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## Studying diabetes through the eyes of a fish: Microdissection, visualization and analysis of the adult tg(fli:EGFP) zebrafish retinal vasculature --Manuscript Draft--

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<b>Abstract:</b>	<p>Diabetic retinopathy is the leading cause of blindness among middle-aged adults. The rising prevalence of diabetes worldwide is already shaping the prevention of diabetic microvascular complications to be one of the key research fields of the next decades. Specialized, targeted therapy and novel therapeutic drugs are needed to manage to upcoming amount of patients with risk of vision-loss. The zebrafish is an established animal model for developmental research questions with a growing relevance to also model metabolic multifactorial disease processes. The advantages of the species allow for optimal visualization, high throughput drug screening approaches combined with the strong ability to knock out genes of interest. Here, we describe a method protocol which will allow an easy analysis of the adult tg(fli:EGFP) zebrafish retinal vasculature as fast read-out in settings of long-term vascular pathologies linked to neoangiogenesis or vessel damage. This is achieved via dissection of the zebrafish retina and whole-mounting of the tissue. Visualization of the exposed vessels is then achieved via confocal microscopy of the green EGFP reporter expressed in the adult retinal vasculature. Correct handling of the tissue will lead to better outcome and less internal vessel breakage to assure the visualization of the unaltered vascular structure. The method can be utilized in zebrafish models of retinal vasculopathy linked to changes in the vessel architecture as well as neoangiogenesis.</p>
<b>Author Comments:</b>	Dear Dr. DSouza,

	<p>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.</p> <p>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.</p> <p>We look forward to a favorable response from you, at your earliest convenience.</p> <p>Yours sincerely, Jens Kroll.</p>
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**TITLE:**

Studying Diabetes through the Eyes of a Fish: Microdissection, Visualization, and Analysis of the Adult tg(fli:EGFP) Zebrafish Retinal Vasculature

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**KEYWORDS:**

Vasculature, diabetes mellitus, complications, visualization, zebrafish, *danio rerio*, retina, hyaloid, protocol, retinopathy, tg(fli:EGFP)

**SHORT ABSTRACT:**

Here, we discuss a method protocol which will allow an easy analysis of the adult tg(fli:EGFP) zebrafish retinal vasculature as a fast read-out in settings of long-term vascular pathologies linked to neoangiogenesis and structural changes.

**LONG ABSTRACT:**

Diabetic retinopathy is the leading cause of blindness among middle-aged adults. The rising prevalence of diabetes worldwide will make the prevention of diabetic microvascular complications one of the key research fields of the next decades. Specialized, targeted therapy and novel therapeutic drugs are needed to manage the increasing number of patients at risk of vision-loss. The zebrafish is an established animal model for developmental research questions with increasing relevance for modeling metabolic multifactorial disease processes. The advantages of the species allow for optimal visualization, high throughput drug screening approaches, combined with the strong ability to knock out genes of interest. Here, we describe a protocol which will allow easy analysis of the adult tg(fli:EGFP) zebrafish retinal vasculature as a fast read-out in settings of long-term vascular pathologies linked to neoangiogenesis or vessel damage. This is achieved via dissection of the zebrafish retina and whole-mounting of the tissue. Visualization of the exposed vessels is then achieved via confocal microscopy of the green EGFP reporter expressed in the adult retinal vasculature. Correct handling of the tissue will lead to

better outcomes and less internal vessel breakage to assure the visualization of the unaltered vascular structure. The method can be utilized in zebrafish models of retinal vasculopathy linked to changes in the vessel architecture as well as neoangiogenesis.

## **INTRODUCTION:**

Diabetes mellitus is a metabolic disease defined by hyperglycemia resulting from dysfunctional insulin secretion or inadequate tissue response to secreted insulin. The WHO estimates that 422 million adults were living with diabetes mellitus in 2014<sup>1</sup> and the prevalence of diabetes worldwide is expected to increase to 8-10% of the population until 2035<sup>2</sup>, making diabetes one of the key research fields in the next decades. Living with chronic high blood sugar leads to long-term microvascular complications, including diabetic retinopathy, nephropathy, and polyneuropathy. The management and prevention of these complications are difficult; indeed, diabetes is becoming the most frequent cause of end-stage renal disease (ESRD), which leads to dialysis<sup>2</sup>, and diabetes is the leading cause of blindness among middle-aged adults<sup>3</sup>.

The initial causes of microvascular damage in the diabetic eye are chronic hyperglycemia, metabolic alterations, as well as certain risk factors (*e.g.*, hypertension, dyslipidemia), leading to vascular endothelial dysfunction, pericyte dropout, and capillary regression that results in acellular vascular sleeves. The resulting retinal ischemia is the cause of neovascularization and increased vascular permeability promoting the development of proliferative diabetic retinopathy (PDR)<sup>4</sup>. Diabetic retinopathy was generally detected in 37% of diabetes patients, while sight-threatening diabetic retinopathy was ascertained in 12% of the screened white European cohort in the UKADS study<sup>5</sup>. The current treatment can only prevent further complications and is not able to fully restore the already induced damage. Panretinal photocoagulation, in addition to glycemic control, is the standard therapy for proliferative diabetic retinopathy (PDR) but affects the adjacent healthy tissue as well. Anti-VEGF interventions show promising results as alternatives to laser treatment<sup>6,7</sup>, but ultimately, specialized, targeted therapy and new drugs are needed to manage the growing number of patients at risk of vision-loss.

The established animal research models of diabetic retinopathy do not share every aspect of human pathophysiology. Utilization of the correct species to address the specific requirements of the scientific research question is one of the most substantial parts of the experimental setup. The zebrafish embryo (*Danio rerio*) is already widely used in developmental research and provides an optimal practical background to knockdown or knockout specific genes via morpholinos or the CRISPR/Cas9 technique<sup>8</sup>. These methods can easily be utilized in zebrafish to investigate genes, which were identified by large scale genome-wide association studies (GWAS), generating insights into particular mechanisms of disease progression and susceptibility<sup>9</sup>. Short generational time, large amounts of offspring, easy and low-cost handling, and growing assay support have increased the relevance of the zebrafish model, especially given its great potential for modeling metabolic disease. Conservation of biological mechanisms in zebrafish has been shown as a basis for pharmacological therapy development. For example, the antidiabetic drug metformin and cholesterol-lowering simvastatin have been shown to “treat” conditions in models of cAMP/dexamethasone-induced high PEPCCK expression and high-cholesterol diet-induced hypercholesterolemia<sup>10-12</sup>. This advancing insight into overarching conserved metabolic

mechanisms is further supported by the growing number of zebrafish diabetes models, which have enabled experiments such as: alternating incubation in glucose solutions, Streptozotocin-induced ablation of beta cells, nitroreductase-mediated beta cell ablation using the prodrug metronidazole, monogenic diabetes mediated via *pdx1* gene knockdown or knockout, as well as models of increased insulin resistance in skeletal muscle<sup>12</sup>. These already established protocols, the above mentioned specifics of the species, and the ability to efficiently manipulate the genome in a large number of samples all demonstrate the advantages of the zebrafish for studying the mechanisms driving complex disease processes as well as the ability to screen for pharmacological interventions.

A general understanding of the basic zebrafish eye anatomy (**Figure 1**) is necessary for the dissector in order to utilize zebrafish as a model for retinal angiopathy. The zebrafish eye has six extraocular muscles, four rectus, and two oblique muscles that insert on the outside of the globe at the sclera<sup>13</sup>. The cornea is the transparent tissue covering the lens and continues directly into the sclera, which forms the outer shell of the eye. The sclera is non-transparent, has a partially light reflecting surface, and is strongly pigmented. The lens itself is more ball-shaped than the human counterpart. The retina consists of three nuclear layers of neuronal cells while the oxygen-providing retinal vasculature is closely associated with the inner ganglion cell layer but does not form a subretinal network. Choroidal vessels, in contrast, lie in between the sclera and retina and are associated with the retinal pigmented epithelium (RPE). This capillary network supplies oxygen to the outer parts of the retina<sup>14</sup>.

