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TITLE:

Monitoring Dynamic Growth of Retinal Vessels in Oxygen-Induced Retinopathy Mouse Model

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SUMMARY:

This protocol describes a detailed method for the preparation and immunofluorescence staining of mice retinal flat mounts and analysis. The use of fluorescein fundus angiography (FFA) for mice pups and image processing are described in detail as well.

ABSTRACT:

Oxygen-induced retinopathy (OIR) is widely used to study abnormal vessel growth in ischemic retinal diseases, including retinopathy of prematurity (ROP), proliferative diabetic retinopathy (PDR), and retinal vein occlusion (RVO). Most OIR studies observe retinal neovascularization at specific time points; however, the dynamic vessel growth in live mice along a time course, which is essential for understanding the OIR-related vessel diseases, has been understudied. Here, we describe a step-by-step protocol for the induction of the OIR mouse model, highlighting the potential pitfalls, and providing an improved method to quickly quantify areas of vaso-obliteration (VO) and neovascularization (NV) using immunofluorescence staining. More importantly, we monitored vessel regrowth in live mice from P15 to P25 by performing fluorescein fundus angiography (FFA) in the OIR mouse model. The application of FFA to the OIR mouse model allows us to observe the remodeling process during vessel regrowth.

INTRODUCTION:

Retinal neovascularization (RNV), which is defined as a state where new pathologic vessels originate from existing retinal veins, usually extends along the inner surface of the retina and grows into the vitreous (or subretinal space under some conditions)[1]. It is a hallmark and common feature of many ischemic retinopathies, including retinopathy of prematurity (ROP), retinal vein occlusion (RVO), and proliferative diabetic retinopathy (PDR)[2].

Numerous clinical and experimental observations have indicated that ischemia is the main

cause of retinal neovascularization[3,4]. In ROP, neonates are exposed to high-level oxygen in closed incubators to increase the survival rates, which is also an important driver for the arrest of vascular growth. After the treatment is done, the retinas of newborns experience a relatively hypoxic period[5]. Other situations are seen in the occlusion of central or branch retinal veins in RVO and damage of retinal capillaries is also observed which is caused by microangiopathy in PDR[2]. Hypoxia further increased the expression of angiogenic factors such as vascular endothelial growth factor (VEGF) through the hypoxia-induced factor-1α (HIF-1α) signaling pathway which in turn guide vascular endothelial cells to grow into the hypoxic area and form new vessels[6,7].

ROP is a kind of vascular proliferative retinopathy in preterm infants and a leading cause of childhood blindness[8,9], which is characterized by retinal hypoxia, retinal neovascularization and fibrous hyperplasia[10–12]. In the 1950s, researchers found that high concentration of oxygen can significantly improve the respiratory symptoms of premature infants[13,14]. As a result, oxygen therapy was increasingly used in premature infants at that time[15]. However, concurrent with the widespread use of oxygen therapy in preterm infants, the incidence of ROP increased year by year. Since then, researchers have linked oxygen to ROP, exploring various animal models to understand the pathogenesis of ROP and RNV[16].

In human, most retinal vasculature development is completed before birth while in rodents the retinal vasculature develops after birth, providing an accessible model system to study angiogenesis in the retinal vasculature[2]. With the continuous progress of the research, oxygen-induced retinopathy (OIR) models have become major models for mimicking pathological angiogenesis resulting from ischemia. There are no specific animal species in the study of the OIR model and the model has been developed in various animal species, including kitten[17], rat[18], mouse[19], beagle puppy[20], and zebrafish[21]. All of the models share the same mechanism by which they are exposed to hyperoxia during early retinal development and then returned to the normoxic environment. Smith et al. observed that exposing mouse pups to hyperoxia from P7 for 5 days induced an extreme form of vessel regression in the central retina and bringing them back to the room air at P12 gradually triggered neovascular tufts, which grew toward the vitreous body[19]. This was a standardized OIR mouse model also named as Smith model. Connor et al. further optimized the protocol and provided a universally applicable method to quantify the area of VO (vaso-obliteration) and NV (neovascularization) in 2009, which increased the acceptance and utilization of the model[22]. OIR mouse model is still the most widely used model now because of its small size, fast reproduction, clear genetic background, good repeatability, and high success rate.

