Reviewer #1:

Manuscript Summary:

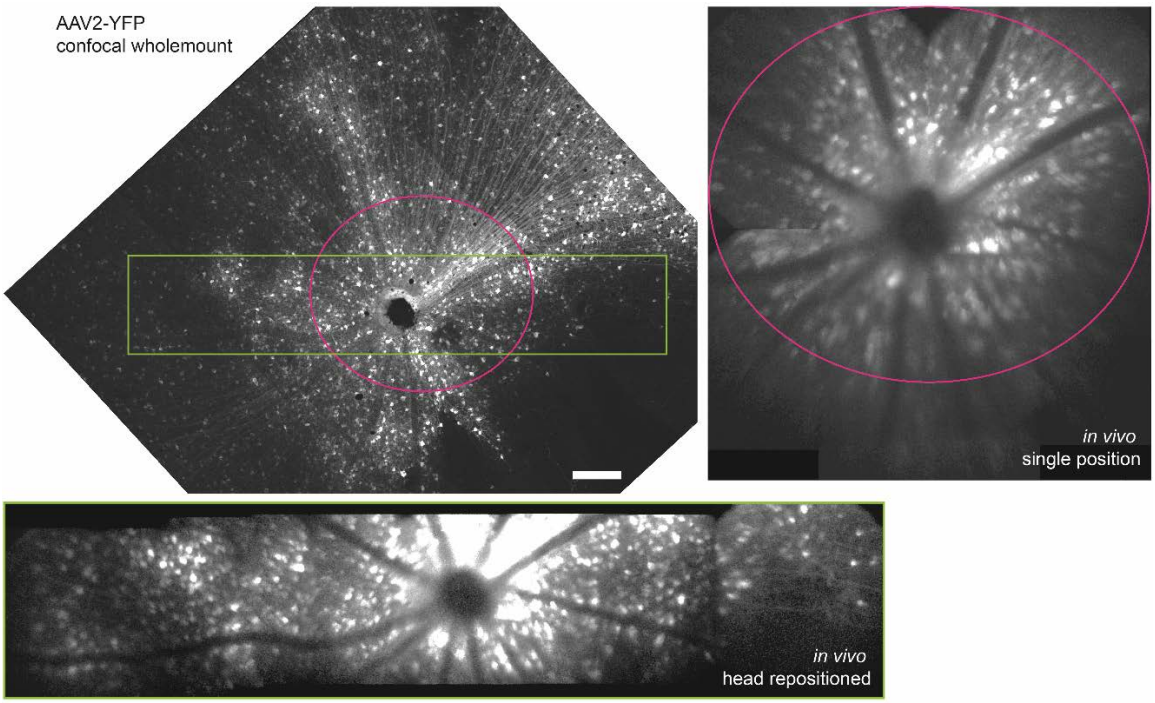
The manuscript „Transpupillary multiphoton in vivo imaging of cellular cohorts in the mouse retina" by Zelun Wang and colleagues gives instructions for in vivo two-photon imaging of the mouse retina with a standard multiphoton microscope. The authors use a simple but effective approach to mitigate the optical impact of the mouse cornea. Several examples of applications in transgenic mice with or without additional injections for fluorophore expression in targeted cells/structures are given.

We thank the reviewer for their accurate assessment of our submission.

Major Concerns:

The instructions are clear and straightforward to follow. The simple approach for cancelling the impact of the anterior optics of the mouse eye may be of interest to many research groups. I am wondering whether the authors have ever experienced any problems when scanning across broad areas of the retina. In this case, the beam might be clipped by the animal's pupil when directed to the very extremes of the scan field.

We have measured the range of imaged area in a single head orientation and the data is presented below. We can image an area of roughly 650 μm^2 in a single head orientation. If we tilt the angle of the head across image acquisitions, we can access a linear range of 2.2 mm, and thus estimate an area of the retina across 3.8 mm^2 is accessible for in vivo imaging with our setup. We have added this information to the text.



Reviewer Figure 1. Imaging field of view. VGlut2-Cre mouse injected with AAV2-EF1α-FLEX-Twitch2b was imaged *in vivo* and the fixed retinal wholemount was then scanned using confocal microscopy (left, YFP signal displayed). A single head tilt position was imaged *in vivo* across multiple fields of view and stitched (right). Additionally, the mouse's head was tilted to extreme positions around a single point of rotation until the fluorescent signal was lost and stitched

across the center of the retina (bottom). The regions containing RGCs were circled in both the *in vivo* and confocal scans. Scale bar = 200 μm.

