Video Article

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

Lucas Moritz Wiggenhauser[1], Katharina Kohl[2], Nadine Dietrich[2], Hans-Peter Hammes[2], Jens Kroll[1]

[1]Department of Vascular Biology and Tumorangiogenesis, Center for Biomedicine and Medical Technology Mannheim (CBTM), Medical Faculty Mannheim, Heidelberg University

[2]V. Medical Clinic, Medical Faculty Mannheim, Heidelberg University

Correspondence to: Jens Kroll at Jens.Kroll@medma.uni-heidelberg.de

URL: https://www.jove.com/video/56674
DOI: doi:10.3791/56674

Keywords: Medicine, Issue 130, Vasculature, diabetes mellitus, complications, visualization, zebrafish, *danio rerio*, retina, hyaloid, protocol, retinopathy, tg(fli:EGFP)

Date Published: 12/26/2017

Citation: Wiggenhauser, L.M., Kohl, K., Dietrich, N., Hammes, H.P., Kroll, J. Studying Diabetes Through the Eyes of a Fish: Microdissection, Visualization, and Analysis of the Adult tg(fli:EGFP) Zebrafish Retinal Vasculature. *J. Vis. Exp.* (130), e56674, doi:10.3791/56674 (2017).

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 and 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.

Video Link

The video component of this article can be found at https://www.jove.com/video/56674/

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[1] and the prevalence of diabetes worldwide is expected to increase to 8-10% of the population until 2035[2], 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[2], and diabetes is the leading cause of blindness among middle-aged adults[3].

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)[4]. 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[5]. 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[6,7], 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[8]. 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[9]. 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 PEPCK expression and high-cholesterol diet-induced hypercholesterolemia[10,11,12]. This advancing insight into overarching conserved metabolic mechanisms is further supported by the growing number of zebrafish diabetes models, through 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[12]. 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[13]. 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[14].

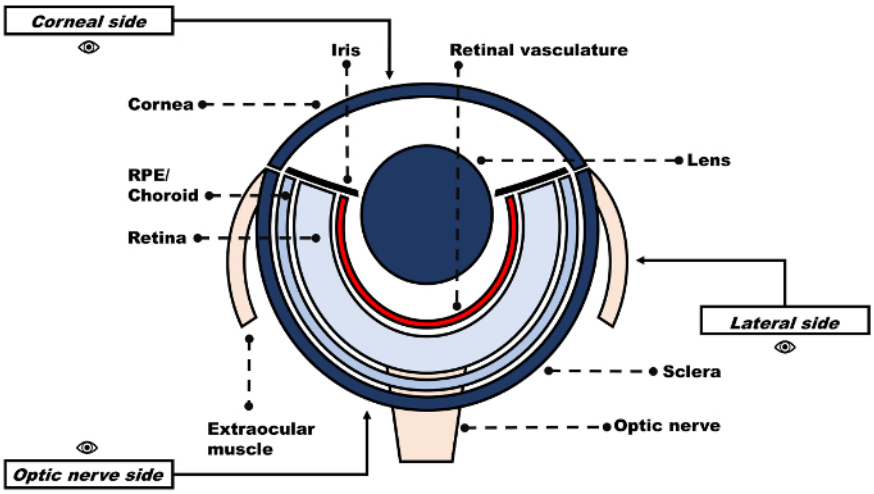

Figure 1: Schematic depiction of the adult zebrafish eye. Please click here to view a larger version of this figure.

Straightforward visualization of the retinal vasculature can be achieved by utilization of the transgenic tg(fli:EGFP) zebrafish line[15]. 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. Prepare fixative fresh every day to ensure optimal preservation of histological tissue integrity.
2. Dissolve 0.2 g of sodium hydroxide (NaOH) in 90 mL double distilled water (ddH_2O) while constantly stirring at room temperature.
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.
4. Dissolve 0.84 g of sodium dihydrogen phosphate (NaH_2PO_4) in the solution and control pH at 7.2 via measurement.
5. Adjust volume to 100 mL with ddH_2O and filter the solution through grade 3 filter paper.
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.

1. Use ethyl 3-aminobenzoate methane sulfonate, also named "tricaine" or MS-222, in a concentration of 0.31 mg/mL for zebrafish euthanasia[16]. 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.
    1. To attain 1,000 mL of 10x egg water, add these salts to 1,000 mL double distilled water (ddH_2O) while constantly stirring in the following order: 10 g NaCl, 0.3 g KCl, 0.4 g $CaCl_2{\cdot}6H_2O$, 1.32 g $MgSO_4{\cdot}6H_2O$.

