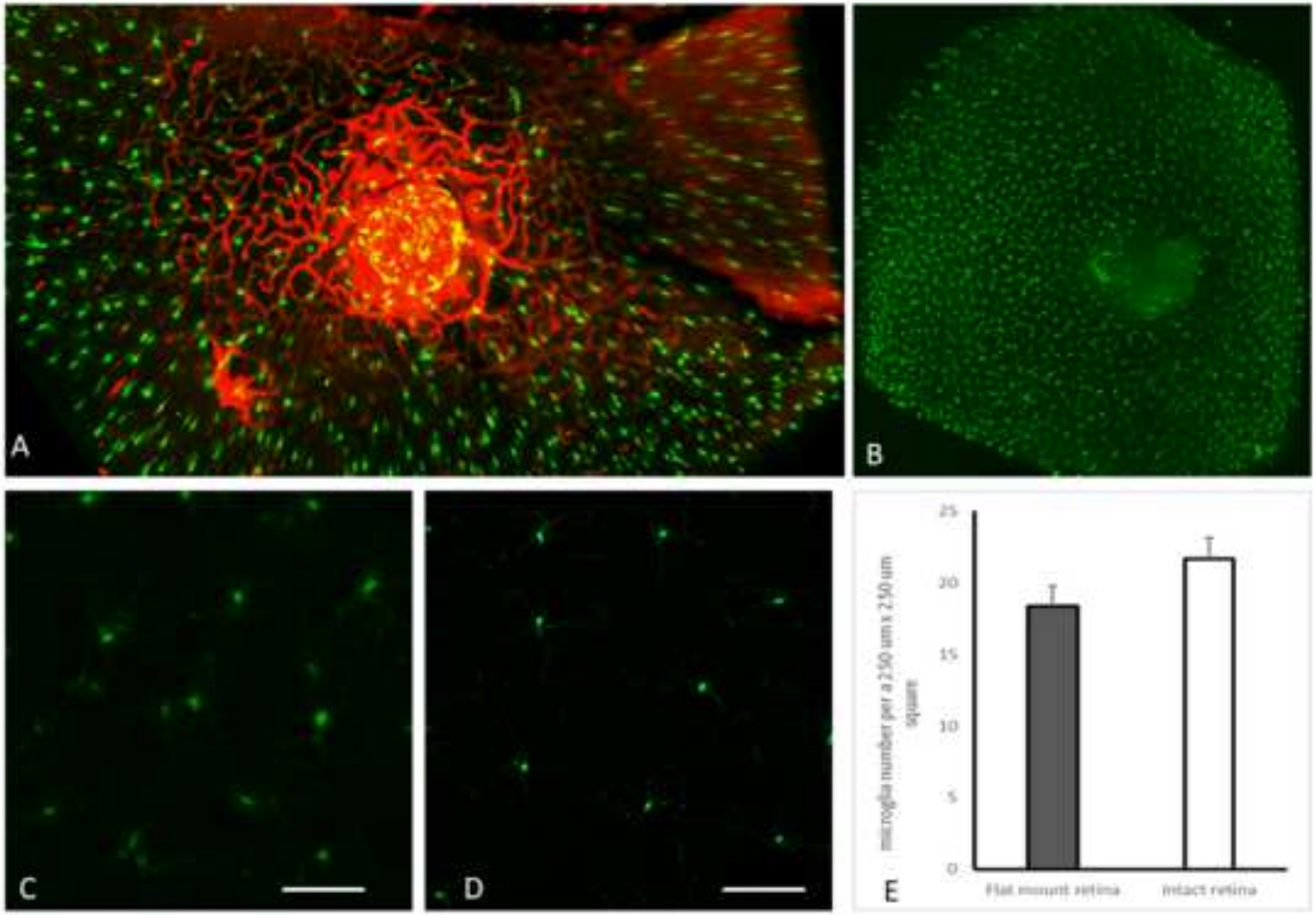
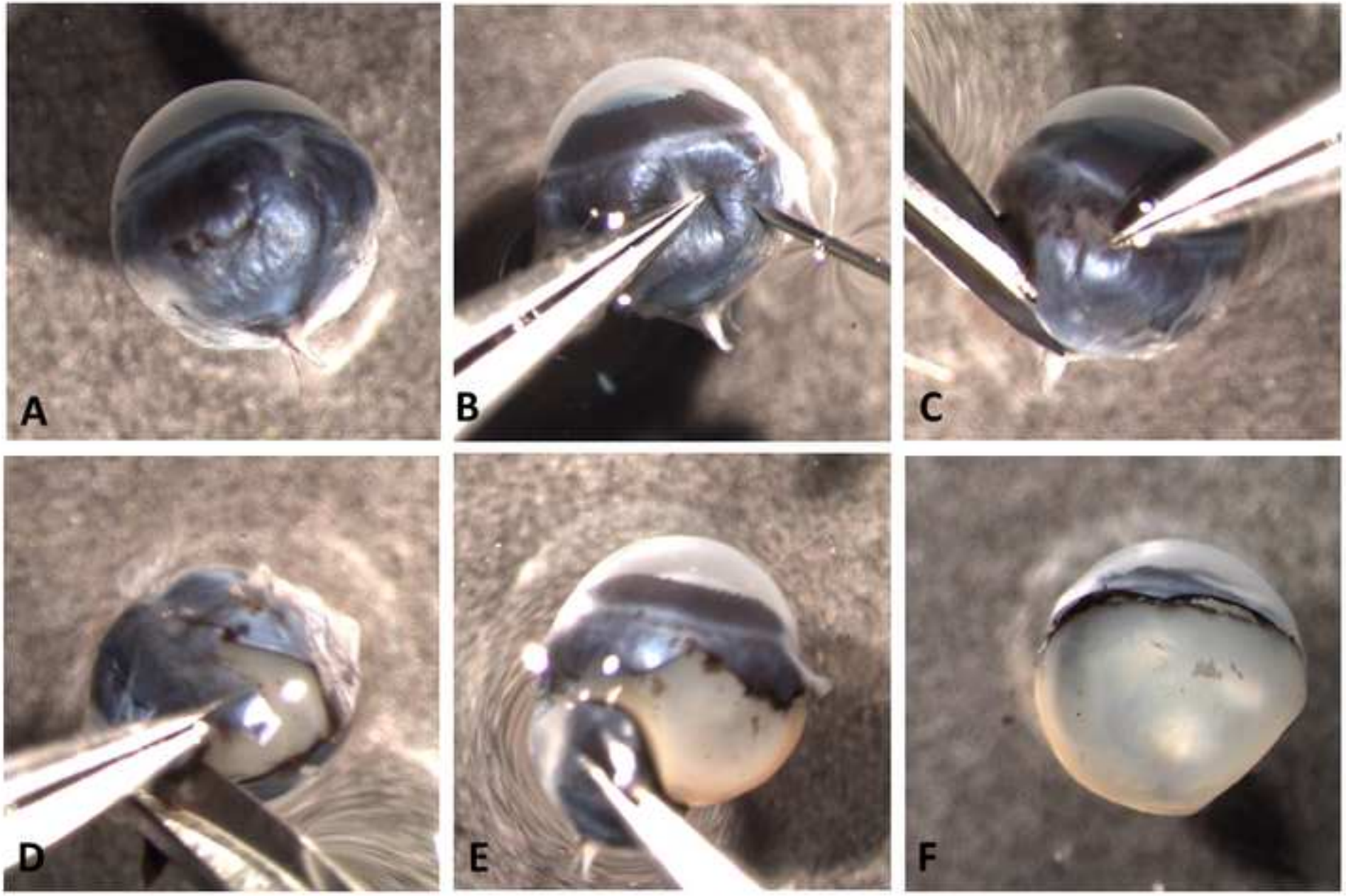
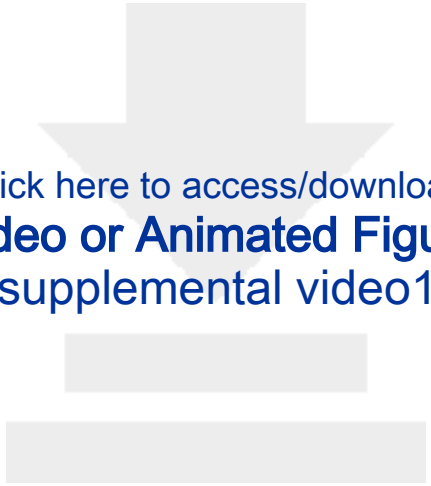