A major point for in vivo imaging would be assessment of retinal function. Do the authors believe that their setup is suitable for this purpose? What are the limitations here? Please discuss.

We have investigated calcium responses in retinal ganglion cells (RGCs) in our preparation using the FRET ratiometric sensor Twitch2b. Similar to previous reports from retinal explants, we have found that onset of laser scanning induces a calcium increase in RGCs that returns to baseline in most neurons (see Figure 8). We attempted to add UV LED stimulation to our microscope setup, but unfortunately we were not able to procure the custom mirrors needed to adapt our light path in the time frame for these revisions. However, the 2-photon power that we use for scanning is within the range of previous reports that have observed light responses, and we can clearly observe transient changes in calcium in RGCs. Thus we believe it would be entirely feasible to image circuit activity with our approach. This information has been added to the manuscript.

Minor Concerns:
Apart from the points above, I only have very few comments and suggestions:
Page 5, Line 71: Maintain numbering format: 1) Adeno-associated virus injections (instead of 1.)

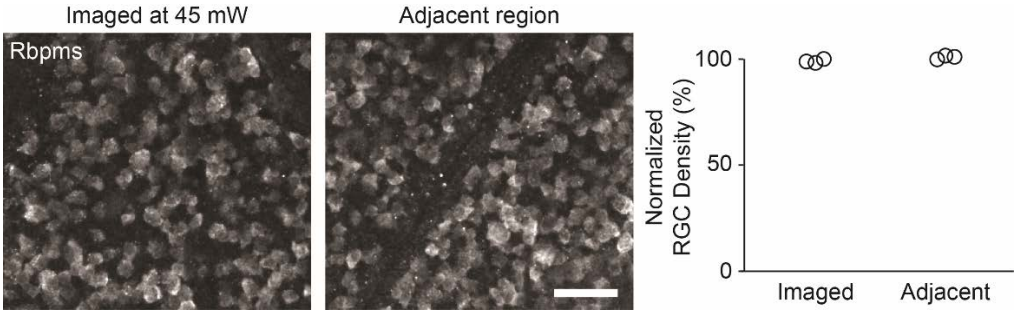
These suggestions have been amended in the manuscript.

Page 7, Section 3 and 4: When trouble shooting Maitai start up problems, people should be referred to the Maitai manual.

As suggested, we have removed commercial language from the manuscript.

Page 8, Line 230-234: As far as I understand, the authors did not specifically test for tissue health or light damage. The reported light levels are still very high and far beyond the safety regime. Please rephrase this section more conservatively. Instead of saying "without damage to the target tissue" use for instance the expression "without immediately visible damage to the target tissue" and only state the lowest laser power, for which damage was observed.

We have quantified RGC survival following chronic imaging by immunostaining wholemount retinae for the RGC marker Rbpms. RGC density was not reduced with repetitive imaging of laser powers up to 45 mW at the pupil. However, imaging from 55 mW and up led to severe loss of RGCs. This information has been added to the manuscript and is presented below.



Reviewer Figure 2. RGC survival after repetitive imaging. Confocal scans of Rbpms immunostained retinal wholemounts from an area that was imaged 3 times on different days at

45 mW, and directly adjacent regions. For quantification, RGC densities from imaged and adjacent pairs were averaged and normalized to account for native variance in RGC density across different regions of the retina. Scale bar = 50 μm.

Page 8, line 251: Remove "leaving"
Page 13, line 515: Add "cell types and structures"
Page 15, line 542: Replace "microscope objective" by "light path"
Page 15, line 545: Replace "pupil" by "light path"
Page 15, line 546: Replace "it does" by "the coverslip and the eye gel do"

These suggestions have been amended in the manuscript.

Page 15, line 557: How did the authors calibrate the size of the scan field to obtain the dimension of the scale bar? What is the lateral and axial resolution of the instrument with their anterior optics hack?

