Dear Dr. DSouza,

Please find enclosed our revised manuscript entitled “Studying diabetes through the eyes of a fish: Microdissection, visualization and analysis of the adult tg(fli:EGFP) zebrafish retinal vasculature” which we would kindly resubmit for publication in JoVE.

We have addressed all concerns raised by the editors and reviewers and highlighted all modifications in our manuscript. Likewise, we include in our submission a version where all filmable content is marked in yellow.

We look forward to a favorable response from you, at your earliest convenience.

Yours sincerely,

Jens Kroll.

**Changes recommended by the JoVE Scientific Review Editor:**  
  
• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Comment: done  
  
• **Animal Use:** Due to the nature of being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in your protocol, please add the following information to your text:  
1) Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.  
Comment: we have included the statement as suggested.

2) Please do not highlight any steps describing anesthesia or euthanasia as these will not be filmed.  
Comment: understood and done.

• **Protocol Language:** Please ensure that all text in the protocol section is written in the imperative tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.  
1) Examples NOT in imperative tense: 1.1, 2.1, 4.9,4.9.1, 6.1,6.2.  
Comment: this has been done accordingly as suggested.

2) Some of your longer steps were split up (4.3, 4.1), please ensure that each step contains no more than 3 related actions.  
Comment: OK

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more details to the following protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.  
1) 2: Which fish strain do you use? At what age?  
Comment: this information has been incorporated in 2.

2) 3.2: The term “euthanize” is preferred over “sacrifice”. How long do you have maintain the fish in tricaine?

Comment: this has been modified and incorporated as suggested in 3.2.

3) 3.3: What do the well plates contain, i.e, is this the fixative from 1.6? There is minor continuity problem here.

Comment: this has been clarified.

4) 3.4 Note: How are the eyes extracted? Perhaps step 4 needs to be referenced here.  
Comment: this has been clarified.

5) 4.1: mention microscope magnification.  
Comment: this information has been added.

6) 4.7,4.8, 4.9: Mention surgical tools use.  
Comment: this information has been added.

7) 4.9: Please describe the RPE removal.  
Comment: this has been clarified.

8) Line 246: Cave?

Comment: this has been clarified.

9) 6.2: Mention fluorescence microscopy and confocal microscopy settings, magnification, excitation, lens NA, emission filter settings etc,  
Comment: done.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE’s instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.  
Comment: understood and done.

o The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.  
Comment: OK

o The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

Comment: OK

o Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

Comment: OK

o Notes cannot be filmed and should be excluded from highlighting.  
Comment: OK

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.  
Comment: we have modified some minor parts in our discussion

• **Figures:**:  
1) Figs 2 to 9 (A,b), 10A-C, 11A-C, 12 A-B: Please provide scale bars, and define them in the figure legend.

Comment: done

2) Please provide each figure as an individual PDF, TIFF, JPEG or PNG files.  
Comment: done

• **Figure/Table Legends:**:  
1) Fig 9: Please expand the legend to fully describe the panels. How were the images acquired, what do they show?

Comment: we have added some additional information.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. An example of commercial sounding language in your manuscript is Fluoromount-G.  
1) Please use MS Word’s find function (Ctrl+F or Command+F (on Mac)), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names.  
Comment: done

• **Table of Materials:** Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as fish strain.  
Comment: we have completed the list as suggested.

• Please define all abbreviations at first use.  
Comment: done

• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.  
Comment: done

• Please print, fill out indicating correct access type (you requested open access during submission but select standard access in the previous license agreement) and sign the appropriate Author License Agreement, then scan and upload it with your manuscript files. https://www.jove.com/files/Author\_License\_Agreement.pdf  
Comment: we have already paid the amount for open access.  
  
• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Comment: OK

**Comments from Peer-Reviewers:**

**Reviewer #1:**  
*Manuscript Summary:*  
In this manuscript Wiggenhauser et al, describe an easy and straightforward approach to analyze the zebrafish retinal vasculature. In general this manuscript is well written and the methods are detailed and well described.  
Comment: we would like to thank the reviewer for the positive comments to our manuscript.

*Minor Concerns:*  
I have only 2 minor concerns:  
-As this is a method article I would suggest that the authors stress or highlight more in the title of the manuscript the methodology used. The current title sounds like a title for a review article.  
Comment: the title has been changed to “Studying diabetes through the eyes of a fish: Stereoscopic microdissection, visualization and analysis of the adult tg(fli:EGFP) zebrafish retinal vasculature”

-Line 72, please add reference to morpholinos and Crispr/Cas9 technique. Line 106, please add reference for tg(fli:EGFP).  
Comment: we have incorporated two new references as suggested.

**Reviewer #2:**  
*Manuscript Summary:*  
This is an exceptionally well written article that provides clear and concise protocols for analysis of the ZF eye for vascular analysis. It is timely and contributes greatly to the field of diabetes research.  
Comment: we would like to thank the reviewer for the positive comments to our manuscript.