Figure1

Click here to access/download;Figure;Figure 1.tif



Video or Animated Figure



Click here to access/download

Video or Animated Figure

New supplemental video1.mp4

Video or Animated Figure

Click here to access/download

Video or Animated Figure

New supplemental video 2.mp4

Table of Materials

Click here to access/download;Table of Materials;Table_of_Materials (2).xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Experimental animal			
CX3CR1-GFP Mouse	The Jackson Laboratory	5582	
Anesthetic			
Dexmedetomidine	Par Pharmaceutical	42023-146-25	
Ketamine	Fresenius Kabi		
Tissue harvesting, fixation, and sample dissection			
cardiac perfusion pump	Fisher scientific	NC9069235	
Cyanoacrylate superglue	amazon.com		
Fine scissors-sharp	Fine Science Tools	14160-10	
Fine tweezers	Fine Science Tools	11412-11	
Paraformaldehyde (PFA)	Electrone microscopy sciences	15710-S	
Phosphate buffered saline (PBS)	Gibco	10010049	
size 1 painting brush	dickblick.com		
straight spring scissors	Fine Science Tools	15000-03	
syringe, needle tip, 27 gauge x 1.25"	BD		
Tubes 1.5 ml, 15 ml, 50 ml	Thermo sceintific		
Tween-20	ThermoFisher	85114	
Immunofluorescent staining			
Anti-mouse collagen IV antibody	Abcam	ab19808	1:200 dilution
Anti-rabbit Alexa Fluor 568	Invitreogen	A-11011	1:200 dilution
Normal goat serum	ThermoFisher	50062Z	10% concentration
Tissue clearing			

2,2′-thiodiethanol (TDE)	Fluka analytica	STBD7772V
Rocking shaker	Fisher scientific	02-217-765
Microscopy		
Fluorescent microspheres	TetraSpeck	T14792
Light sheet fluorescent microscope (LSFM)	Zeiss	Z1
Microglia enumeration		
ImageJ	National Institue of Health	

Response to editorial and reviewers' comments:

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. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

- We proofread our manuscript to ensure there are no spelling or grammar issues. We appreciate all the comments and suggestions.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

- The revised manuscript is re-formatted as instructed.

3. Please provide an email address for each author.

- Email addresses are provided.

4. Please make the title concise and remove hyphens, colons etc.

- The title is revised as suggested.

5. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Presented here is a protocol …"

- The short summary is revised as suggested.

6. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

- The long abstract is revised as suggested.

7. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s) without brackets.

- In-text reference numbers are changed as suggested.

8. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Zeiss Light sheet Z.1 microscope, ZEN Black acquisition software, Abcam ab19808, Sigma Aldrich182 #88561, (ZEN 2014 SP1 version 9.2.0.0, arivis Vision4D, Imaris, etc

- We revised the imaging and image processing steps and removed all the microscope specific details and commercial names as suggested.

9. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.

- Numbering is revised as instructed.

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

- The manuscript is revised as suggested.

11. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

- The manuscript is revised as suggested.

12. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

- The manuscript is revised as suggested. The protocol only contains action items and notes in the revised version.

13. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.

- The manuscript is revised as suggested.

14. Please ensure you answer the “how” question, i.e., how is the step performed?

- The manuscript is revised as suggested.

15. Any age and sex specific bias of the mouse used? How do you perform euthanasia in your lab?

- No age and sex preference for the purpose of this study. We specified this in the revised manuscript.

16. We cannot have supplementary checklist of materials in the manuscript. Please include all

materials in the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the table in alphabetical order.

- Supplementary checklist of material is removed from the manuscript. Supplementary checklist Excel file is updated considering the suggestions.

17. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

- We revised the manuscript as suggested. The protocol section in the manuscripts passes the 2.75-page limit. However, this section now includes many “NOTE” that if removed, the filmable content would fall within the 2.75-page limit.

18. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

- Suggested changes are made. A new paragraph is added to the discussion explaining the representative results.

19. Please include a figure or a table in the Representative Results showing the effectiveness of your technique backed up with data.

- New figure 3 represents orthogonal projection images as requested by the reviewers. It also includes microglia quantification comparing the presented method and flat mount preparation.

20. 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].”

- All the results and figures/videos are original. No copyright permission is needed.

21. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

- The discussion is revised to include these suggestions.

22. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate the journal titles.

- References are revised as suggested.

23. Please include a still Figure 3 to show the effectiveness of the technique and a video/movie file can be added along.

- A new figure 3 includes representative still images and a comparison between the current protocol and flat mount preparation.

Reviewers' comments:
Reviewer #1:
Overall: The authors present a protocol for tissue clearing and commercial light sheet imaging of retinas. They make multiple claims of novelty, but reference papers (in particular, Refs (5) and (15)) that show results that surpass those of the authors. These references also contradict the authors claims that multiple tissue clearing protocols are incompatible with the goals of their studies. A recent preprint also contradicts these claims and provides quantitative data (10.1101/2020.05.06.081463)