We estimated the pixel dimensions in our scanfield by correlating in vivo imaged regions with confocal images of the same areas in fixed retinal wholemounts. We have further corroborated the scale of our *in vivo* images by injecting 1 and 2 μm diameter microspheres into the retina and imaging these *in vivo*. The axial resolution is quite variable dependent on if the imaging field is in the center or peripheral region of the lens, as this greatly affects parallax. Unfortunately, microspheres injected into the vitreous are not stationary, thus we were not able to estimate axial resolution. This information has been added to our manuscript as Figure 7, which describes our imaging dimensions.

Page 16, line 579: Remove "designed for imaging other regions of the central nervous system"

This suggestion has been amended in the manuscript.

Page 17, line 612 et sqq.: What about the limitation that the animal pupil acts as a field stop? See comment about beam clipping.

Pupil dilation is absolutely critical for *in vivo* imaging and the fact that we cannot image the entire range of the retina would indicate there are some limitations due to beam clipping. We believe the efforts presented above in Reviewer Figure 1 define these limitations.

Reviewer #2:
This manuscript describes a procedure for two-photon (2P) fluorescence imaging of the mouse retina in the intact, living eye. 2P imaging in situ is of considerable current interest for addressing questions pertaining to progressive retinal disease but has been challenging because of the eye's optic properties including refraction from cornea and lens. The procedure described here is straightforward and uses readily available technology. The provided images substantiate the method and give fair proof of principle, applied to neuronal, glia, and vascular imaging. I have the following requests for additional detail and recommendations.

We thank the reviewer for their positive assessment of our submission.

Line 92: cut the glass pipette using what? Please provide more detail - scalpel blade? Razor blade? Iris scissors?

We are using a razor blade to cut the glass pipette. This information has been added to the protocol.

Line 110: Common, apparent refinement of this procedure would be to use Proparacaine 1% ophthalmic solution. One drop in each eye suffices and suppresses all eye lid and corneal reflexes within several seconds. Consider including this at least as an alternative approach.

We appreciate this suggestion, and have added this as an alternative in the protocol.

Much of the methods description is highly system specific, for example, line 253 references 'PMT C', the laser is presumed to be Spectraphysics MaiTai. Try to make it more generally applicable by leaving these details out and focusing on the function. 'Start up ultrafast pulse laser and ensure mode-locking'. 'Configure fluorescence emission collection path to sample green (505-525nm) light using appropriate dichroic filter sets (for example: XX, YY, ZZ; Chroma, US) and a photomultiplier tube (Hamamatsu XXX)'.

Line 309: assume that all samples are important. Describe 'best practice' procedures, only.

These are a very good points, we have made the descriptions as generic as possible throughout.

Line 446: Instead of clicking the "X" button to close the main controls and shut down ScanImage, consider using of the built-in 'scim_exit' function by typing that in the MATLAB command window. This lets ScanImage stop and 'clean up' all running processes and close all program windows in an organized manner.

We attempted this on our system, but it did not function as described. We would appreciate more information about this command. We have also removed mention of ScanImage to eliminate commercial language from the protocol.

Reviewer #3:
Manuscript Summary:
In-vivo 2-photon imaging of mouse retina is a challenge. In the manuscript authors describe an experimental protocol, which should allow to perform this experiment. Unfortunately, experimental approach, protocol and manuscript itself are full of weakness. The only one modification from the standard two-photon imaging microscope is an exchanging of the objective, which is not properly use according to specification. Described imaging protocol is danger for potential user. Authors don't understand terms which they are using, and they have no idea about the data which they collect. This manuscript Is not presenting any scientific value in this form.

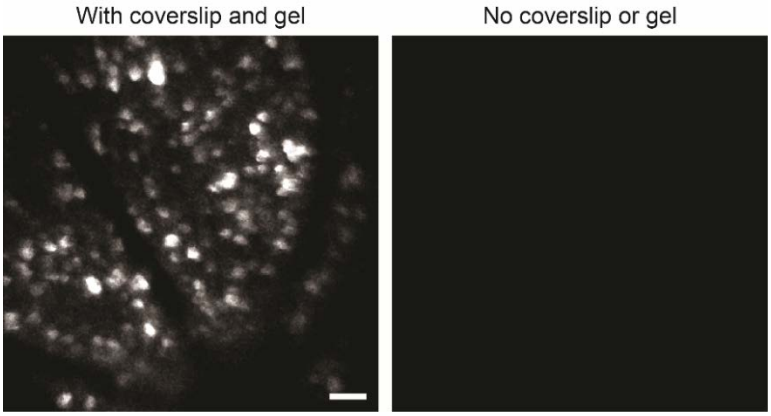
We disagree wholly with Reviewer 3’s assessment of our manuscript.