3. Fixation of Zebrafish Tissue

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

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

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**).

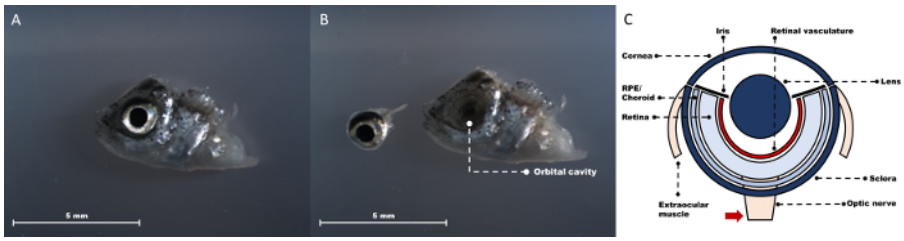

Figure 2: Removal of the adult zebrafish eye. Corneal side view with the eye still in the orbital cavity and intact optic nerve **(A)**. Corneal side view with detached eye **(B)**. Schematic depiction of the zebrafish eye at this step **(C)**. Please click here to view a larger version of this figure.

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**).
    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.

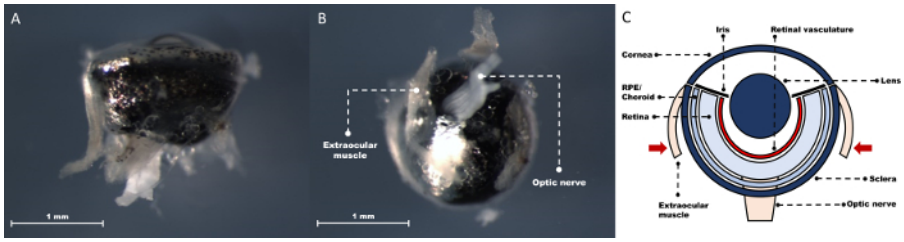

Figure 3: Adult zebrafish eye with extraocular muscles in focus. Lateral view with attachment of an extraocular muscle to the left outer rim of the ocular globe in focus **(A)**. Optic nerve side view with extraocular muscles symmetrically flanking the optic nerve **(B)**. Schematic depiction of the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

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.

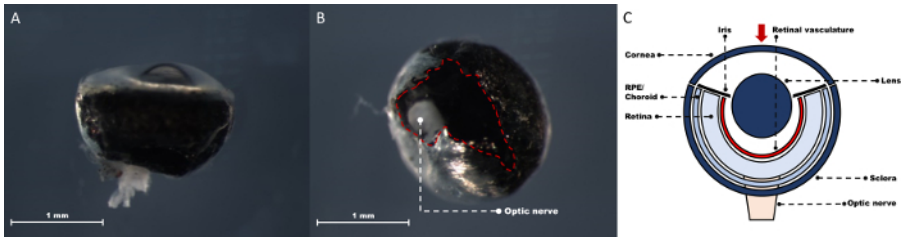

Figure 4: Adult zebrafish eye after removal of all extraocular muscles. Lateral view with the cleared outer rim of the ocular globe visible **(A)**. Optic nerve side view with optic nerve in the middle. The light-reflecting sclera is not covering the whole area around the nerve (red dashed line) **(B)**.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

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**).

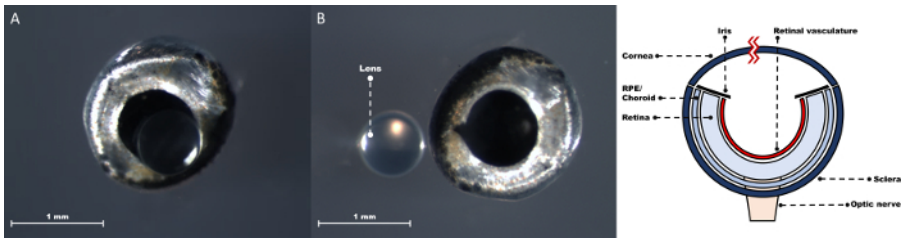

Figure 5: Adult zebrafish eye in the process of lens removal. Corneal side view with the lens pushed through the corneal tear **(A)**. Corneal side view with the lens beside the ocular globe **(B)**. Schematic depiction of the zebrafish eye at this step **(C)**. Please click here to view a larger version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

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**).
    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.