We appreciate the comments from the reviewer informing us about a preprint published since the original version of our manuscript was prepared. In the revised version of the manuscript, we cited the most recent publication while attempting to demonstrate how our methodology and findings are still novel considering all the references cited by the reviewers. Specifically, we believe that our method addresses some of the shortcoming of the existing methods such as the surgical dissection of the outer opaque and pigmented coatings without disturbing anterior retina and hyaloid vasculature. In addition, a combination of all these modifications that we apply to the previously described methods eliminates a need for bleaching, thus allows imaging of innate and in-vivo staining as well as the use of transgenic animals for study of retina pathology while maintaining the integrity of the imaging probe.
We cited Chang et al.'s paper in our revised manuscript. Chang et al.'s publication (10.1101/2020.05.06.081463) is an elegant paper with superb result. However, a major difference between our technique and Chang et al.'s paper is in our dissection method. Although Chang et al. do not describe their dissection method in detail, what appears from the figures is that they removed the cornea and the lens when dissecting sclera and choroid. Removing cornea and lens disturbs the peripheral retinal vasculature and hyaloid vessels. As we elaborated more clearly in the revised manuscript, removing outer pigmented and opaque layers of eyeball (sclera, choroid, and RPE) and leaving cornea and lens unperturbed enables us to capture images of retina and hyaloid vasculature in their natural status. The ability to image intact retina

and vitreous using our protocol is a highly desirable feature for whole retina imaging, has not been described in previously published literature.
In addition, our method eliminates a need for bleaching (as Chang et al. and Prahst et al.'s methods do), and thus allows imaging of innate and in-vivo staining. With all these, we believe our method adds to the current state of knowledge and has merits for publication.

There are no quantitative metrics to show that this protocol is optimal over existing protocols. An optimized retinal preparation protocol paired with an honest discussion of pros and cons would be of value to the community. Unfortunately, the lack of quantitative data is this protocol makes it difficult to evaluate the claims of the authors. To improve the protocol, authors need to rewrite their discussions to better capture the state of the field, eliminate the claims of novelty, and provide quantitative metrics for researchers to utilize as benchmarks. I suggest the authors review the existing tissue clearing and light sheet literature to adapt the benchmarks proposed in the community already.

- We revised our discussion based on these very constructive suggestions and comments from reviewer #1 and removed claims of novelty while addressing the gap that our methodology could fill as related to high resolution optical imaging of intact retina. We also expanded our original manuscript's discussion about the pros and cons of our method.
  We recognize that our manuscript does not offer major quantitative data, however, we believe this is a protocol video report and the lack of quantitative data does not undermine the originality as well as the expected impact of our method. Nevertheless, following the suggestions of the reviewer, we added a new figure to our report (Figure 3) comparing the ability of our method to that of conventional flat mount preparation for enumerating microglia.

Detailed review:

Line 41: TDE exchange is well known to disrupt the structure of tissue. To what degree does that occur here?

- We have not observed obvious structural disruption of retinal tissue after TDE clearing. Our goal was not to compared our TDE exchange method with other clearing methods, however, using TDE exchange, vascular tissue, microglia, astrocytes (not presented here), and cell nuclei (not presented here) did not show obvious deformation or disruption.

Line 44: This method is not novel. Displacing water with a higher RI media dates back to Spalteholz work in 1911.

- We are sorry for the confusion. Our method's originality lies on optimizing a combination of previously described methods for intact whole retina imaging. We

hope that the reviewer agrees with us that with the exception of one publication that was published while our manuscript was under preparation and peer-review process, we have made references to the relevant previous studies using tissue clearing agents. We hope that the revised manuscript is more clear in describing a new method for high resolution whole retina imaging that uses a combination of surgical dissection of outer coatings of the eyeball without removing cornea and the lens, TDE clearing method, and LSFM and demonstrates the advantage of our method in enabling retinal and hyaloid vasculature in their entirety and in a way that has not been described previously.

Line 62-64: Ref (5) used cleared retinas and LSFM. The entire point of this paper was that flat mounts give a distorted view of the retina

- We are sorry for the confusion. As the reviewer noted, retinal structure distortion is less pronounced with intact whole retina imaging methods compared to retina flat mount preparation. We acknowledged Prahst et al.'s work in the original manuscript and in the revision and indicated that they used tissue clearing and LSFM. However, our method adds to what was published by Prahst et al. (Ref 2 in the revision). Unlike the method described by Prahst et al. (previous reference #5), we did not remove anterior segment of the eye, thus enabling peripheral retinal and hyaloid vasculature imaging. In a new video that we added to the revised manuscript, we show how the hyaloid vessels can be visualized with the method described in our manuscript. In addition, unlike the methods by Henning et al. and Vigouroux et al., our method eliminate use of bleaching, thus enabling the imaging of innate fluorophores such as GFP. So, we hope that the reviewer agrees with us that our method is original and addresses a need that has not been met previously.

Line 68-69: Ref (5) uses LSFM, not serial section reconstruction.