Major Concerns:
1. Title "Transpupillary multiphoton in vivo imaging of cellular cohorts in the mouse retina" is suggesting, that there will be presented in-vivo imaging on neural activity in retina, because for that the 2 or 3-photon imaging is in use in neuroscience. There are other, much better methods for a just acquiring static images of cells anatomy. Authors in discussion (lines 620-626) describe it. So, there is no sense to use a relatively hight-power, low resolution 2P imaging for anatomical tracking of retina cells.

We have updated the title to reflect 2-photon imaging

2. Authors mentioned in discussion that "The only added components are an unusually long working distance objective to allow for the optical relay of the mouse lens, and a planar coverslip with transparent gel to partially correct for the cornea". First, what they want to correct?

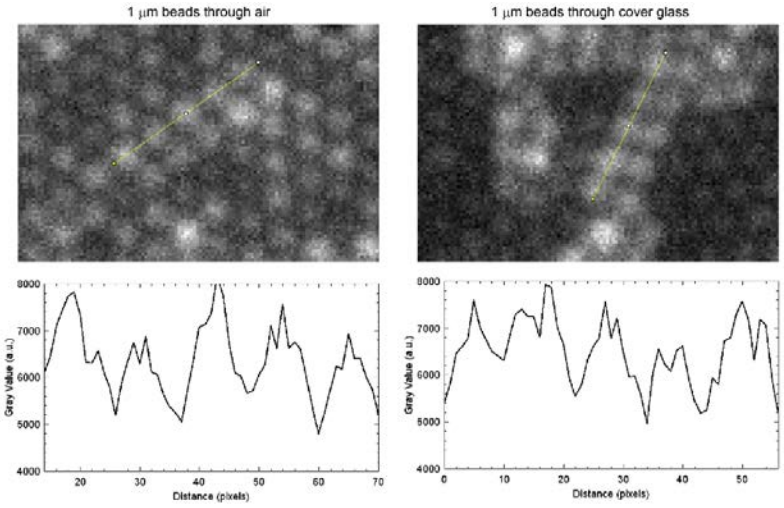
Below is an image of our setup without the eye gel and coverslip to correct for the properties of the cornea.



Reviewer Figure 3. Corrective components are required for imaging. *In vivo* fluorescence in RGCs was imaged as described with the methods in this manuscript (left). The same region was then imaged after removing the coverslip and gel from the eye and attempting to optimize z-position (right). Scale bar = 50 μm.

Second, according to the information form an incomplete list of materials, this objective is Mitutoyo WE715042319, from Edmund Optics (catalogue number 46-404). This is long working distance air objective, dedicated for cutting and trimming of semiconductor wafer and circuits (ideal for semiconductor and telecommunication inspection, or for laser cutting with common Nd:YAG lasers). And authors are using this objective, combine with coverslip, transparent gel and mouse eye. Authors probably never heard about refractive index mismatch, causing terrible distortion of excitation PSF (creating giant aberration). Combination of objective/air/coverslip/gel/eye optic patch is an excellent example of bad understanding principle of optics.

The aberration of the “air” objective mismatch through coverglass is trivial compared to the aberration caused by the mouse lens and cornea. Below is an image of 1 μm microspheres imaged through air and through our coverglass. There is little distortion cause by this mismatch.



Reviewer Figure 4. Comparison of resolution imaging with and without coverglass. One micron diameter green fluorescent microspheres were air dried to a coverglass used in our protocol. The microspheres were then imaged with our 2-photon setup either through the air only (left) or through the coverglass (right) using the same scan settings. Pixel intensity histograms of the lines in each image are shown below.

Further, eye gel paired with either a contact lens or a coverglass imaged using an ‘air’ microscope objective is standard procedure for *in vivo* imaging of the mouse eye. Eye gel is required for imaging beyond 10-15 minutes to prevent the mouse cornea from desiccating, causing corneal opacity. We have described this aspect of the procedure in greater detail including a method to reliably position the coverslip in a fashion that we found significantly easier and more reproducible than more often reported contact lens application.