In mice, retinal vascularization starts after birth with the ingrowth of vessels from the optic nerve head into the inner retina toward the ora serrata. During normal retinal development, the first retinal vessels sprout from the optic nerve head around birth, forming an expanding network (the primary plexus) that reaches the periphery around postnatal day 7(P7)[23]. Then the vessels start to grow into the retina to form a deep layer, penetrate the retina, and

establish a laminar network around the inner nuclear layer (INL) as in human[24]. By the end of the third postnatal week (P21), deeper plexus development is almost completed. For the OIR mouse model, vascular occlusion always appears in the central retina because of the rapid degeneration of a large number of immature vascular networks in the central region during hyperoxia exposure. So, the growth of pathological neovascularization also occurs in the mid-peripheral retina, which is the boundary of the non-perfusion area and the vascular area. However, human retinal vessels have almost formed before birth. As for premature infants, the peripheral retina is not completely vascularized when exposed to hyperoxia[25,26]. So vascular occlusion and neovascularization mainly appear in the peripheral retina[27,28]. Despite these differences, the mouse OIR model closely recapitulates the pathologic events that occur during ischemia-induced neovascularization.

The induction of the OIR model can be divided into two phases[29]: in phase 1 (hyperoxia phase), retinal vascular development is arrested or retarded with occlusion and regression of blood vessels as a result of the decline in VEGF and the apoptosis of endothelial cells[24,30]; in phase 2 (hypoxia phase), the retinal oxygen supply will become insufficient under room air conditions[29], which is essential for neural development and homeostasis[19,31]. This ischemic situation usually results in unregulated, abnormal neovascularization.

Currently, the commonly used modeling method is alternating high/low oxygen exposure: Mothers and their pups are exposed to 75% oxygen for 5 days at P7 followed by 5 days in room air till P17 demonstrated comparable results[22], which is the endpoint of OIR mouse model induction. (**Figure 1**). In addition to simulating ROP, this ischemia-mediated pathological neovascularization can also be used to study other ischemic retinal diseases. The main measurements of this model include quantifying the area of VO and NV, which are analyzed from retinal flat mounts by immunofluorescence staining or FITC-dextran perfusion. Each mouse can be studied only once because of the lethal operation. At present, there are few methods to observe dynamic changes of retinal vasculature continuously during the process of vascular regression and pathologic angiogenesis[32]. In this paper, we provide a detailed protocol of OIR model induction, analysis of retinal flat mounts as well as a workflow of fluorescein fundus angiography (FFA) on mice which would be helpful to gain a more comprehensive understanding of vascular dynamic changes during two phases of the OIR mouse model.

PROTOCOL:

All procedures involving the use of mice were approved by the animal experimental ethics committee of Zhongshan Ophthalmic Center, Sun Yat-sen University, China (authorized number: 2020-082), and in accordance with the approved guidelines of Animal Care and Use Committee of Zhongshan Ophthalmic Center and the Association Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

1. Induction of mouse OIR model

1.1. Use mice with a lower rate of congenital malformation of the eyes, e.g., C57BL/6J mice, and mate them at a ratio of male/female = 1:2. Get the pups born on the same day and start to induce the OIR model at P7. Record the bodyweight of mouse pups strictly before modeling.

NOTE: Note the day of birth as P0. Record the weight of each mouse regularly. The bodyweight of newborn pups is very important during the induction of OIR as the sensitivity of mice in different states to oxygen is different. Exclude the pups more than 5 g at P7 to ensure comparable results.

1.2. Provide a suitable living environment for nursing mothers and their pups, such as setting the temperature at 23 °C ± 2 °C, controlling the humidity at 40%–65%, alternating 12 h of light and 12 h of darkness every day, adding some cotton wool to the cage for nesting, ensuring adequate sterilized food and water, and keeping them in individually ventilated cages (IVC).

NOTE: Add enough food to the bottom of the cage. Add boiled eggs and autoclaved melon seeds regularly to provide adequate nutrition for the nursing mothers.

1.3. Monitor the level of humidity and temperature inside the chamber. Control the humidity between 40% to 65% and keep the temperature at 23 °C ± 2 °C.

1.4. Check the oxygen supply with oxygen sensors, maintain a constant oxygen level at 75% and control the oxygen flow rate at 0.5-0.75 L/min. Put 50 g soda lime at the bottom of the chamber to absorb excessive CO2 and maintain CO2 values below 3%[22].

1.5. Monitor the behaviors of nursing mothers such as nest-building behavior, biting their pups, and refusing lactation at least once a day. Eliminate nursing mothers with poor motherhood.

1.6. Place the P7 pups (male and female) and their nursing mothers into an oxygen chamber in which the oxygen level is 75% for 5 days to P12. Avoid unnecessary opening of the chamber during the period of model induction. Ensure that there are extra surrogate mothers for replacement, in case the nursing mothers die due to lung injury while in hyperoxia.

NOTE: To ensure the comparability of the experiment, restrict the number to 6–8 pups for each mother. Pay attention to the potential problem of oxygen toxicity, which causes the death of some nursing mothers. Prepare some surrogate mothers, e.g., 129S1/SvImJ for replacement and use them only if necessary. It is not recommended to replace nursing mothers as a routine, as this will lead to frequent opening of an oxygen chamber, resulting in unstable oxygen levels and maternal aggression.