*Major Concerns:*  
None  
  
*Minor Concerns:*  
None  
  
*Additional Comments to Authors:*  
This is a very impressive article that is extremely well written.

**Reviewer #3:**  
*Major Concerns:*  
I have no concerns about your manuscript. All steps are clearly described, critical points are emphasized and limitations are mentioned.  
Comment: we would like to thank the reviewer for the positive comments to our manuscript.

*Minor Concerns:*  
The part "c" of your figures with the preparation steps should emphasize the current dissection step better (may be an additional colour).   
Comment: this has been changed accordingly; we have included in each figure c some additional information as indicated.

*Additional Comments to Authors:*  
I have no major concerns about your manuscript. All steps are clearly described, critical points are emphasized and limitations are mentioned.

**Reviewer #4:**  
*Manuscript Summary:*  
The manuscript provides a detailed explanation of how to dissect and flatmount a retina from an adult fish  
Comment: we would like to thank the reviewer for the positive comments to our manuscript.

*Major Concerns:*  
Since the title of this manuscript specifically says that this method allows the study of diabetes in fish, the authors have to add examples of what the vasculature looks like after a pathological challenge, either by applying any of the diabetes models mentioned in the introduction or by knocking out a gene involved in blood vessel formation, for example using CRISPR/Cas9; including the protocol to apply such a model or, if a protocol paper already exists, at least cite it.  
Comment: we have incorporate references to pathological changes mediated through hypoxic and hyperglycemic conditions in the zebrafish retina as requested in the discussion of the article (line 432-456).

It is quite important that the authors offer an example of the parameters that can be quantified, for example in fig 9 and 10, eg number of branchpoints, etc...  
Comment: we have incorporated new Figure 12 as an example how to quantify the parameters.

Moreover, it would be helpful for any reader if the authors could share their experience and provide a list of antibodies that have proven to work in their hands for immunostaining of the fish retinal vasculature and surrounding structures.  
Comment: We have not performed any antibody staining on the retinal vasculature so far and we are not aware of published data form other groups. Yet, in our previous publications we have used an anti-GFP staining in fli:EGFP zebrafish embryos/larvae (please refer to Urbich et al. Blood 2012).

The authors should expand or explain better the topic discussed in the first paragraph of the discussion as at the moment. I.e.: what do the observations from references 20, 21 and 22 mean in relation to diabetic retinopathy? Why is the progression to PDR missing? Authors should describe what such progression is.  
Comment: We have incorporated these references to show that the zebrafish vasculature is susceptible to hyperglycemia and diabetic conditions leading to different vascular alterations. We are not aware of PDR data in zebrafish; yet this is the concept of our work which aims to analyze different stages of diabetic retinopathy in zebrafish. In order to make this more clear, we have slightly modified the first paragraph of our discussion.

*Minor Concerns:*  
Line 48: remove "to"  
Comment: this has been corrected accordingly.

Line 55: it's the capillary regression that generates ischemia: change to "...pericyte dropout and capillary regression that results in acellular vascular sleeves."  
Comment: this has been corrected accordingly.

Line 87: "advantages of the species": not clear, please amend.  
Comment: this has been corrected accordingly.

Line 106: change to "The green fluorescent protein expressed under.."  
Comment: this has been corrected accordingly.

Line 160: add reference to Figure 2A here  
Comment: this has been corrected accordingly.

Line 166: not clear: why do the authors mention the eyes when eyes have not been dissected yet at this stage? Replace "eyes" with "heads" ?  
Comment: this has been corrected accordingly.

Line 247-8: not clear: please rephrase  
Comment: this has been corrected accordingly.

Line 263: retina misspelling  
Comment: this has been corrected accordingly.

Line 280: the authors should first describe how to section and stain eye sections with HE before discussing the results in figure 11 and 12  
Comment: we have incorporated the protocol for section and HE staining as suggested.

Line 297: why should there be any angiogenic sprouting in an untreated adult? If the authors think that the fine structures they see in fig10C are filopodia from new growing vessel sprouts, then they should provide a much higher magnification image to prove that. Given that Fli-GFP also labels macrophages, the authors should rule out that those fine structures are not microglia/macrophages.  
Comment: we have included new Figure 12. This higher magnification clearly shows that those fine structures are angiogenic sprouts originating out of an existing blood vessel and not microglia/macrophages.

Figure 12: the fluorescent signal is not clear: everything looks green! Please replace. Is that because of GFP imaging after HE staining? If so, the authors should just combine GFP imaging with fluorescent nuclear labellnig (such as dapi)  
Comment: Unfortunately, the zebrafish eye has a very strong autofluorescence (independently of HE and fixation). Therefore, it would not make sense to replace the existing image. Yet, we would prefer to include the image because it shows from a different perspective where the retinal blood vessels are located.