3. On experimental protocol, section 2: Microscope Setup, line 167, authors suggest pressing Enable button on the shutter controller as one of the first action, before turning on femtosecond laser and acquisition software. In each of the laser safety courses, the fundamental message is that the last mechanical shutter should be enabled only in a time of recording !!!!!!!! Behaviour presented by authors is extremely dangerous for the microscope users and other people in the same room. In a case of any problem with laser and microscopy hardware or software, this shutter is preventing user from a direct exposure of laser light, especially if as in this manuscript the eyepieces are in use, and there is a step for a hand exchange of filter cube.

We appreciate that Reviewer 3 has spotted this error and have adjusted the protocol accordingly.

4. Authors are trying to describe in detail all the steps on the microscope, but unfortunately only one information about this experimental setup is that it is a Hyperscope from Scientifica. This setup can be purchased with different configuration, so without schematic draw and info about principle components (scanning mirrors, PMTs, scan and tube lens, dichroic filters) it is extremely difficult to follow the guidance. This information should be included in a method paper.

We have added a schematic of our optical configuration to the manuscript as Figure 1.

5. I don't understand for what is a point 5: Measuring laser power. It is important to do this once when You install Your setup or make any modification on it, but not every day. If the authors have a problem with stability of the excitation power, I would recommend contacting with the service.

6. Authors in general are describing a lot of possible problems with this experimental setup (lines 181, 191, 198 and others). If this problem shows up on any setup, I will advise to contact with the service.

We wanted to provide a detailed description of measuring the laser power for the protocol. We have updated the manuscript accordingly.

7. In line 242 authors are mentioning abut possibility to PMT damage during cubes filter cubes changing and advise to do this in darkness. As a standard procedure, PMTs should be off for all the time when you are not recording, so I can't understand how it is possible that PMTs can be damaged? Are You trying to keep them on, with enabled shutter and modify optical patch in darkness?

As described in the protocol, the PMTs are off during filter changes. It is best practice not to expose PMTs to room light even without power across them.

8. Line 365. "obtain a flat imaging area", "retina is not flat with respect to the imaging light patch". Retina is lot flat due to the anatomy.In 2-photon microscopy shape of imaging area is related with a shape of excitation patch. I don't know how it looks like on this setup. according to a not standard objective and terrible distortion of the excitation PSF. In the method paper, author should present example of the image, which they want to obtain by adjusting "z-position of the stage until the fluorescence cells or structures in the retina come into focus".

We trust that a researcher can determine if their sample is in optimal focus.

9. Authors are collecting z-stack of images, and to create volumetric display of image data they use a maximum intensity projection function. It makes no sense. 2-photon microscopy operate on spatially limited excitation spot. Main advantage of this method is to acquire data from a tiny z-plane. In the authors approach, what will happen if there will be z-spatial overload of the two or more cells? Collecting of a 2-photon data z-stack allow to create a 3D reconstruction of cell anatomy (of course if the z-step is selected correctly), and this should be presentation of this data.

We use the maximum intensity projection function for display purposes. We have clarified this in the manuscript.

10. Line 486: "it may be beneficial to visualize changes to the image over time". I think that it will be beneficial to the authors if they will understand that 2-photon imaging microscopy is dedicated for that.
11. Line 617: "An alternative approach to correct this at the system level is adaptive optics which allows for subcellular resolution in the retina". An alternative approach to correct aberration will be using proper objective to create diffraction limited spot.

We have found that in order to image through the mouse pupil *in vivo* requires a microscope objective with a working distance of at least 10 to 10.5 mm. If Reviewer 3 could present a microscope objective that has such a working range, allows for 2-photon excitation wavelengths and emission collection of visible light, and has a higher numerical aperture than the one we describe in our set-up, we would be grateful for this information.

Minor Concerns:
1. Title "Transpupillary multiphoton in vivo imaging of cellular cohorts in the mouse retina" Authors are using 2-photon imaging, which of course is a kind of multiphoton imaging, but this term is suggesting that authors are trying to use 3-photon excitation for the experiment.

We have adjusted the title to indicate 2-photon imaging.

2. Line 106 - what is the max speed of centrifuging?

We have included the centrifugation speed.