[Place FIGURE 1 here]

Straightforward visualization of the retinal vasculature can be achieved by utilization of the transgenic *tg(fli:EGFP)* zebrafish line<sup>15</sup>. The green fluorescent protein expressed under control of the *fli* promotor in endothelial cells of the vasculature is the basis for the visualization via a laser scanning microscope in the later steps. This is achieved via dissection of the zebrafish retina and whole-mounting of the tissue. This transgenic model provides a fast vascular readout without any application of intravascular labeling or whole-mount immunohistochemistry. To analyze diabetic retinopathy in zebrafish, a step by step and standardized preparation routine should be used by every dissector. The following preparation protocol provides other research groups the option to easily evaluate vascular changes in the exposed vessels of the adult zebrafish eye and provide guidance to establish an optimized dissection routine for the zebrafish retina.

## **PROTOCOL:**

All procedures, including steps related to animal subjects, have been approved by the Animals Ethics Committee (Regierungspräsidium Karlsruhe) and follow the animal care guidelines of Heidelberg University.

### **1. Preparation of Fixative (4% PFA/PBS)**

1.1. Prepare fixative fresh every day to ensure optimal preservation of histological tissue integrity.

1.2. Dissolve 0.2 g of sodium hydroxide (NaOH) in 90 mL double distilled water (ddH<sub>2</sub>O) while constantly stirring at room temperature.

1.3. Add 4 g paraformaldehyde (PFA) and stir until the solution is completely clear for at least 5-10 min to assure depolymerization of macromolecules.

Caution: PFA is toxic, handle with care.

1.4. Dissolve 0.84 g of sodium dihydrogen phosphate (NaH<sub>2</sub>PO<sub>4</sub>) in the solution and control pH at 7.2 via measurement.

1.5. Adjust volume to 100 mL with ddH<sub>2</sub>O and filter the solution through grade 3 filter paper.

1.6. Store 4% PFA/PBS on ice.

## **2. Preparation of Euthanasia Solution**

Note: The following steps were done with ABTL wild-type zebrafish strain at a general age of 6-8 mon.

2.1. Use ethyl 3-aminobenzoate methane sulfonate, also named “tricaine” or MS-222, in a concentration of 0.31 mg/mL for zebrafish euthanasia<sup>16</sup>. Utilize 1x egg water, used to raise zebrafish embryos, as solvent for the euthanasia solution. Generally, control the correct anesthesia depth before working with sedated fish by demonstrating loss of equilibrium and touch reaction loss.

2.1.1 To attain 1,000 mL of 10x egg water, add these salts to 1,000 mL: double distilled water (ddH<sub>2</sub>O) while constantly stirring in the following order: 10 g NaCl, 0.3 g KCl, 0.4 g CaCl<sub>2</sub>•6H<sub>2</sub>O, 1.32 g MgSO<sub>4</sub>•6H<sub>2</sub>O.

## **3. Fixation of Zebrafish Tissue**

3.1. Transfer 6 mL 4% PFA/PBS solution per sample into the wells of six-well plates in advance.

3.2. Euthanize the adult zebrafish (previously maintained on a standard light-day cycle, *e.g.*, 12 h: 12 h) 2 h after lights turn on in the morning, by placing them in the tricaine solution and wait until they have reached euthanasia. Following the cessation of opercular movement, which can take up to 10 min.

3.3. Take the fish out of the tricaine euthanasia solution and put them on fresh paper towels. Dry the fish and use a scalpel to cut the heads behind the operculum (see **Figure 2A**). Transfer the heads directly into the prepped well plates, which contain the freshly prepared fixative.

3.4. Store the well plates containing the fish heads at 4 °C overnight for at least 24 h to assure the fixative penetrates the deeper retinal layers.

Note: Zebrafish heads can be stored for a maximum of 48 h in 1x phosphate buffered saline (PBS) at 4 °C before preparation without a relevant loss of fluorescence signal strength. Increased latency can lead to loss of tissue integrity and reduced picture quality, and thus should be avoided.

#### 4. Preparation of the Zebrafish Retinal Vasculature

4.1. Fill a petri dish up to a third with heated 2% agarose and wait until the agar is firm. Cover the agar plate with 1x PBS to create a workspace to dissect the eyes.

Note: This will allow both preservation of preparation tweezers and low pressure on the tissue while dissecting, reducing the possibility of structural damage. All further preparation steps should be performed in this workspace utilizing #5 straight forceps with fine tips under a dissecting microscope with additional epi-illumination.

4.1.1. While dissecting, use magnification between 4.0x and 6.0x to allow for both overview and detail-oriented assessment of the tissue.

4.2. Transfer the fixed sample into the petri dish and, by holding the head at the cut surface with one tweezer, insert another pinned-together tweezer below the eyeball at the orbital cavity. Slowly open the tweezer below the eye and grip the optic nerve, then carefully tear and detach the eyes (**Figure 2B**).

[Place FIGURE 2 here]

4.3. Remove any of the four rectus and two oblique extraocular muscles that are still connected to the eye as well as residual extraocular tissue connecting the eye to the orbital cavity (compare **Figure 3** and **Figure 4**).

4.3.1. Achieve this by holding back the eye through a half-closed tweezer and, gripping the structure with the other tweezer, softly rip it off with a diametric movement. Since gripping the eyeball directly leads to internal vessel breakage, this technique is appropriately gentle to preserve the vasculature.

[Place FIGURE 3 here]

4.4. Use a 27G (0.4 x 19 mm) disposable needle to puncture the cornea (**Figure 4C**, red arrow) at the outer range. Through this opening hold the cornea with both tweezers and slightly tear it open. Afterwards carefully work to create a centered tear approximately the size of the respective pupil.

[Place FIGURE 4 here]

4.5. Apply pressure on the corneal side of the ocular globe (bulbus oculi) at the outer corneal edge above the iris. This will create a small dent and push the lens to the height of the corneal tear. Run the tweezers under the lens and remove it (**Figure 5A**).

[Place FIGURE 5 here]

4.6. Turn the eye upside down to the optic nerve facing the researcher. Note that the sclera and cornea are connected and form the fibrous tunic of the eye bulb to protect the cup-shaped retina. (**Figure 6**).

4.6.1. This shell, consisting of the cornea and sclera, is also called “corneosclera” and spares out the area around the optic nerve (**Figure 4B**, red dashed line). Insert a needle at this discontinuation once to create an opening between the sclera and the retina.

[Place FIGURE 6 here]

4.7. Using this access with both tweezers, carefully rip the sclera axially into stripes to increase the opening around the optic nerve. Be careful to keep the corneosclera intact at the transition to the corneal side circumference (see **Figure 6A**).

4.8. Hold the sclera with one tweezer and, by grabbing the optic nerve with the other and pulling away, wholly remove the corneosclera from the eye and discard it (**Figure 6**). Try to sever any connections before separating the corneosclera and the remaining intraocular tissue, as attachment to other structures will be a critical point; this step will provide a cup-shaped structure consisting of the uvea and retina containing the retinal vasculature (**Figure 7**).

4.9. Create a rupture in the choroidal/RPE layer by holding a 27G needle sideways to the remaining cup while scraping the outer surface with the rim of the needle tip. Use the rupture as an access point to get a grip on the choroidal/RPE layer and rip it with both tweezers into stripes, but keep the connection to the iris intact.

4.9.1. Afterwards, run one tweezer under the iris and go around in a circuit from the outside while creating tension by pulling at the discontinued choroidal/RPE layer to achieve detachment of the combined structure.