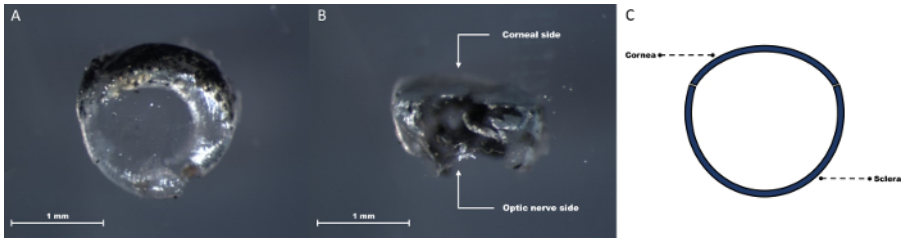

Figure 6: Corneosclera consisting of sclera and cornea, which is disconnected from the remaining intraocular tissue. Corneal side view showing the continuation of the translucent cornea into the pigmented sclera **(A)**. Lateral view focused on the scleral part of the corneosclera **(B)**. Schematic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

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**).
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**).
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.
    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.
    2. If parts of the iris cannot be removed, utilize a 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.
    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 upper part of 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 step will thus preserve retinal vascular integrity, but lead to damage of the outer retinal layers.

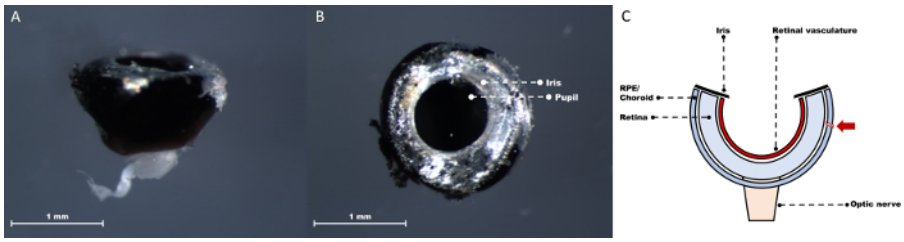

Figure 7: Adult zebrafish eye after removal of the corneosclera. Lateral view shows the remaining intraocular tissue with intact iris and optic nerve **(A)**. Corneal side view with iris in focus **(B)**. Schematic depiction of the zebrafish eye at this step **(C)**. Please click here to view a larger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

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.

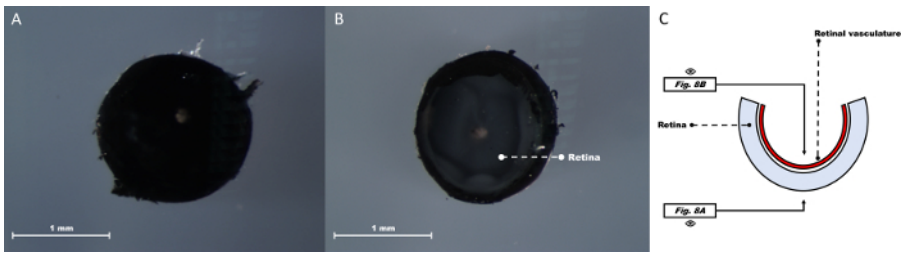

Figure 8: Adult zebrafish eye after removal of RPE/choroid and truncated optic nerve. Optic nerve side view shows the retina with a truncated optic nerve **(A)**. Corneal side view with a direct look onto the most inner layer of the retina **(B)**. Schematic depiction of the zebrafish eye at this step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

5. Mounting of the Retinal Vasculature

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.
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**).
3. Suck up the leftover PBS with a fine piece of paper. Be careful not to touch the retina.
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. Minimize movement of the coverslip and seal the cover with clear nail polish.

6. Visualization of the Retinal Vasculature

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.
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_2O 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.

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.
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.
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.

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.
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.
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_2O, 1x4 min in Mayer's hematoxylin, 1x10 min in ddH_2O, 1x2min in 0.5% eosin, 1x30 s in ddH_2O, 1x30 s in 80% ethanol, 2x30 s in 96% ethanol, 3x1 min in 99% ethanol, and 1x1 min in xylol.
7. Take the glass slide out of the xylol and quickly cover the tissue with mounting media. Avoid to let the tissue dry out completely, then place a coverslip on top and seal 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.