- We are sorry for the confusion. In our manuscript (line 66-68 original manuscript), we referred to Prahst et al.'s finding that flat mounting distorted retinal vessels organization compared to whole retina imaging. We never claimed that Ref 5 used serial section reconstructions. In line 68-70 (original manuscript) we suggested that although reconstructing images from sectioned tissue may partially address tissue distortion, section reconstruction is subject to error as suggested by Ref 6 and 7. In response to the reviewer's concerns, we rewrote the introduction to eliminate such confusion.

Line 70-73: This is exactly what Ref (5) and (15) already show.

- We don't agree with this comment but we acknowledge that we were not clear enough in our writing. In both Ref 5 and Ref 15, the cornea and the lens were removed for preparing the whole retina samples. This inevitably disrupts the hyaloid vasculature. Again, we revised the manuscript to avoid this confusion.

Line 75: Dissection + clearing disturbs the retina from the native state.

- Unfortunately, it appears that we have failed to clearly describe the advantages of our method compared to the previously described techniques for whole retina imaging. Again, we want to emphasis that in our method, we don't remove the anterior segment of the eye (cornea and lens and vitreous) and we don't make any cuts in peripheral retina meaning that our dissection and clearing steps results in significantly minimal perturbation and disruption of intact whole retina. Nevertheless, we recognize that fixation and clearing steps may cause tissue shrinkage or expansion to an unknown degree; but these are inherent limitations that all histologic studies using fixation and clearing techniques are subjected to.

Line 88-96: This is an incorrect summary of optical clearing methods. Recent reviews such as doi: 10.1016/j.neuron.2020.03.004 and doi: 10.1038/s41583-019-0250-1 may help the authors understand the state of the field. Additionally, Refs (5) and (15) show endogenous fluorophore retention and exogenous labeling in cleared whole retinas imaged with LSFM.

- We greatly appreciate the suggestion made by the reviewer and believe that incorporation of the references cited by the reviewer enhances the presentation of our manuscript. Thus, we revised our manuscript to address the reviewer's concern related to the visualization of endogenous fluorophores. We revised our brief introduction to optical clearing using the additional references that the reviewer helped us to recognize.

Line 108: This method is not novel.

- As stated in our response to other comments above, we revised the introduction as well as the discussion components of our manuscript hoping that we have adequately addressed the reviewer's concern.

Line 127: Many universities prefer to avoid cervical dislocation now.

- Our IACUC protocol still allows us to perform cervical dislocation.

Line 166-171: There needs to be quantification of how well this passive labeling method works. Typical passive labeling in thick tissue leads to incomplete penetrance. Please see doi: 10.1038/nbt.3641 and doi: 10.1038/s41467-020-15906-5. There needs to be troubleshooting and quantification steps on how to select antibodies as per the above papers for a protocol.

- We agree with the reviewer that this should have been elucidated better. We have tested multiple antibodies and the method in this manuscript is the optimized final method. We believe that the ability to see all the three vascular layers including deep vascular plexus and hyaloid vasculature (supplemental video 2 in the revised submission) validates the efficiency of our labeling antibody to penetrate deep into the retinal tissue. We revised the methods and discussion parts of the manuscript considering the reviewer's concerns.

Line 172-190: This entire protocol will change the shape of the retina. Please provide quantification of the distortion so that users can calibrate.

- We appreciate the reviewer's comment. We don't have any method to quantify tissue changes imposed by tissue fixation and clearing methods. We added this to the limitations of the method to address the reviewer's concern.

Line 191-392: Tying the imaging protocol to a specific imaging system and commercial software is not helpful to the majority of users. Please provide a generic sample mounting protocol, such as those prepared in Ref (5) and (15). Additionally, please provide quantitative metrics for the quality of the imaging, such as bead measurements (pre/post deconvolution), Fourier Ring Correlation measurements of image quality, and defocus values throughout the retina.

- We appreciate the reviewer's comments. We revised our protocol and removed any references to the specifics of any commercial imaging system we used in our study.

Line 449-453: This method does not overcome limitations of already published work that the author's have referenced.

- We appreciate the reviewer's comment and recognize that our method doesn't overcome "all the limitations" of the current methods. We revised the manuscript to acknowledge our methods advantages and limitations compared to the existing methods.

Line 468-477: The authors need to prove such a claim, since it is not supported by the literature and references the authors provide.

- We acknowledge that our argument was not well supported here. We expanded this part of discussion and added new citations to support it.

Line 479-488: The authors reference multiple studies that used LSFM and clearing in the introduction, but not here.

- We added LSFM citations to this section.

Reviewer #2:

Manuscript Summary:

The authors of the manuscript "Intact Whole Retina Imaging - An Optical Clearing and Light Sheet Microscopy Approach" present a novel method for volume imaging of a cleared mouse retina, which in that way was not reported so far. In contrast to at least two whole-mouse eye clearing methods, this protocol focuses only on the retina and the vitreous, providing the basis for more focused analyses on these structures, if outer ocular layers, such as the choroid, are not of interest. In my opinion this protocol is an important addition to currently available protocols for retinal research.