Note: The iris must be disconnected so as to not obstruct the fluorescence while visualizing the vessels (**Figure 8B**). Grabbing the iris directly to remove it can lead to extensive vessel damage, especially at the inner optic circle (IOC), since the iris is still connected to the surrounding tissue. The choroidal layer and the retinal pigmented epithelium (RPE) can be separated and often retain a connection to the iris, which allows for an easy secondary removal via creation of a natural detachment at one point.



[Place FIGURE 7 here]

4.9.2. If parts of the iris cannot be removed, utilize another natural breaking point the adult zebrafish eye exhibits inside the photoreceptor layer (PL), in a similar fashion to step 4.9, to remove the iris.

4.9.3. Scrape over the outside of the remaining cup-shaped retina to induce discontinuations in the PL above the breaking point (**Figure 11C**, black arrow). Use the created access to remove the layer while keeping the possible connection to the iris intact. Afterwards, run the tweezers under the iris and go around in a circuit as mentioned before to detach the combined structure.

Note: One should exercise patience as the whole step 4.9 can be difficult, but this care will achieve a better vascular outcome than straightforwardly grabbing the iris at the rim of the pupil or forcing an opening by destroying connections to the choroidal/RPE or photoreceptor layer without their respective removal. Following this protocol will thus preserve retinal vascular integrity, but lead to damage of the outer retinal layers and should be considered when more than retinal vessels as readouts are of interest to the experimenter.

4.10. Use a microdissection spring scissor with a straight 2.5 mm cutting edge to cut the optic nerve as close as possible to the retina; this will allow for a better flat-mounting of the tissue.

[Place FIGURE 8 here]

## **5. Mounting of the Retinal Vasculature**

5.1. Wash the dissected retina two times for 5 minutes in 1x PBS. To transfer the retina after truncation of the optic nerve, utilize a lab spatula with a micro spoon end to avoid direct handling of the exposed tissue.

5.2. Place a drop of PBS onto a glass slide and transfer the retina into the droplet. Use a tweezer to keep the tissue in place while cutting the cup-shaped structure with a scalpel to create a flat four-petal or five-petal shape depending on the retinal size (see **Figure 9**).

5.3. Suck up the leftover PBS with a fine piece of paper. Be careful not to touch the retina.

5.4. Coat the flat-mounted retina in mounting media and cover with a coverslip. Be attentive not to create foam, as air bubbles can distort visualization of the retinal vessels.

5.5. Seal the cover with clear nail polish.

## **6. Visualization of the Retinal Vasculature**

6.1. Keep the time gap between preparation and visualization as short as possible to reduce image detail loss through fluorescence signal loss. Store the prepared retinal vasculature mounts

at 4 °C to reduce signal loss, if direct visualization cannot be achieved, as degradation of image quality slowly becomes visible 48 h after preparation.

## 6.2. Visualize the retinal vasculature mounts via fluorescence microscopy or laser scanning confocal microscopy.

Note: Vessel visualization by fluorescence microscopy was achieved at 2.5x magnification with an exposure time of 2.0 s, gain of 6.0x and gamma settings of 1.67. For confocal microscopy, an Ar-laser (488 nm/20 mW) at 20% power was utilized in combination with a TD 488/543/633 excitation filter, a 20x/0.7 NA multi-immersion objective with ddH<sub>2</sub>O as immersion media, and emission filter settings of 505-560 nm. Confocal pictures are composed of 4x4, 4x5, or 5x5 singular images combined through a tile scan depending on the retinal size.

## 7. HE Sections of the Zebrafish Retina

Note: For HE sections of the zebrafish retina, prepare the tissue as described in the protocol until step 4.5 and afterwards skip directly to step 7.1.

7.1. Wash the enucleated eyes (after step 4.5) two times for 5 minutes in 1x PBS. Afterwards, transfer the tissue into 70% ethanol (EtOH). At this point, prepared eyes can be stored at 4 °C until paraffin embedding.

7.2. Wash the tissue the following way to dehydrate it before paraffin embedding: 2x15 min in 80% ethanol, 2x15 min in 90% ethanol, 3x15 min in 96% ethanol, 3x15 min in 99% ethanol, 2x15 min in acetone/99% ethanol (1:2), 3x15 min in acetone. Keep the tissue in paraffin at 62 °C overnight.

7.3. Heat up fresh paraffin to 62 °C and pour it into embedding molds. Transfer the tissue into the molds directly and, at a fast pace, control correct orientation of the eyes for the desired section. Afterwards, apply an embedding cassette to the mold and cover with additional heated paraffin.

7.4. Remove the embedding molds after paraffin blocks have cooled down. Cut the paraffin blocks at a microtome into 10 µm sections and float them out on a 45 °C water bath on the water surface long enough for them to flatten completely.

7.5. Catch the sections on a glass slide and drain them vertically to remove excess water before drying them overnight in an oven at 45 °C.

7.6. Process the sections further with the following schedule to deparaffinize and HE stain: 4x1 min in xylol, 1x1 min in 99% ethanol, 1x1 min in 96% ethanol, 1x1 min in 80% ethanol, 1x1 min in 70% ethanol, 1x1 min in ddH<sub>2</sub>O, 1x4 min in Mayer's hematoxylin, 1x10 min in ddH<sub>2</sub>O, 1x2min in 0.5% eosin, 1x30 s in ddH<sub>2</sub>O, 1x30 s in 80% ethanol, 2x30 s in 96% ethanol, 3x1 min in 99% ethanol, and 1x1 min in xylol.

7.7. Let the glass slide dry completely before covering and sealing the stained tissue.

### REPRESENTATIVE RESULTS:

Here we demonstrate two typical morphological examples of the retinal vasculature in adult tg(fli:EGFP) zebrafish: once visualized with a fluorescence microscope (**Figure 9A**) and once with a confocal laser scanning microscope (**Figure 9B**). The revealed retinal structure shows a highly-organized pattern. Strong autofluorescence is seen in the zebrafish retina, when visualized with a fluorescence microscope. Thus, a visualization of only the vascular layer via a confocal scan is advised to reduce background fluorescence and increase contrast. For faster picture acquisition or situations where qualitative data are enough, the fluorescence microscope is an attractive alternative.

[Place FIGURE 9 here]

The optic artery penetrates the retina at the optic nerve head and, in most samples, spreads into 5-7 main vessels (**Figure 10A**). The main vessels then branch into a succession of arcades and connect to the inner optic circle (IOC), also called the circumferential vein (CV), encircling the optic disc in the periphery of the flat mounted retina (see **Figure 10B**). The IOC is the venous vessel that drains the arterial blood at the inner rim of the ocular globe (bulbus oculi). The zebrafish retinal vasculature also shows areas of high vascular activity with branching of the capillaries and angiogenic sprouting (**Figure 10C**). This vascular activity is mostly situated in near proximity to the IOC. It is important to notice that the same eye can demonstrate both high and low vascular activities in different regions of the retina (compare **Figure 10B** and **Figure 10C**). In general, the retinal vasculature of the zebrafish shows more avascular space and fewer capillaries in between the main branches if compared to, for example, the retina of mice<sup>17</sup>. Both eyes of each sample should be analyzed because the vasculature may vary in between and singular inspection can lead to bias.

[Place FIGURE 10 here]

A highly-conserved architecture of neuronal layers is already present in zebrafish from approximately 72 h post fertilization (hpf) onwards<sup>18</sup>. The adult zebrafish retina shows the following layers from inside out (**Figure 11**): ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptor layer (PL), and the retinal pigmented epithelium (RPE). The estimation of vascular parameters from retinal vasculature visualization is shown in **Figure 12**. The inner retinal vasculature is situated on the ganglion cell layer (GCL) (**Figure 13B**, white box) while the choroidal capillaries would be associated with the retinal pigmented epithelium (RPE).

[Place FIGURE 11 here]

### FIGURE AND TABLE LEGENDS:

**Figure 1: Schematic depiction of the adult zebrafish eye.**

**Figure 2: Removal of the adult zebrafish eye.** Corneal side view with the eye still in the orbital cavity and intact optic nerve (A). Corneal side view with detached eye (B). Schematic depiction of the zebrafish eye at this step (C).