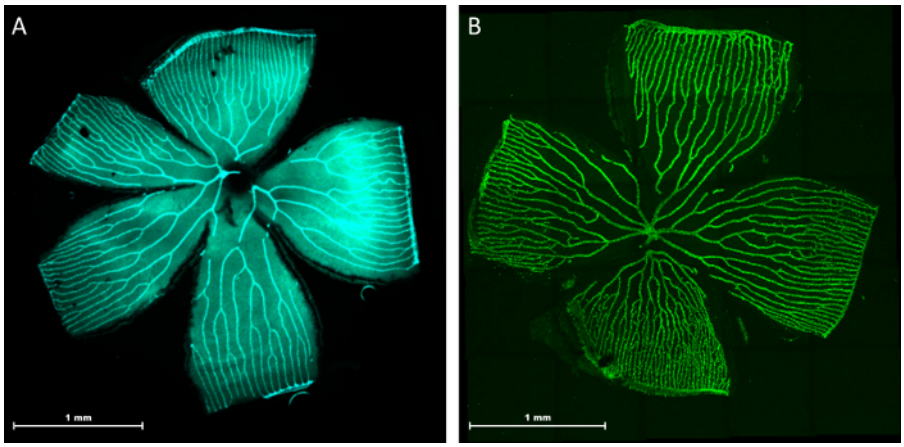

Figure 9: Representative pictures of adult tg(fli:EGFP) zebrafish retinal vasculature. Two typical morphological examples of the retinal vasculature are shown: The central optic artery spreads into 5-7 main vessels, which then branch into a succession of arcades. All further vessels drain into the circumferential 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)**. Please click here to view a larger version of this figure.

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[17]. Both eyes of each sample should be analyzed because the vasculature may vary in between and singular inspection can lead to bias.

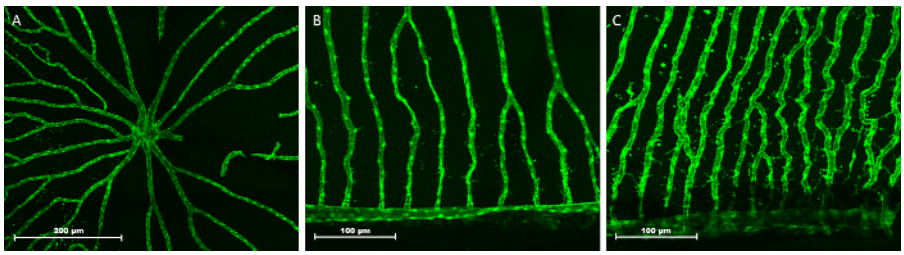

Figure 10: Magnified areas of adult tg(fli:EGFP) zebrafish retinal vasculature visualization. The optic artery branching into main vessels **(A)**. Retinal capillaries in an area of low vascular activity connecting to the inner optic circle (IOC) at the bottom of the picture **(B)**. Retinal capillaries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

A highly-conserved architecture of neuronal layers is already present in zebrafish from approximately 72 h post fertilization (hpf) onwards[18]. 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).

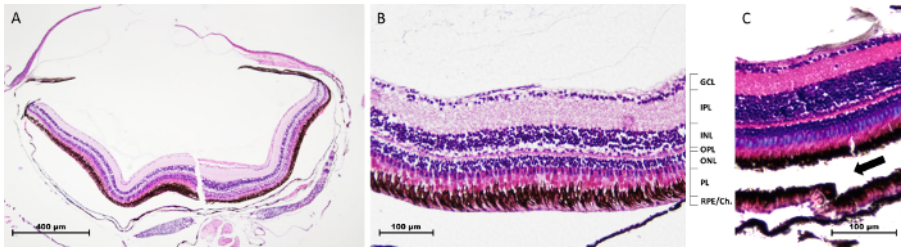

Figure 11: HE staining of the adult zebrafish eye. Cross-sectional overview of the whole eye after removal of the lens **(A)**. Retinal layers of the adult zebrafish eye[19] **(B)**. Indication (black arrow) of the natural breaking point in the PL **(C)**. Please click here to view a larger version of this figure.

Discussion

Models of hypoxia-induced retinal neoangiogenesis show increased numbers of branching points, angiogenic sprouts, total vascular area and decreased intercapillary distance in zebrafish[20]. These findings support the idea that the zebrafish is susceptible to diabetic microvascular complications[21], 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[22]. Alternating immersion in glucose solutions for 30 days also decreases the thickness of the IPL and INL[23]. 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[24]. 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[25]. 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.