3. Items described in lines 165, 166, 167 and other should have specify model name, not only producer.

We have added specifics for these items.

4. Line 198 - which version of ScanImage (not scanimage) ate You using?

We have added the ScanImage version to the Materials List.

5. All the safety warnings, line the one from line 206, should go to the beginning of procedure description.

We have moved and enhanced the safety warnings to the front of the procedure.

6. Line 252 - what is a "single wavelength emission imaging"? Are the authors measuring laser emission? Using a monochromator? Spectrometer?

This section has been removed from the protocol.

7. Line 362 - how authors are checking if the sample fluorescence is insufficient bright? Is there any scale for that?

Sufficient brightness at this step is subjective and used only for locating the target area to be imaged. We have added a description of when it may be beneficial to increase LED stimulation power.

8. Line 286 - authors are not describing how they operate on the change of z-dimension during imaging. Is this a piezo, hand or motored moved engine?

We have added the motorized objective to the microscope description.

9. Line 547 - I am not sure of what You are creating, but it is not a lens, it is a just an immersion of gel.

Any non-light absorbing liquid that is not flat at the point of light entry and/or exit will create a lens.

10. Line 557 - there are no scale bar on Fig. 2.
11. Line 562 - the same as above.

We have added scale bars to all figures.

12. Line 580 - 20 mm is not an unusual working distance for air objective.

It is for a relatively high NA objective. We tested using ‘standard’ air objectives, which typically have an NA of at best 0.25 and were not able to image on our setup. We have added this information to the manuscript.

13. Line 582 - correct of what?

We have clarified this in the text.

14. Line 583 - Based on what authors claim that ACs and RGCs are the main neuronal components of the retina?

We have clarified this in the text.

15. Line 612 - a limitation of this approach is the fact that aberrations are created, not corrected.

The aberrations inherent to the cornea and lens are far greater than any issues caused by coverglass or eye gel.

16. Line 616 - what is the out of focus light in 2P excitation?

We have clarified this in the text.

17. There is no info about field of view in this configuration.

We have measured the range of imaged area in a single orientation and the data is presented below. We can image an area of roughly 650 μm^2 in a single orientation. If we tilt the angle of the head across a single rotational axis, we can access a linear range of 2.2 mm, and thus estimate an area of the retina across 3.8 mm^2 is accessible for in vivo imaging with our setup (See Reviewer Figure 1). We have added this information to the text.

18. List of materials should be upgraded on all of the 2-photon setup important elements (acquisition software, PMTs, scanning mirrors etc.)

We have added these components to the list of components/reagents.

Reviewer #4:
Manuscript Summary:
In the manuscript entitled "Transpupillary multiphoton in vivo imaging of cellular cohorts in the mouse retina", Wang et al. presented a protocol of using standard two-photon microscope for in vivo imaging of mouse retina. The current form of the manuscript needs a major revision to be acceptable for the publication in JoVE.

Major Concerns:
#1: The reported protocol proposed minor adaptations to a commercial two-photon microscope system for in vivo retinal imaging. However, it ignored the fact that the imaging quality in terms of resolution and fluorescence intensity are inevitably degraded by the optical aberration of mouse eye. Therefore, a major question that must be clearly addressed is what exactly can be interpreted from the low-resolution images acquired by the two-photon imaging system. For example, the authors claimed that fluorescence intensity of cell soma can be easily quantified (5th paragraph in the discussion section). However, the fluorescence intensity are significantly affected by the mouse eye aberration which could vary from animal to animal or measurement time to time. The effect can be seen in Figure 2 that although the same imaging site can be relocated in time-lapse imaging based on the vascular landmarks, the fluorescence intensity and distribution of retinal ganglion cells between the two imaging sessions are largely different (especially the bottom row of Figure 2). Without correction of the aberration, the quantification of fluorescence intensity of cells is not reliable. In the revised manuscript, I would suggest the authors focus on cell counting that does not require high imaging resolution. The authors could demonstrate a detailed procedure of in vivo RGC or other neurons counting using an image processing tool, such as Fiji.

We agree with the reviewer and have updated our manuscript accordingly. While we do believe intensity based measurements can be applied to acute experiments where head position is maintained constant across images, in samples that are examined chronically, assessments relying on ratiometric read outs are far more reliable in this system.