**Figure 3: Adult zebrafish eye with extraocular muscles in focus.** Lateral view with attachment of an extraocular muscle to the left outer rim of the ocular globe in focus (A). Optic nerve side view with extraocular muscles symmetrically flanking the optic nerve (B). Schematic depiction of the zebrafish eye at this step (C).

**Figure 4: Adult zebrafish eye after removal of all extraocular muscles.** Lateral view with the cleared outer rim of the ocular globe visible (A). Optic nerve side view with optic nerve in the middle. The light-reflecting sclera is not covering the whole area around the nerve (red dashed line) (B). Schematic depiction of the zebrafish eye at this step (C).

**Figure 5: Adult zebrafish eye in the process of lens removal.** Corneal side view with the lens pushed through the corneal tear (A). Corneal side view with the lens beside the ocular globe (B). Schematic depiction of the zebrafish eye at this step (C).

**Figure 6: Corneosclera consisting of sclera and cornea, which is disconnected from the remaining intraocular tissue.** Corneal side view showing the continuation of the translucent cornea into the pigmented sclera (A). Lateral view focused on the scleral part of the corneosclera (B). Schematic depiction of the removed corneosclera (C).

**Figure 7: Adult zebrafish eye after removal of the corneosclera.** Lateral view shows the remaining intraocular tissue with intact iris and optic nerve (A). Corneal side view with iris in focus (B). Schematic depiction of the zebrafish eye at this step (C).

**Figure 8: Adult zebrafish eye after removal of RPE/choroid and truncated optic nerve.** Optic nerve side view shows the retina with a truncated optic nerve (A). Corneal side view with a direct look onto the most inner layer of the retina (B). Schematic depiction of the zebrafish eye at this step (C).

**Figure 9: Representative pictures of adult tg(fli:EGFP) zebrafish retinal vasculature.** Two typical morphological examples of the retinal vasculature are shown: The central optic artery spreads into 5-7 main vessels, which then branch into a succession of arcades. All further vessels drain into the circumferential vein (CV) limiting the outer part of each petal. Visualization via fluorescence microscopy at 2.5x magnification (A). Visualization of the retinal vasculature by confocal laser scanning microscopy through a combined 5x5 single image tile scan (B).

**Figure 10: Magnified areas of adult tg(fli:EGFP) zebrafish retinal vasculature visualization.** The optic artery branching into main vessels (A). Retinal capillaries in an area of low vascular activity connecting to the inner optic circle (IOC) at the bottom of the picture (B). Retinal capillaries in an

area of high vascular activity connecting to the IOC (C).

**Figure 11: HE staining of the adult zebrafish eye.** Cross-sectional overview of the whole eye after removal of the lens (A). Retinal layers of the adult zebrafish eye<sup>19</sup> (B). Indication (black arrow) of the natural breaking point in the PL (C).

**Figure 12: Example presentation of vascular parameters as readout of retinal vasculature visualization.** Measurement of intervascular distance (red double arrow) near the inner optic circle (IOC) (A). Three branching points (red circles) near the IOC (B). Visualization of zebrafish retinal vasculature with an enlarged area (red box) showing a sprouting vessel (C). Vessel diameter measured over a certain distance (white double arrow) from the central artery (white cross) (D). Vessel density is the percentage of retinal area occupied by overlaying vessels (diagonal red lines) (E).

**Figure 13: Comparison of HE staining and autofluorescence in the adult zebrafish retina.** Retinal vasculature in focus above the GCL (A). Retinal vasculature (white box) showing green an EGFP signal and retinal layers exhibiting strong autofluorescence (B).

## DISCUSSION:

Models of hypoxia-induced retinal neoangiogenesis show increased numbers of branching points, angiogenic sprouts, total vascular area and decreased intercapillary distance in zebrafish<sup>20</sup>. These findings support the idea that the zebrafish is susceptible to diabetic microvascular complications<sup>21</sup>, as key findings of later diabetic retinopathy include hypoxia-mediated neoangiogenesis, which is strongly linked to VEGF expression. Hyperglycemia-induced changes in the zebrafish retina lead to increased thickness of the vessels but maintain the overall patterning<sup>22</sup>. Alternating immersion in glucose solutions for 30 days also decreases the thickness of the IPL and INL<sup>23</sup>. The direct influence of glucose metabolites in zebrafish as metabolic proponents of vasculopathy was also already shown. Additional vascular hyperbranching was observed in the trunk vasculature of zebrafish embryos after incubation with methylglyoxal<sup>24</sup>. At the moment, there is no animal model which provides all the key criteria of diabetic retinopathy. Early changes are often found, but the progression to proliferative diabetic retinopathy is missing<sup>25</sup>. The zebrafish also falls into this definition, as we have only seen either hypoxia-mediated neoangiogenesis or hyperglycemia-induced changes until now. The current findings support the idea that the zebrafish is susceptible to hyperglycemia-mediated vascular changes and as an animal model may potentially show a progression to proliferative diabetic retinopathy. Depending on the strength and exposure to the hyperglycemia-mediated effect, the right experimental model could lead to ischemia in certain areas of the zebrafish retina and promote neoangiogenesis as the key criteria of proliferative diabetic retinopathy. However, as the zebrafish is a comparably new player in the field of modeling long-term microvascular pathologies, upcoming diabetes models in zebrafish will provide further information and clarify its importance in respect to other models and their pathologies. For example, an in-cross of tg(gata1a:DsRed) zebrafish with red-labelled erythrocytes into the tg(fli:EGFP) line could be used to simultaneously visualize potential intraocular bleedings in future zebrafish models showing microaneurysms as a predictor of progression to PDR.

Since the retinal vasculature progresses into a succession of arcades, evaluation of the intervascular distance, number of branching points and the total vascular area are related to the distance from the central optic artery. To avoid bias in the assessed vascular parameters, a point of orientation is required. The IOC is such a structure and is highly relevant since areas of vascular activity lie in direct spatial proximity. For consistent assessment, the retinal scan should be divided into multiple rectangular image sections with a consistent distance to the IOC. The entire retina should be analyzed and images numerically symmetrically distributed. The zebrafish retina shows areas with high and low capillary density and an unequal distribution of image sections can lead to additional bias.

[Place FIGURE 12 here]

To measure the intervascular distance, one needs to set a certain distance to the IOC as standard (**Figure 12A**, white double arrow) and draw an imaginary horizontal line (**Figure 12A**, horizontal white line) parallel to the IOC. The distance between the vessels on this line added up and arithmetically averaged equals the intervascular distance. Branching points are counted inside each image whenever a vessel splits and more than one vascular lumen continue from the point of origin. This also includes horizontal connections between capillaries. It is important to stay consistent with analysis and decide which branching points to count and keep these rules throughout the whole experiment to reduce unneeded variation. Vascular sprouting, as demonstrated in **Figure 12C**, is another parameter that can be counted in each image to evaluate influence on the retinal vasculature. Angiogenic sprouts do not follow the arcade-like succession between central artery and IOC and focus near the outer parts of the retina. To assess certain vessel diameters, a point of orientation is always needed from which a set distance marks the measurement spot. The central artery's origin inside the retina provides such guidance for the main stem vessels (**Figure 12D**, white cross). The area occupied by vessels (**Figure 12E**, diagonal red lines) as a percentage of the whole retinal area is the vascular density and directly correlates to the avascular area.

A complete confocal scan of one sample should be composed of multiple high-detail pictures to allow visualization of small capillary hypersprouting. To optimize time and resources spent on this step, an automated tiling procedure should be used with a general scan depth for every tile. Uneven mounting of the retina can greatly increase the time spent to scan the vasculature with a confocal microscope. In an optimal approach one would like to only scan the vascular layer (**Figure 13B**, white box), but partial inclusion of the GCL is often necessary. Leaving an excess length of the optic nerve after truncation, as well as the cuts to achieve the flat-mount being too short, can lead to uneven mounting.