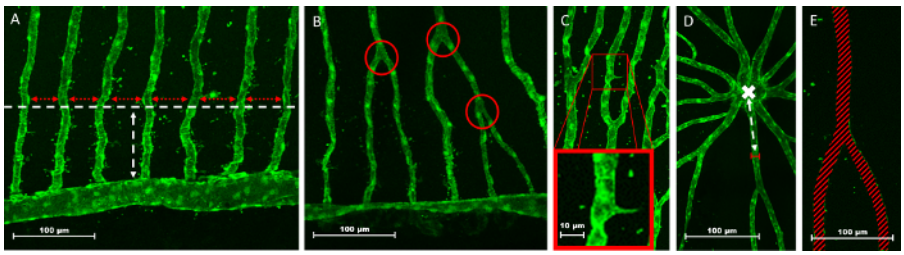

Figure 12: Example presentation of vascular parameters as readout of retinal vasculature visualization. Measurement of intervascular distance (red double arrow) near the inner optic circle (IOC) **(A)**. Three branching points (red circles) near the IOC **(B)**. Visualization of zebrafish retinal vasculature 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)**. Please click here to view a larger version of this figure.

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 indirectly 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 tilling 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.

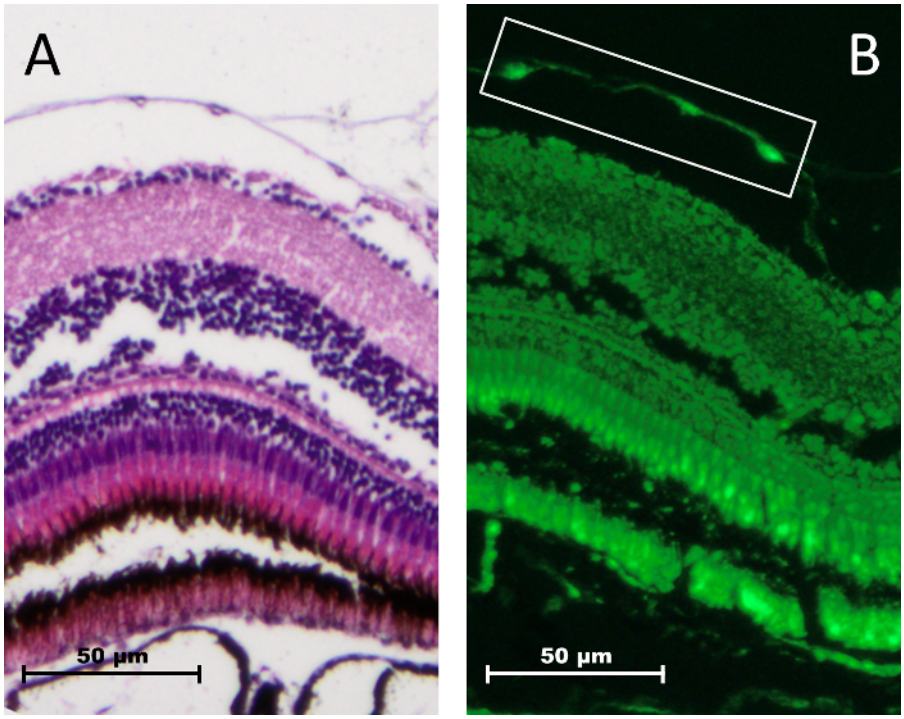

Figure 13: Comparison of HE staining and autofluorescence in the adult zebrafish retina. Retinal vasculature in focus above the GCL **(A)**. Retinal vasculature (white box) showing green an EGFP signal and retinal layers exhibiting strong autofluorescence **(B)**. Please click here to view a larger version of this figure.

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.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors would like to thank Katrin Bennewitz and Marlene Hausner for zebrafish husbandry and technical assistance. The authors acknowledge the support of the Core Facility Live Cell Imaging Mannheim at the Centre for Biomedicine and Medical Technology Mannheim (DFG INST 91027/9-1). This study was supported by Deutsche Forschungsgemeinschaft (International Research Training Group 1874/1 "DIAMICOM", project SP5 and SP9; Collaborative Research Centre SFB1118, project B1 and Collaborative Research Centre SFB/TR23 project Z5).