However, a some points must be addressed, before this manuscript will be suitable for publication.

- We are very pleased to receive a very encouraging and positive feedback from Reviewer#2 recognizing the unique contributions that our work can make to the development of a novel protocol for high resolution imaging of intact retina. Below, we attempted to address the constructive comments along the concerns of Reviewer #2.

Major Concerns:
The movie of the cleared mouse retina is impressive, as it allows a global look at the target structures. However, an extra figure must be presented, which shows the three target structures (microglia, retinal vessels, hyaloid vessels) in a higher magnification and resolution. With the provided material, I cannot say whether the target structures are stained and imaged in a sufficient resolution to conduct quantification analyses.

- We greatly appreciate the reviewer's comment and recommendation. In response to this comment, we added a new figure 3 that includes still images of retinal microglia and vessels with improved resolution and quantification.

- Minor Concerns:
  General comments:

  All SI-units should have a space between number and unit
  I am no native speaker, but I have the feeling that some phrasings are a bit clumsy. Maybe you should thoroughly revise the text before submitting the revised version (e.g. L166).
  Something in your paragraph numbering went wrong.
  - We appreciate the comment. We revised the manuscript and used a professional editor to edit the manuscript.
-

  Minor comments:
  L40 prevent
  - Corrected.
-

  L43 Should be phrased more precisely, because there are at least two whole-eye clearing methods, which allow detailed investigation of immunofluorescently labeled structures in the retina in its native structural organization. Better would be to distinguish between retinal flat mount techniques and clearing, such as "Retinal flat mount techniques disrupt the three-dimensional, native organization of target structures and currently available whole-eye clearing protocols are probably not compatible with endogenous fluorescent molecules such as GFP."
  - We appreciate the comment. We revised the manuscript following the guidance provided by reviewer #2.
-

  L48 the

- We appreciate the comment.

- L84 You should mention the two basic methods for tissue clearing: water-based and solvent-based and cite the respective primary literature (e.g. iDISCO for solvent-based and CLARITY for water-based).
  - We appreciate the comment. We revised the manuscript following the guidance provided by reviewer #2.

- L85 You write about tissue clearing methods to visualize intact tissues like brain and eye but then again about retinal flat mounts, which is not congruent. Why not especially cite references where whole brains and eyes were cleared? One new eye-clearing paper was not cited in this manuscript: Vigouroux et al. eLife 2020;9:e51275. DOI: https://doi.org/10.7554/eLife.51275
  - We agree that this section was not clear. We revised the manuscript to address the reviewer's concern. We added the suggested citation.

- L86 retinal pigment epithelium
  - We appreciate the comment.

- L90 Pan et al (2016) even cleared an entire adult mouse with the uDISCO method, but eyes were still uncleared. Should be cited (https://www.nature.com/articles/nmeth.3964).
  - We appreciate the comment. We added the suggested citation.

- L93 add: Vigouroux et al. eLife 2020;9:e51275.
  DOI: https://doi.org/10.7554/eLife.51275
  - We appreciate the comment. We added the suggested citation.

- L93 Don't begin a sentence with "But".
  - We appreciate the comment. This section is completely revised.

- L95 retinal tissue or only retina.
  - We appreciate the comment. We incorporated the suggested changes.

- L95 in the protocols published by Henning et al. (2019) and Vigouroux et al (2020), no apparent damage to the retina and increased autofluorescence was observed by bleaching.
  - We modified the text to avoid the apparent confusion.

- L96 autofluorescent
  - We appreciate the comment. We incorporated the suggested changes.

- L99 the protocol by Henning et al. is a whole intact eye clearing protocol.
  - We appreciate the comment. We made the required change here.

- L100 tissue clearing is performed after immunolabeling according to the protocol.
  - We appreciate the comment. We made the suggested change here.