[Place FIGURE 13 here]

As the proper preparation of the zebrafish retinal vasculature needs prior training, a small sample size with untrained dissectors and strongly varying preparation results are a main limitation of the technique. While the preparation of tg(fli:EGFP) zebrafish eyes gives fast insight into the state

of the vasculature, the technique still utilizes around 20 min of work time per retina for an experienced researcher. All of these preparation steps for the retinal vasculature must be performed under a dissecting microscope and dissectors need to stay concentrated the whole time as a careless step can potentially induce vessel breakage. The dissector should practice regularly as prolonged absence from preparation reduces a dissector's likelihood of maintaining vessel integrity.

Furthermore, additional readout via immunohistochemistry (IHC) is still limited, as only a small number of antibodies validated on human and rodent tissue are working with zebrafish. Experimenters interested in IHC are advised to search for zebrafish-specific antibodies, especially when working with new targets. Alternatively, it is recommended to use additional zebrafish reporter lines which are useful to study cells beyond the vasculature in the eye. This strategy, however, is time consuming, as it takes a few months to generate adult zebrafish lines which carry multiple transgenic reporters.

Nonetheless, the zebrafish poses a unique array of advantages. They are relatively small and reproduce readily. They can grow to adult stages quickly and their embryos are optimal for drug screening. The field is readily growing and more literature is becoming accessible. With the ability to generate gene knockouts at a rapid speed and an abundance of transgene fluorescence reporter lines, the choice for zebrafish is only restrained by the chosen research question.

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#### **DISCLOSURES:**

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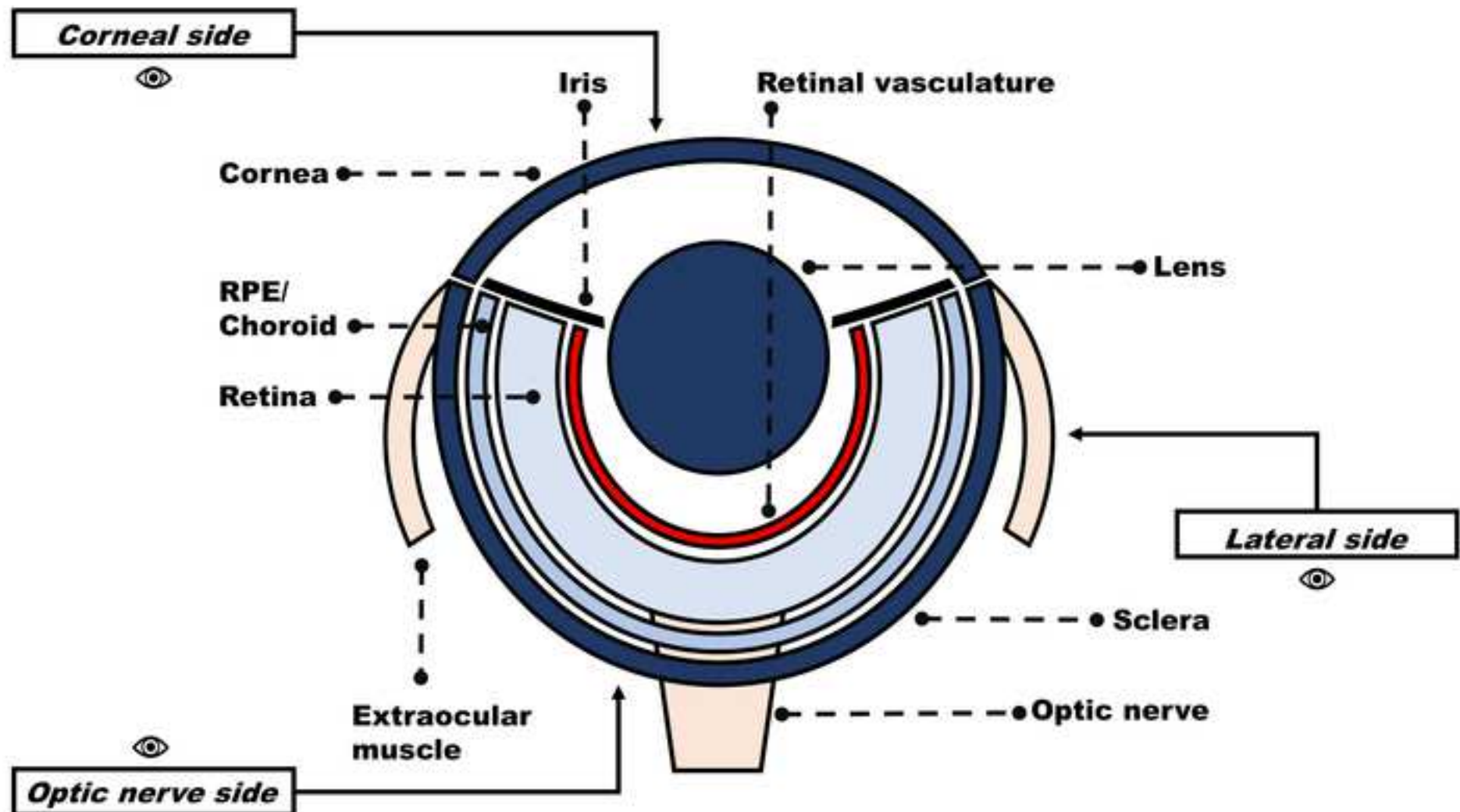
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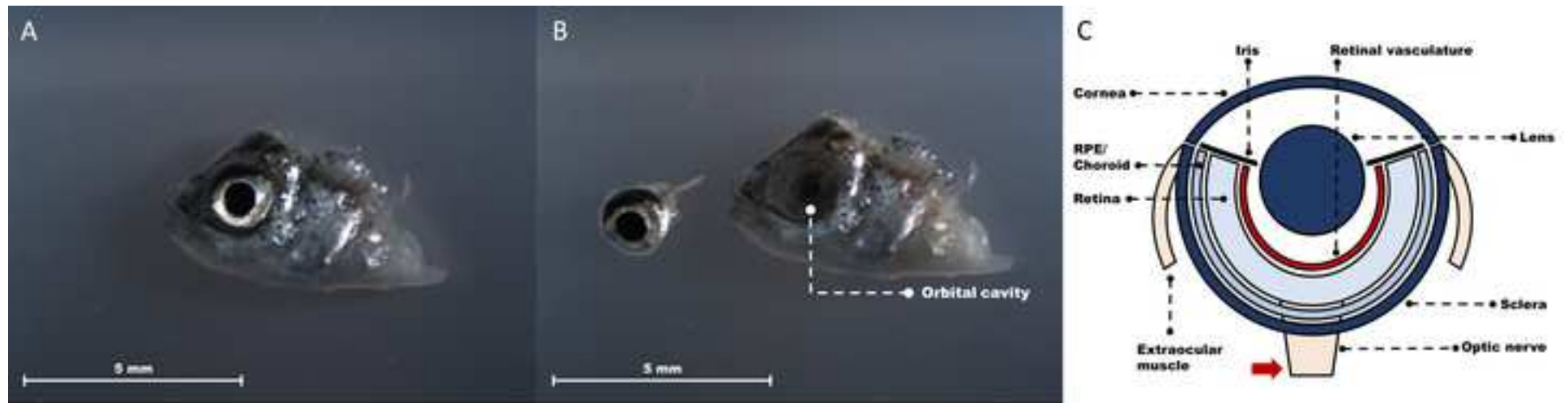