References

1. WHO. *Global report on diabetes.* 88 (2016).
2. Lim, A. Diabetic nephropathy - complications and treatment. *Int J Nephrol Renovasc Dis.* **7**, 361-381 (2014).
3. Yau, J.W. *et al.* Global prevalence and major risk factors of diabetic retinopathy. *Diabetes Care.* **35** (3), 556-564 (2012).
4. Cheung, N., Mitchell, P., & Wong, T.Y. Diabetic retinopathy. *The Lancet.* **376** (9735), 124-136 (2010).
5. Raymond, N.T. *et al.* Higher prevalence of retinopathy in diabetic patients of South Asian ethnicity compared with white Europeans in the community: a cross-sectional study. *Diabetes Care.* **32** (3), 410-415 (2009).
6. Heng, L.Z. *et al.* Diabetic retinopathy: pathogenesis, clinical grading, management and future developments. *Diabet Med.* **30** (6), 640-650 (2013).
7. Writing Committee for the Diabetic Retinopathy Clinical Research, N. *et al.* Panretinal Photocoagulation vs Intravitreous Ranibizumab for Proliferative Diabetic Retinopathy: A Randomized Clinical Trial. *JAMA.* **314** (20), 2137-2146 (2015).
8. Jao, L.E., Wente, S.R., & Chen, W. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proc Natl Acad Sci U S A.* **110** (34), 13904-13909 (2013).
9. Sharma, K.R. *et al.* ELMO1 protects renal structure and ultrafiltration in kidney development and under diabetic conditions. *Sci Rep.* **6**, 37172 (2016).
10. Baek, J.S., Fang, L., Li, A.C., & Miller, Y.I. Ezetimibe and simvastatin reduce cholesterol levels in zebrafish larvae fed a high-cholesterol diet. *Cholesterol.* **2012**, 564705 (2012).
11. Elo, B., Villano, C.M., Govorko, D., & White, L.A. Larval zebrafish as a model for glucose metabolism: expression of phosphoenolpyruvate carboxykinase as a marker for exposure to anti-diabetic compounds. *J Mol Endocrinol.* **38** (4), 433-440 (2007).
12. Gut, P., Reischauer, S., Stainier, D.Y.R., & Arnaout, R. Little Fish, Big Data: Zebrafish as a Model for Cardiovascular and Metabolic Disease. *Physiol Rev.* **97** (3), 889-938 (2017).
13. Kasprick, D.S. *et al.* Microanatomy of adult zebrafish extraocular muscles. *PLoS One.* **6** (11), e27095 (2011).
14. Gestri, G., Link, B.A., & Neuhauss, S.C. The visual system of zebrafish and its use to model human ocular diseases. *Dev Neurobiol.* **72** (3), 302-327 (2012).
15. Lawson, N.D., & Weinstein, B.M. In vivo imaging of embryonic vascular development using transgenic zebrafish. *Dev Biol.* **248** (2), 307-318 (2002).
16. Matthews, M., & Varga, Z.M. Anesthesia and euthanasia in zebrafish. *ILAR J.* **53** (2), 192-204 (2012).
17. Bell, B.A. *et al.* Retinal vasculature of adult zebrafish: in vivo imaging using confocal scanning laser ophthalmoscopy. *Exp Eye Res.* **129**, 107-118 (2014).
18. Avanesov, A., & Malicki, J. Analysis of the retina in the zebrafish model. *Methods Cell Biol.* **100**, 153-204 (2010).
19. Gramage, E., Li, J., & Hitchcock, P. The expression and function of midkine in the vertebrate retina. *Br J Pharmacol.* **171** (4), 913-923 (2014).
20. Cao, R., Jensen, L.D., Soll, I., Hauptmann, G., & Cao, Y. Hypoxia-induced retinal angiogenesis in zebrafish as a model to study retinopathy. *PLoS One.* **3** (7), e2748 (2008).
21. Jorgens, K., Hillebrands, J.L., Hammes, H.P., & Kroll, J. Zebrafish: a model for understanding diabetic complications. *Exp Clin Endocrinol Diabetes.* **120** (4), 186-187 (2012).
22. Alvarez, Y. *et al.* Predominant cone photoreceptor dysfunction in a hyperglycaemic model of non-proliferative diabetic retinopathy. *Dis Model Mech.* **3** (3-4), 236-245 (2010).
23. Gleeson, M., Connaughton, V., & Arneson, L.S. Induction of hyperglycaemia in zebrafish (Danio rerio) leads to morphological changes in the retina. *Acta Diabetol.* **44** (3), 157-163 (2007).
24. Jorgens, K. *et al.* High tissue glucose alters intersomitic blood vessels in zebrafish via methylglyoxal targeting the VEGF receptor signaling cascade. *Diabetes.* **64** (1), 213-225 (2015).
25. Lai, A.K., & Lo, A.C. Animal models of diabetic retinopathy: summary and comparison. *J Diabetes Res.* **2013**, 106594 (2013).