1.7. Bring the pups and their nursing mothers back to the room air at P12 and monitor the weight of all the pups continuously until P17. Group the pups based on the weight to ensure that each experimental group has a similar weight distribution.

2. Preparation of retinal whole mounts and immunofluorescence staining

2.1. Record the bodyweight of the pups. Sacrifice the pups by an overdose of anesthetic (1% pentobarbital sodium 20 mL/kg) or CO2 inhalation.

2.2. Use curved scissors to release the connection between eyeballs and orbital tissue. Then, put curved forceps into the posterior part of the eyeball, clamp the optic nerve, and quickly lift the eye out from the orbit. Wash the eyeballs in a pre-cooled 1x phosphate buffer saline (PBS) to remove the hair and blood from the surface of the eyeballs.

2.3. Place the cleaned eyeballs in a 2 mL microcentrifuge tube filled with 4% paraformaldehyde (PFA) and incubate for 15 min at room temperature on a shaker at a speed of 12–15 revolutions per minute (rpm) (initial fixation).

CAUTION: Paraformaldehyde is known to be allergenic, generally toxic, and extremely cytotoxic. Follow the safety instructions strictly and avoid inhalation and skin contact.

2.4. Use a culture dish and put a drop of 1x PBS into the central part and perform the following steps under a dissecting microscope and place one eyeball in this drop. Hold the eyeball with a pair of forceps and carefully puncture the cornea at the corneal limbus using a 1 mL syringe needle. Insert the tip of the scissors into this hole and cut off the cornea carefully along the cornea limbus. Be careful not to cut the retina.

2.5. Remove the iris and lens with a pair of forceps. Then place the remaining eyecup in the 4% PFA and fix again for another 45 min at room temperature on a shaker at a speed of 12–15 rpm (secondary fixation).

2.6. Use a culture dish and put a drop of 1x PBS into the central part. Place the fixed eyeball in this drop. Hold the eyeball with a pair of forceps. Gently separate the retina and sclera layers using two forceps. Place the tip of the scissors between the retina and sclera layers and cut the sclera toward the optic nerve. Peel the sclera off the retina and obtain the retinal cup.

NOTE: Hold the posterior cup by the optic nerve with forceps, then use the curved end of another forceps to press down on the sclera at the optic nerve head and gently massage out the retina in a forward sweeping motion as an alternative to release the retina.

2.7. Use forceps to release the connection between radial hyaloid vessels and peripheral retina, clamp the root of the hyaloid vessels which is close to the optic nerve head, and cut

the hyaloid vessels off carefully.

2.8. Use a 2 mL pipette with the tip cut off to transfer the retinal cup. Place the retinal cup into one well in a 48-well plate and wash it for 3 x 5 min with 1x PBS at room temperature on a shaker at a speed of 12–15 rpm.

2.9. Incubate the retinal cup in a mixed solution of 1% Triton X-100 (in PBS) and 5% normal donkey serum (in PBS) overnight at 4 °C.

2.9.1. Alternatively, block and permeabilize retinas at room temperature for 1 h as an alternative. Change blocking serum according to the source of the secondary antibody.

2.10. If labeling the retinal vasculature using Isolectin B4, incubate the retina in a well of 48-well plate with 0.1% normal donkey serum (400 µL) and IsolectinB4-594 (1:400) overnight at 4 °C on a shaker at a speed of 12–15 rpm.

NOTE: If labeling the blood vessels with other markers, such as CD31, or labeling other cells, use specific primary antibodies to label them.

2.11. Incubate the retina with 1:100–1:500 specific primary antibodies (in 400 µL 0.1% normal donkey serum) at 4 °C on a shaker at a speed of 12–15 rpm for 48 h. (optional)

2.12. After returning to the room temperature, wash the retina with 0.1% PBST (0.1% TritonX-100 in PBS) for 3 x 20 min on a shaker at a speed of 12–15 rpm.

2.13. Incubate the retina with 1:1,000 secondary antibodies (in 400 µL 0.1% normal donkey serum) overnight at 4 °C on a shaker at a speed of 12–15 rpm. (optional)

2.13.1. Alternatively, incubate the retina with high-affinity secondary antibodies at room temperature for 1 h.

2.14. Incubate the retina with DAPI (1:1,000) at room temperature for 20–25 min to label the nucleus.

NOTE: Test the optimal dilution ratios for all the antibodies used in steps 10–11 and 13–14 in pre-experiment.

2.15. Wash the retina for 3 x 30 min with 0.1% PBST on a shaker at a speed of 12– 15 rpm at room temperature.

2.16. Transfer the retinal cup to a clean slide with the opening facing upward. Cut the retina radially at the 3, 6, 9, and 12 o'clock positions from peripheral to central by cutting

approximately 1–1.5 mm away from the optic nerve head.