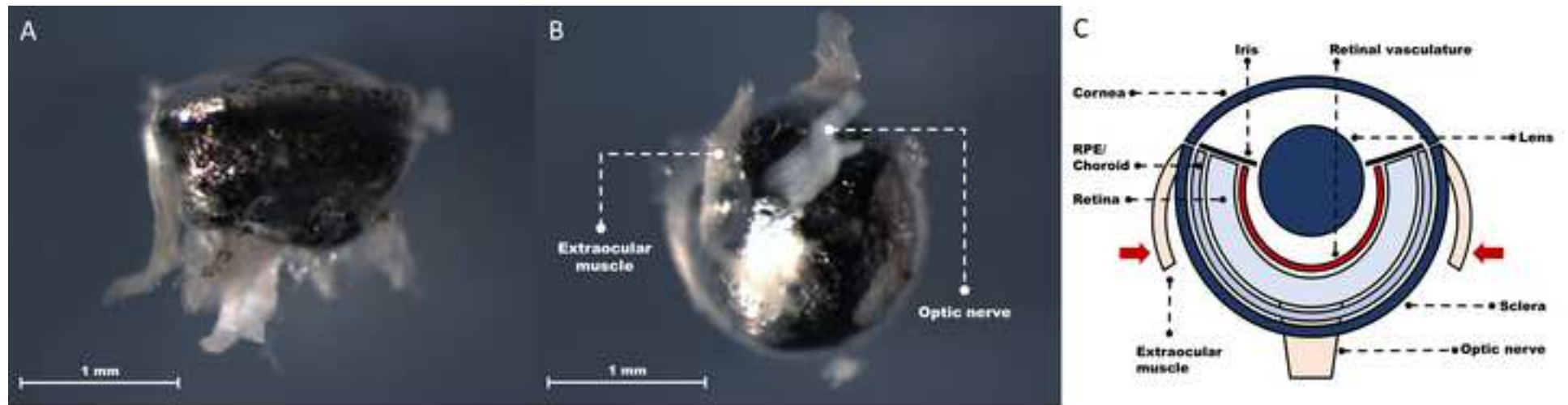
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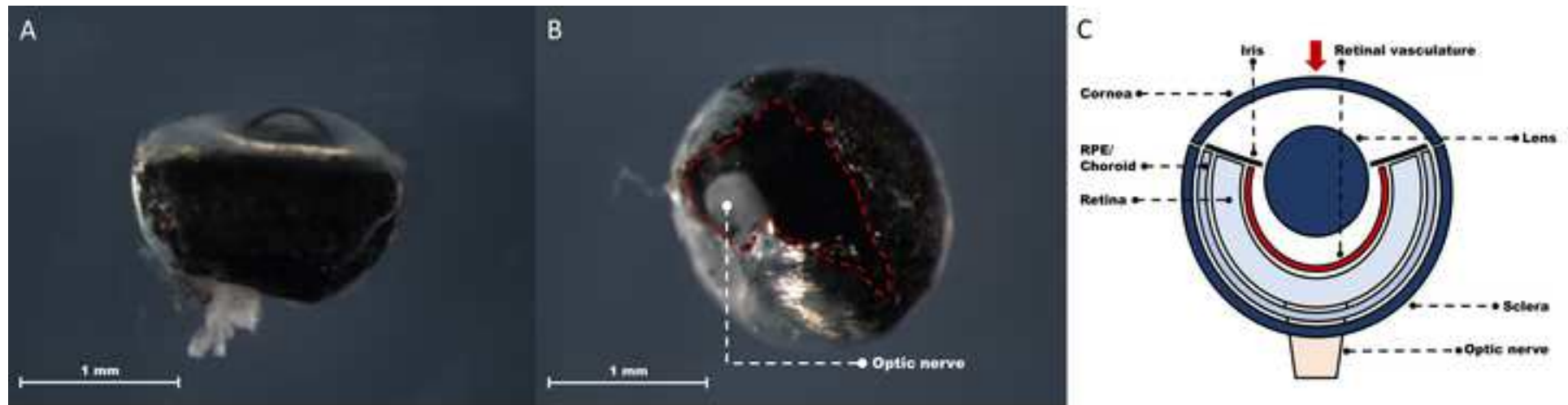


Figure 5

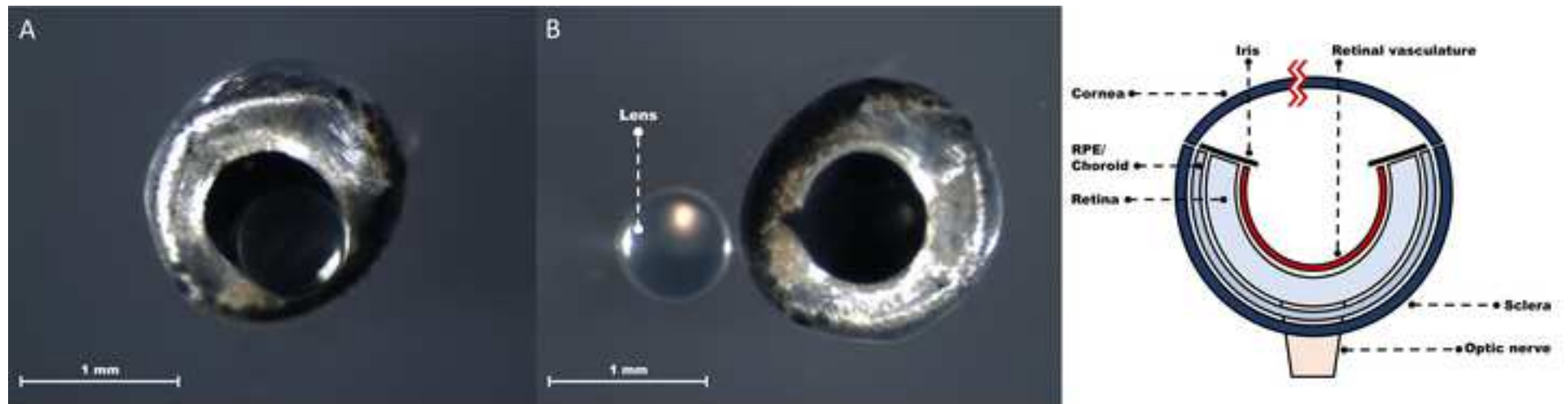
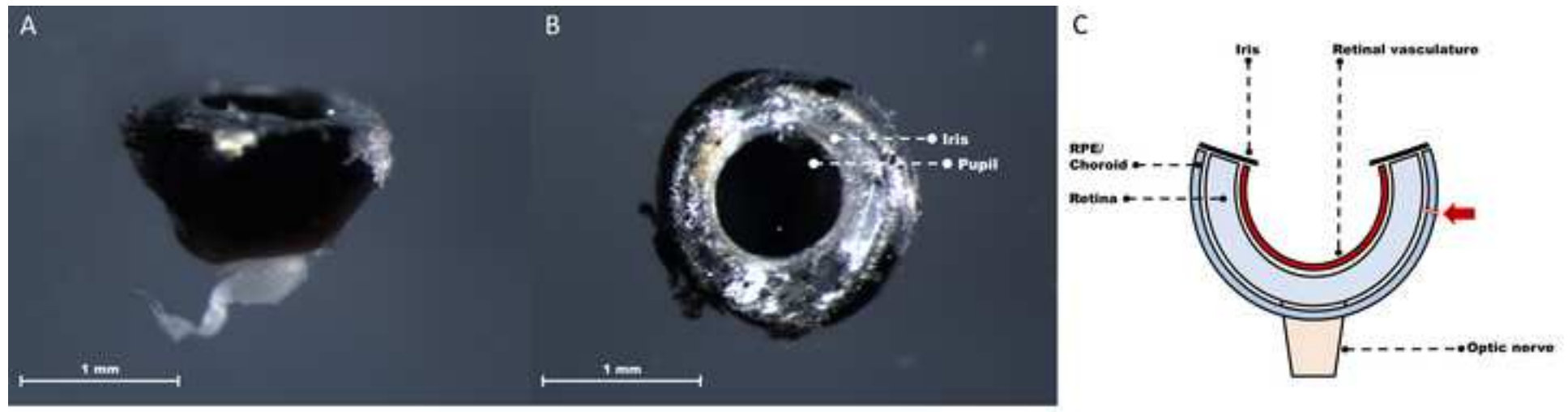