- L101 the anterior segment was not removed.
    - We appreciate the comment. We made the corrections.
- L101f. this is redundant, of course tissue clearing was performed before LSFM.
    - We appreciate the comment. We made the corrections.
- L104 GFP-fluorescence (space between two words); "too" sounds weird here
    - We appreciate the comment. We added the suggested changes.
- L104 applying bleaching sounds weird, why not only "after bleaching"; retina/RPE/choroid/sclera
    - We appreciate the comment. We added the suggested changes.
- L104 You should point out that the currently available whole-eye clearing methods (Henning et al; Vigouroux et al) are well-suited when the interaction between choroidal vessels/RPE/retina and/or the visual pathway are of interest. On the other hand, your method is advantageous for researchers interested in the retina/vitreous only.
    - We appreciate the comment. We revised the manuscript to highlight this point.
- L110 GFP-tagged
    - We appreciate the comment. We added the suggested changes.
- L110 I am not sure how this clearing method was optimized for retina. Does it mean that clearing with TDE is better than other clearing methods or that it was modified compared to other TDE clearing protocols? None of these options was mentioned in the manuscript and also not validated.
    - We revised the manuscript to avoid the confusion. We removed “optimized” in the revision.
- L111 microglial
    - We revised this section.
- L124 bracket wrong
    - We appreciate the comment. We made the correction.
- L132 why not use heparin in PBS to prevent clotting of the blood? It's just a suggestion to obtain better perfusion results. Maybe it could be included as an advice for people having trouble with perfusion.
    - We appreciate this comment. We don’t have any experience with this method so we prefer not to mention it in the manuscript.
- L136 was any step included to mark the orientation of the eye? If not, this should be included as an advice for people who need this information afterwards (e.g. when quantifying opsin expression).

- We appreciate the comment. We included a comment about the marking in the revision.

-

L141 ° C

- We made the suggested change.

-

L148 do you have any advice to ensure that you cut between RPE and retina? Any specific angle of the scissor or specific blade length of the scissor which makes it easier to precisely cut between retina and RPE without damaging the retina?

- RPE is usually peeled off of outer retina during dissection. It is not uncommon to have small patches of RPE left on the retina. These patches can be removed easily with gentle robbing by size 1 panting brush. We added this explanation to the revision.

-

L151 did you remove the optic nerve and if so when?

- The optic nerve was removed when dissecting the eye. We added this to the revised manuscript.

-

L166 weird phrasing. Suggestion: "Perform incubation with the first antibody overnight at 4 °C. Here, we used an anti-mouse collagen IV antibody".

- We appreciate the comment. We revised this line to make it clear.

-

L166 in what buffer were the antibodies diluted? What dilution?

- We described how we prepared antibody dilutions and the diluting buffer to the revised manuscript.

-

L169 "…with a fluorescently-labeled secondary antibody. Here we used an…"

- We made the suggested changes.

-

L170 why two incubation times? Dependent on what? Antibody buffer?

- We revised the manuscript to eliminate the confusion.

L177 Something went wrong here. In Staudt et al, the reference you cite here, no brain tissue was rendered transparent. Only cell culture experiments were conducted.

- We appreciate the comment. We revised this section and corrected the mistake here.

L182 Did you also test higher concentrations without better clearing results?

- Yes, we did the higher concentrations. With concentrations above 60% (70% and above) retina becomes less transparent and image quality deteriorates.

L187 Incubation times at each dilution step?

- As noted in the original and revised manuscript, incubation time at each concentration is 2-4 hours.

L197 To my knowledge, for confocal microscopy of cleared samples, a selfmade

imaging chamber is needed. Refer to the iDISCO protocol (https://doi.org/10.1016/j.cell.2014.10.010).

- This is a correct observation. Imaging chambers and mounting platforms vary based on the specific configuration of the microscope system used. While we removed the technical details about our specific microscope in the revised manuscript (as suggested by the editor and reviewers), we added a new cartoon (as suggested below) to the revised manuscript that shows the mounting platform for the specific imaging set up that we used.

L204ff. a picture would help to understand how to prepare the chamber.

- We appreciate this suggestion. We revised figure 2 by adding a new cartoon that shows our microscope's imaging chamber and our sample mounting method.

L216 imaging media's

- We appreciate the comment, however, we removed the technical details about our specific microscope in the revised manuscript, so this section has changed.

L226 60 % TDE in PBS?

- Yes, TDE concentrations are v/v in PBS as explained in the beginning of this section.

L233 pipette tip should be cut and the opening widened with a second pipette tip to avoid sharp edges

- We appreciate the comment. We added this suggestion to the revised protocol.

L240 do you mean Fig.2c? It is really hard to see, an arrow would help to see where the needle is. Figs.2a,b, and d were not mentioned in the text so far.

- We appreciate this comment. We revised figure 2 and added a cartoon that shows our mounting platform in more details. Arrows have been added to show the details.

L242 how is the syringe placed and fixed within the imaging chamber? (photo?)

- A revised figure 2 shows the syringe and needle that are used for mounting the sample in the imaging chamber. We hope the reviewer finds this revision helpful in explaining our method.