Figures 3,4,6 and 7: please add an arrow and a lower font "B" in panels A to help understand the orientation of panel B relative to panel A  
Comment: we have included in each figure arrows which mark some anatomical structures of the eyes and therefore now help better to understand orientation in each figure.

Figure 5: panels A and B should be inverted as current panel B happens before panel A.  
Comment: this has been modified accordingly.

Figure 8: provide a brighter version of panels A and B as they look identical at the moment.  
Comment: this has been modified as suggested.

Figures 9,10,11 and 12: scale bars are missing  
Comment: this has been modified accordingly.

**Reviewer #5:**  
*Manuscript Summary:*  
In this manuscript, the authors describe a technique to visualize zebrafish retinal vasculature using the transgenic EGFP-fli1 line. This technique can be applied to a wide variety of uses, though the authors specifically focus on its relevance to the observation of vasculature complications associated with prolonged hyperglycemia (diabetic retinopathy). Though GFP fluorescence can be easily observed in zebrafish larvae, because they are transparent, the pigment present in adults makes this problematic. Thus, the technique described by the authors provides a means to observe fluorescent labeling in adults. This is an important technique. There are a few comments that need to be addressed, as well as some minor corrections.  
Comment: we would like to thank the reviewer for the positive comments to our manuscript.

*Major Concerns:*  
1. Are the adults used light or dark adapted prior to fixation? Adaptational state can make a significant difference with regard to the ease with which the retina can be separated from the sclera.  
Comment: We have used fish which were kept under a standard light cycle, e.g. 12 hours light and 12 hours dark. All fish were sacrificed in the morning, two hours after the light switch on.

2. Why not simply visualize the vasculature through the lens? Presumably this is because visualization through the lens will only show central vasculature and not peripheral vessels. Can the authors speak to differences in image quality, extent, etc with their preparation vs. keeping the eye intact and visualizing vasculature through the lens.  
Comment: Yes, we expect a higher imaging quality of a flat mounted retina as compared to keeping the eye intact, although we have not systematically compared both procedures. Likewise, a flat mounted retina is better accessible for further manipulations (antibody staining) and analyses (quantification).

3. Please provide more detail with regard to the dissecting tools. For example, what type of scissors are 'microdissection scissors'? Are these spring/iris scissors? Straight blade or curved? The 'sharp needle' - what size is that? 000? What size forceps do you use? #5? Are the forceps straight blade or curved? Etc. The materials list at the end is helpful, but these details would also be useful in the text itself.  
Comment: We have added this information as requested into the Material section.

4. The authors are advised to take care with their directional/anatomical terms. For example, referring to 'top' and 'bottom' of the eye is misleading. The authors should consider 'corneal side' or 'optic nerve side' or 'lateral' and 'medial', as these terms are more clear. More appropriate anatomical terms should be used in the text, figures, and figure captions. It may also be helpful to include these directions on panel C in each figure. This panel is a great addition to each figure, and indicating direction will help to better orient the reader, particularly when orientation in either panel A or B may not be readily obvious. A good example for this is Figure 6 where it is difficult to orient how the images in panels A and B go with the drawing in panel C.

Comment: We have clarified the anatomical terms and provide a direction for better orientation.

5. Building on the above comment, the authors use terms such as 'shell' and 'cup'. It is appreciated that these are descriptional terms, however, they are also colloquial or lab-based terms. For clarity, the authors could (1) add a qualifier to these terms, such as 'shell (sclera)' and/or (2) include their term on their figures (in panel C).  
Comment: We have clarified the anatomical terms.

6. Figures 11 and 12 show H&E stained retinal sections. How are these prepared?  
Comment: We have added the protocol how the sections where prepared.

*Minor Concerns:*  
1. section 4.9, line 233: "…Afterwards, take of the iris…" should be "Afterwards, take off the iris…"  
Comment: this has been modified accordingly.

2. discussion, line 403: "…To optimize time, resources spend on this step…" should be " To optimize time and resources spent on this step…"  
Comment: this has been modified accordingly.

3. discussion, line 422: "…human and rodent tissue is working…." Should be "…human and rodent tissue are working…"  
Comment: this has been modified accordingly.

4. Figure 4 caption, line 322: Panel B shows 'the light reflecting sclera is not covering the whole area around the optic nerve'. This is difficult to see in the figure. The authors should consider circling the area around the optic nerve that is not covered by the sclera or adding an arrow to this panel for clarity.  
Comment: we have highlighted the area as suggested.

5. Figure 5. Should panels A and B be switched? Doesn't the image in B occur before the image in A when you do the dissection?  
Comment: this has been modified as suggested.

6. Would it be possible to include a box on Figure 10A to indicate where the higher magnification panels B and C are located with respect to central retina? If consistent, this would be useful information to indicate.  
Comment: Unfortunately not, because panels in b or c are not seen in a.