Figure 6







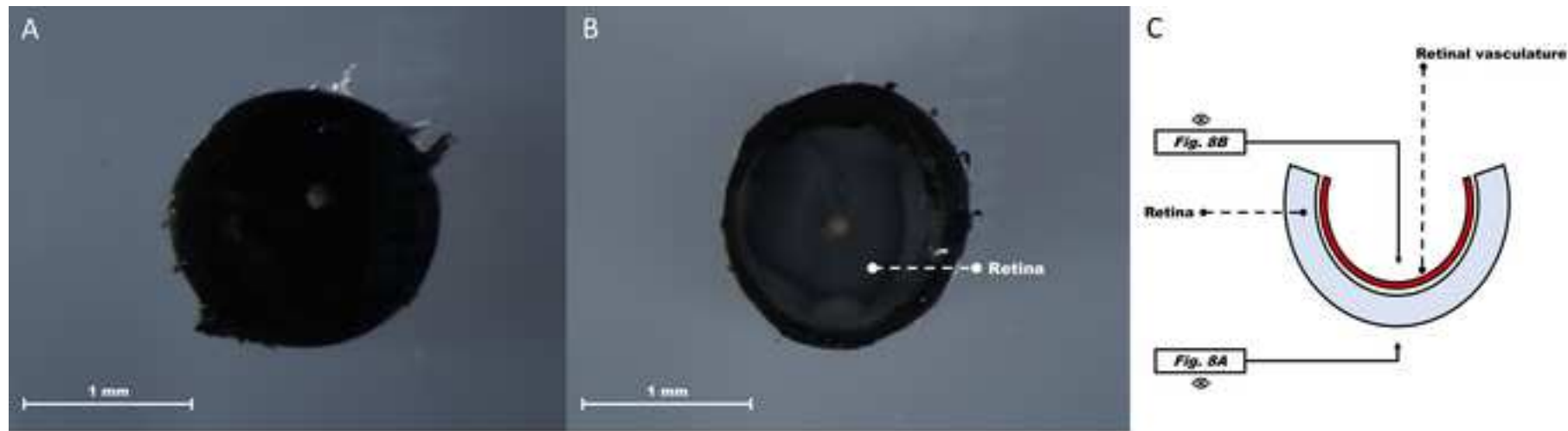
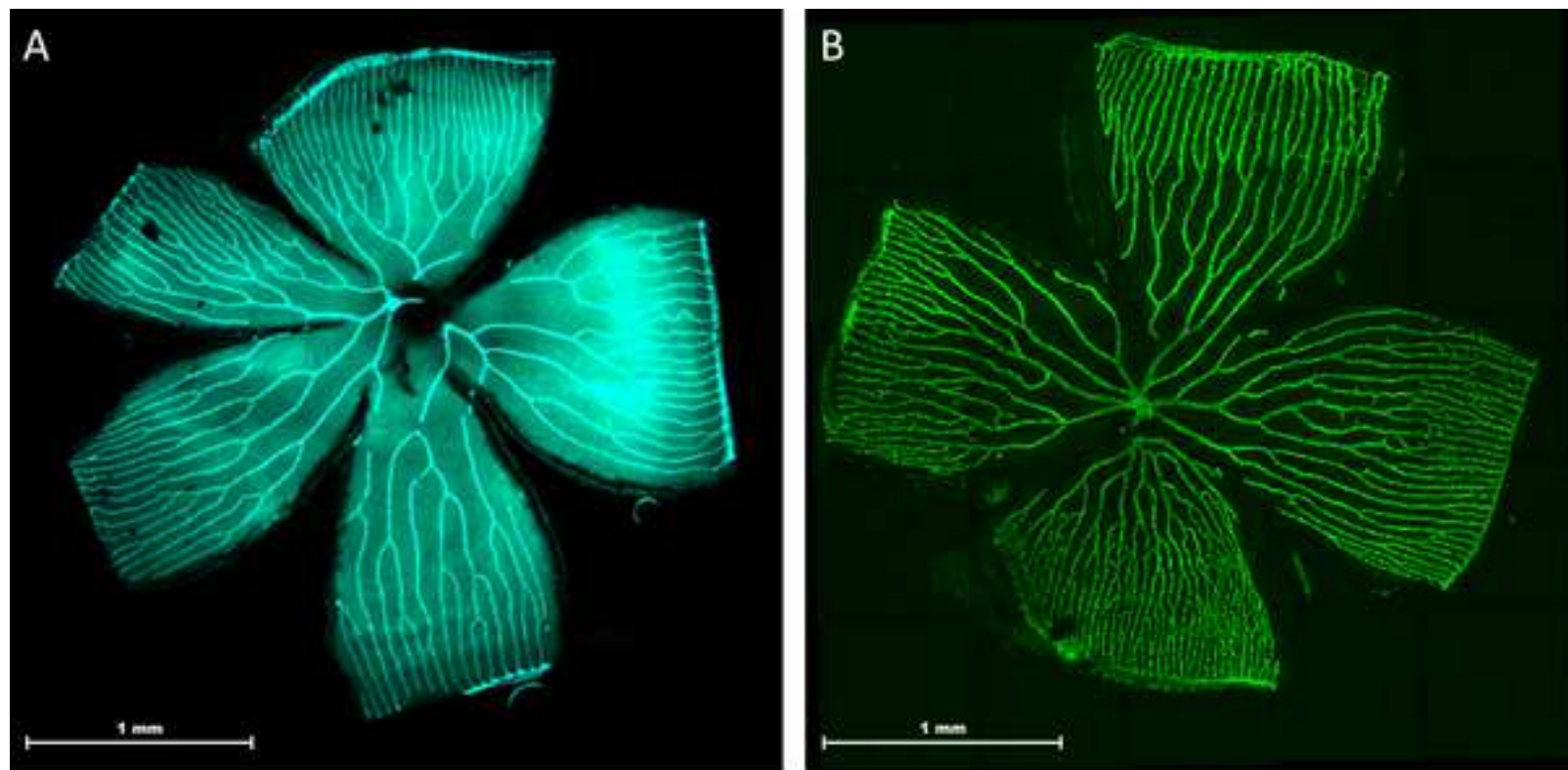
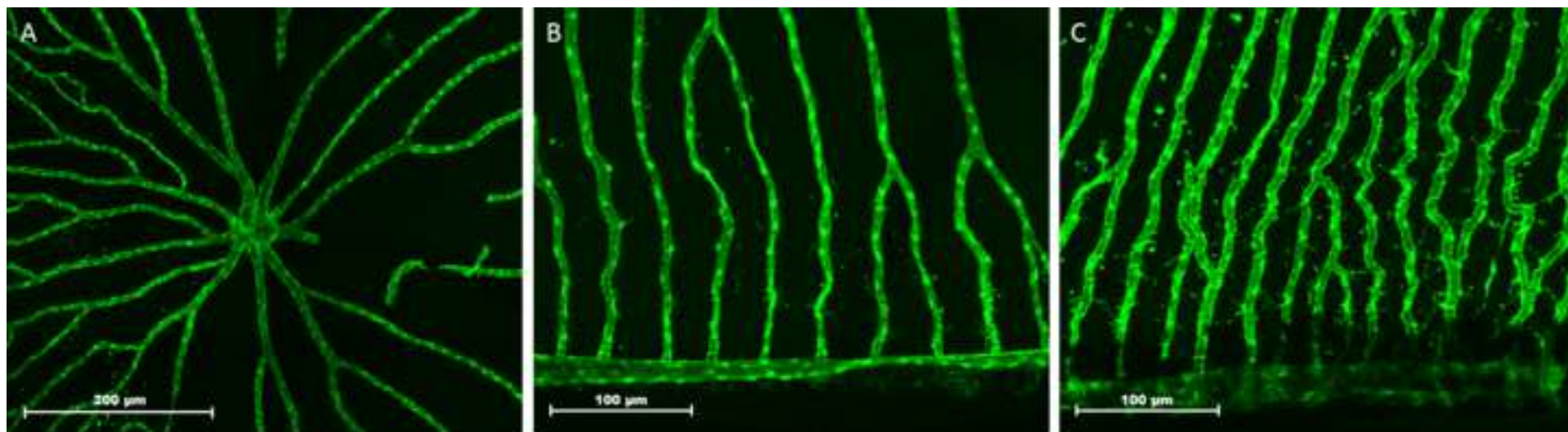
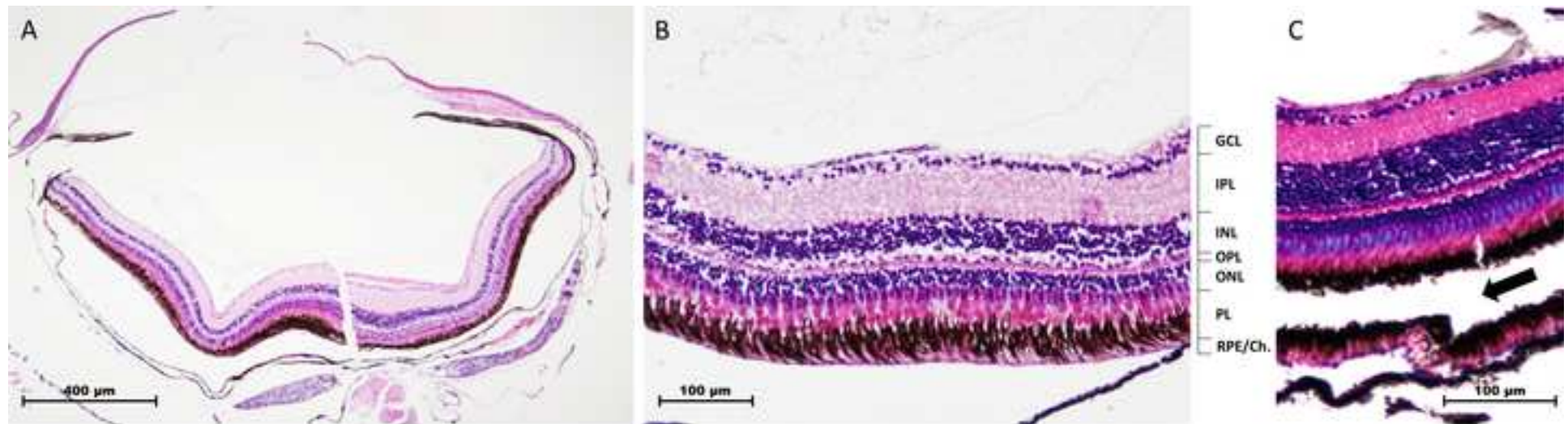