#2: As correctly pointed out by the authors, one major advantage for two-photon retinal imaging is to use NIR laser that can avoid stimulation of intrinsic visual response of retinal neurons. Since the authors used a calcium sensor, Twitch2b, for visualization of retinal ganglion cells and amacrine cells, it would be of great interest that the authors can demonstrate the functional calcium imaging capability apart from structural imaging.

We have investigated calcium responses in RGCs in our preparation using the FRET ratiometric sensor Twitch2b. Similar to previous reports from retinal explants, we have found that onset of laser scanning induces a calcium increase in RGCs that returns to baseline in most neurons (see Figure 8). We attempted to add UV LED stimulation to our microscope setup, but unfortunately we were not able to procure the custom mirrors needed to adapt our light path in the time frame for these revisions. However, the 2-photon power that we use for scanning is within the range of previous reports that have observed light responses, and we can clearly observe transient changes in activity in RGCs. Thus we believe it would be entirely feasible to image circuit activity with our approach. This information has been added to the manuscript.

#3: For the microglia imaging, one of the most important goal is to examine the inflammatory activity in the retina by direct visualizing the typical amoeboid morphology of reactive microglia. In Figure 4, however, the detailed morphology of most microglia in the field of view were not clear. This prevents rigorous judgment on microglia states and thus is unable to identify inflammatory activity at an early stage of eye diseases. The limitation, again due to poor resolution, should be pointed out and discussed in the manuscript.

To address this point, we have performed excitotoxic lesions of the retina by injecting NMDA intravitreally in CX3CR1-GFP transgenic mice. In line with the literature, we see microglia with amoeboid morphology as compared to untreated retinae. This data has been added to the manuscript.

#4: In line 230, the authors stated that maximal laser power without causing photodamage to the retina is ~50mW in their two-photon imaging system. However, the microscope system used by others could be different. I would suggest the authors to work out a general procedure or criterion to determine the laser power threshold of a microscope with different optical configuration for safe retinal imaging.

We have further characterized the damage (or lack thereof) caused by our *in vivo* imaging setup and present this data above in Reviewer Figure 2. This information has also been added to the manuscript. Additionally, we have added a section to the discussion describing strategies for laser tolerance.

5: In line 435, as pointed out by the authors, the clarity of mouse eye is crucial for high-quality retinal imaging. How did they quickly identify whether the mouse eye is still in good and transparent condition during a continuous imaging experiment? How to restore the clarity of a drying or opaque eye?

Development of cataracts cannot be restored and is usually caused by improper intravitreal injection. Corneal drying will often occur if the non-imaged eye is not properly maintained. We have added a method to maintain the non-imaged eye during experiments. Corneal opacity due to desiccation can resolve on its own over the course of a few days. We have added this information to the manuscript.

Minor Concerns:
#6: In the 3rd paragraph of the discussion section, the CAG promoter has not been used in the experiments.

We have edited this accordingly.

#7: The calibration of scale bar of the retinal images should be also discussed in the protocol.

We estimated the pixel dimensions in our scanfield by correlating in vivo imaged regions with confocal images of the same areas in fixed retinal wholemounts. We have further corroborated the scale of our *in vivo* images by injecting 1 and 2 μm diameter microspheres into the retina and imaging these *in vivo*. This information has been added to our manuscript as Figure 7, which describes our imaging dimensions.

#8: In line 252, a 605/70 band pass filter was used for the collection of Evans Blue signal. In fact, the emission peak of Evans blue is ~680nm. Why wasn't a band pass filter centered at 680 nm used?

This was an error on our part, we have updated the description of the optical components to image Evans Blue.

#9: To minimize the stress of mice, a contact lens would be a better choice. The authors simply placed a coverslip on the eye which would introduce extra pressure to the mouse eye and more importantly increase the eye aberration.

In our initial experiments, we tested the use of a contact lens and found it much more challenging to work with because it is not stable and extremely difficult to orient perpendicular to the microscope objective. It is important to note that the coverslip does not touch the mouse eye. It is maintained in a microscope holder at a small distance above the cornea. This should cause less stress to the eye since a contact lens would rest on the cornea. We have updated our description to make this clear including adding panels to Figure 2, and added comparisons with contact lens imaging to the Discussion.