L249 There are numerous ways to setup a LSFM for imaging, therefore it should be pointed out that imaging can be conducted according to the standard imaging protocol of each institute. In my opinion it is not necessary to describe the imaging steps in detail (maybe as supplementary data?). If you decide to keep the imaging

paragraph in detail, you should try to organize it neater. Maybe by giving each subbullet a title or including screenshots of each step (if that is possible without violating copyrights). This could help not very experienced users to follow.
Critical steps that are important for whole-eye imaging should be emphasized, of course.
This could be a decision, which you should discuss with the editor, since I don't know how detailed such software-specific paragraphs should be written for JOVE.

- We really appreciate this comment. We revised the imaging section as suggested while avoiding mentioning microscope brand.

L338 same comment as for paragraph "Setting up imaging parameters in ZEN software and light sheet alignment".

- We appreciate this comment. We hope the reviewer finds it useful that the revised manuscript is focused more on the sample preparation method and less on outlining the specific details of the microscope.

L346 from

- We made the correction as suggested.

L355 200/200 referring to?

- We removed the machine/software specific instructions from the revision as suggested by the reviewers and the editor.

L 343 it is confusing that you number the subbullets in each paragraph sometimes with letters sometimes with numbers. Should be done consistently.

- We appreciate the comment. We made the correction and a uniform numbering system is used in the revision.

L397 what did you use to anesthetize the animals?

- We added the anesthetics to the list of supplements.

L408 more information on the Vannas scissor used in your study would be great, especially the length of the blade.

- We added the details describing our tools to the supplies list.

L413 more information on the antibody dilution buffer, serum, tween-20, and triton x-100.

- We provided more details in the list of supplements.

L425 manufacturer?

- We added more details to the list of supplements.

L435 retinal pigment epithelium

- We corrected the manuscript.

L435 "pigmented and/or opaque layers" (because the sclera or choroid are not pigmented)

- We made the suggested changes.

L440 Figs. 2a,b,d not mentioned in text. An actual photo of the eye in the imaging chamber would be great to get an impression of how the syringe/eye construction is placed and fixed in the imaging chamber.

- We referred to Figure 2a, b, d in the revised manuscript. Regarding an actual photo of the eye in the imaging chamber, since the first author has moved to another institute, we are not able to take a new picture from the mounting chamber at this point. However, we will add a figure/video using JOVE videography team if the manuscript is accepted and we proceed with making the video.

L440 retinal pigment epithelium

- We corrected the manuscript.

L444 (Movie): Besides detailed, high resolution images of the three main structures (see my general comments), you should include text in your movie depicting the visible structures including the color you have chosen for these structures.

- We appreciate the comment. We added a new figure 3 that includes high resolution still images and a comparison between the current technique and conventional flat mount preparation.

L449 the method you are presenting in this manuscript is obviously elegant to perform volume imaging of retinal and vitreous structures. However, you should be careful with using phrases like "overcomes all the limitation of the existing methods", because you did not perform any quantitative analysis. Therefore, you could conclude that immunofluorescent visualization of an intact retina including some target structures is possible with your method, but to overcome "all limitations of the existing methods", you must prove the applicability of your method on quantitative analysis. Otherwise you cannot conclude that your method is sufficient to replace any other method. Moreover, if a researcher is interested in the interaction of RPE/choroid and retina, other methods (such as Henning et al, Vigouroux et al) would be much more suitable. Therefore, your method is really powerful when it comes to retinal research on immunofluorescent basis, especially compared to retinal flat mounts, but it must be proven that quantification is possible with your clearing protocol.

- We agree with the reviewer. We revised the manuscript as suggested.

L452 where did you show hyaloid vessels?

- We added a new video that shows hyaloid vessels.

L461 also cite Vigouroux et al (2020)

- We added the suggested citation.

L469 concentration

- We corrected this in the revised manuscript.

L470 it would be great when you could include a figure showing an uncleared vs. cleared specimen to see the clearing efficiacy

- Since the first author has moved to another institute, we are unfortunately not able to take new pictures at this time.

L483 what is meant by "to be imaged from its inner retina side"? Since EyeCi is a whole-eye clearing method, the eye is imaged as a whole. Or do you refer to another publication, then the reference is missing.

- We revised this section to avoid confusion.

L487 since no quantitative analysis were conducted, this cannot be concluded. Please phrase more carefully.

- We agree with the reviewer. We removed this sentence.

L488 thickness 2x

- We made the correction

L490 no hyloid vasculature shown?

- We added a new video that shows hyaloid vessels.

Reviewer #3:

Manuscript Summary:

The manuscript is well written and nicely describes the methods. In my point of view the title is a bit misleading as the novelty lies in the preparation of the retina and not the clearing or imaging. The imaging is anyways a bit sparse.

- We appreciate the positive feedback from reviewer #3. We revised the title to address the concerns of this reviewer. Also, we added more figures aiming to more clearly demonstrate the capability of our method.