Figure 9







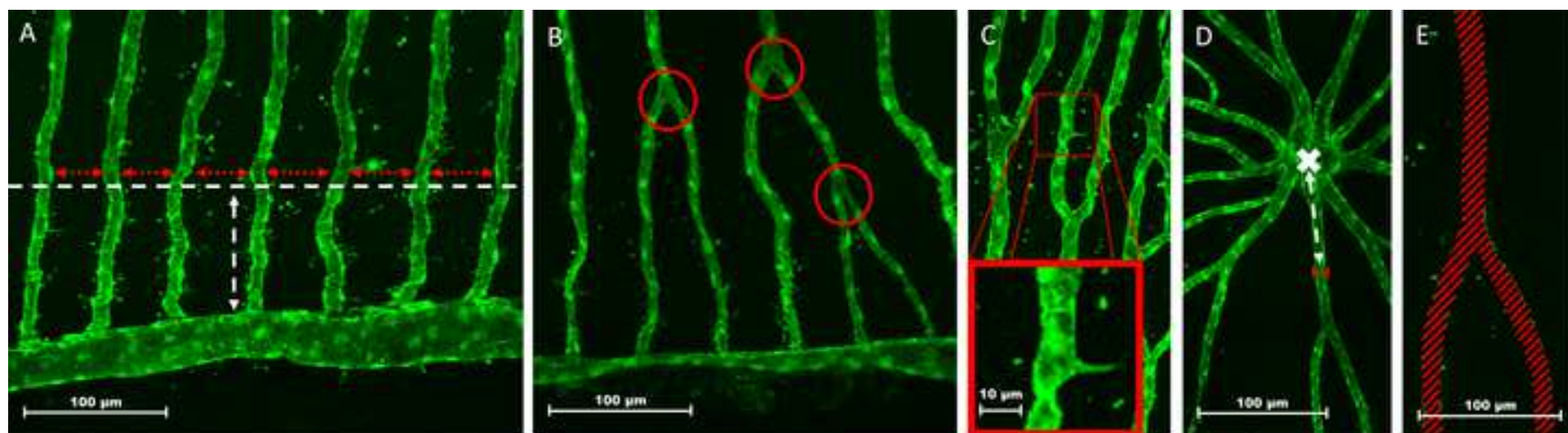
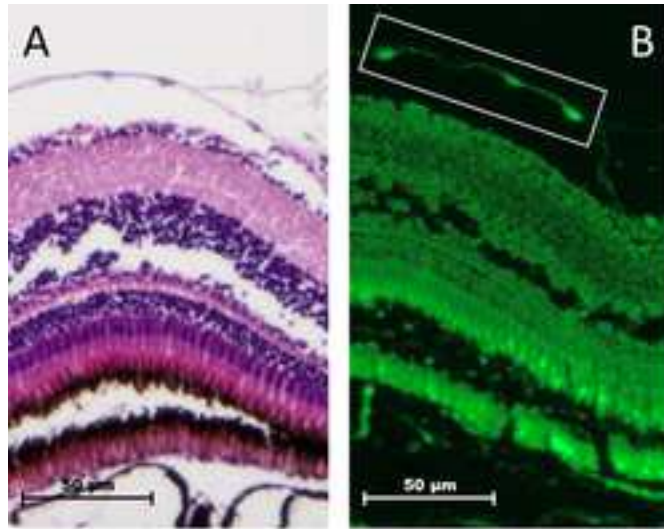




Figure 13



Name of Material/ Equipment	Company
NaOH	Roth
KCl	Merck
CaCl <sub>2</sub> *6H <sub>2</sub> O	Roth
MgSO <sub>4</sub> *7H <sub>2</sub> O	Merck
Paraformaldehyd	Roth
Sodium dihydrogen phosphate	Roth
Ethyl 3-aminobenzoate methanesulfonate salt (MS-222,Tricaine)	Sigma
PBS	Roth
Agarose	Roth
Fluoromount-G	Thermo-Fischer
Petri dish	Greiner Bio one
Six-well plate	StarLab
Needle	MSG Praxisbedarf
Micro Tweezer	World Precision Instruments
Microdissection Scissor	World Precision Instruments

Glass slide	Carl Roth
Coverslip 22mmx22mm	neoLab
Scalpel	MSG Praxisbedarf
Epi-Illumination	Leica
Fluorescence stereomicroscope MZ10 F	Leica
Confocal laser-scanning microscope SP5 DS	Leica
Stereomicroscope M80	Leica
Zebrafish line: Tg(fli:EGFP), ABTL wildtype	NA
Mayer's hematoxylin	Dr. K. Hollborn & Söhne
0.5% eosin	Dr. K. Hollborn & Söhne
99,9% ethanol	Roth
Paraffin	Merck
Xylol	Roth
Acetone	Emsure
Microtome RM 2165	Leica



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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  $\mu\text{L}$ , 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

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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 labelling (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-flil1 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 descriptive 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 were 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.