2.17. Add a few drops of 1x PBS to rinse the retina three times. Use air-laid paper to dry and flatten the retina. Add a drop of mounting medium (see **Table of Materials**) to the center of the coverslip and stop adding it until the diameter of the droplet increases to half of the coverslip. Quickly turn over the coverslip and place it on top of the outspread retina. Avoid forming bubbles.

2.18. Take images of the retinal flat mounts or store and protect the slides from light at 4 °C.

3. Analysis and quantification of retinal flat mounts

NOTE: For the OIR mouse model, the researchers often record the area of central retinal vascular occlusion and peripheral retinal pathological neovascularization during P12–P25. Previous studies have shown that the central avascular area of the retina reaches the maximum at P12 and gradually shrinks from P13 to P17; at the same time, the retina of OIR mice reaches the peak of neovascularization area at around P17[22,29]. From P17, neovessels gradually regress and functional vessels regrow into the avascular area. The retinal vasculature basically returns to normal at P25[33].

3.1. Take images of retinal flat mounts by a fluorescence microscope (see **Table of Materials**) with 10x objective lens. First, choose the DAPI channel and set the optic nerve head in the center of the visual field. Then, adjust other channels and focus on the superficial vasculature of the retina. Check **Tiles** in a photo software (see **Table of Materials**) and set the number of photos that need to be stitched. Click on **Start Experiment** to capture the whole retina.

3.2. Use an image processing program (see **Table of Materials**) to quantify the area of vaso-obliteration (VO) and neovascularization (NV) after immunofluorescence staining.

3.2.1 First, click on the **Magic Wand Tool** and set an appropriate tolerance according to the difference in brightness and move the cursor to the background and click the mouse. Then, choose the **Select Inverse** to obtain a basic outline of the retina. Use the **Lasso Tool** to further outline the details of the retina. Using the **Histogram** function, record the pixel value of the whole retina and write it down or generate a table in a database program.

3.2.2 Divide the retina image into four quadrants. In each quadrant, use the L**asso Tool** to draw the VO area (**Figure 2A–C**), and use the **Magic Wand Tool** to select the NV area (**Figure 2D–F**). Through the pixel information in the histogram, calculate the pixel ratio of VO and NV to the whole retina, that is, the percentage of VO or NV area relative to the whole retina.

NOTE: There is also an open-source and fully automated pipeline for the quantification of VO and NV areas in OIR images using deep learning neural networks (http://oirseg.org/), which

provides a reliable and time-saving way for researchers as well as unifies the standard of the quantification[34].

3.3. Record pixel information in a spreadsheet table, which is convenient for subsequent analysis.

4. *In vivo* imaging with fluorescein fundus angiography (FFA)

NOTE: For OIR mice, both FITC perfusion and immunofluorescence staining can only be used for one time as the death of experimental animals. Compared with this, one of the advantages of FFA is the observation of the dynamic changes of mouse retinal vessels during development and pathological state *in vivo*[35,36].

4.1. Weigh the pups before anesthesia.

4.2. Anesthetize pups by intraperitoneal injection of 0.3% pentobarbital sodium at a dose of 10–20 mL/kg.

NOTE: For mice within 1 month, pay attention to the anesthetic doses. Use lower concentrations and doses of anesthetic to reduce the death of mice caused by anesthesia. After the pups are anesthetized, use a small heating pad to maintain the body temperature. Hypothermia not only affects pups' physiological function, but also leads to changes in crystallin and accelerates the development of cataracts.

4.3. Use 20 µL mydriatic eye drops (0.5% tropicamide + 0.5% phenylephrine hydrochloride) for each pup and wait for 5 min to achieve long-lasting pupil dilation (**Figure 3A,B**).

4.4. Bring the anesthetized pups in front of the imaging device (see **Table of Materials**). Click on the mode of **Infrared Fundus Imaging (IR)** to adjust the optic nerve head to the center of the screen. Keep the pups on a small heating pad, place the pups in a stable position, and use artificial tears regularly to maintain moisture in the cornea.

NOTE: When observing one eye of the pups, do not forget to protect the other eye. Use Hypromellose eye drops to prevent the cornea from whitening due to dryness.

4.5. After intraperitoneal injection of 0.15 mL 0.5% fluorescein sodium salt solution, click on the **FA** button and the **Injection** button immediately on the touch panel of the imaging device to start timing. Record the images after 3 min when the blood circulation of the retina enters the venous phase and observe the retina no less than 6–8 min.

NOTE: After intraperitoneal injection of fluorescein sodium salt solution, the skin, mucosa, and urine of the pups show obvious yellowish green. Most of the fluorescein is excreted by

the pups within a day. Injecting the fluorescein sodium intraperitoneally every other day for six times does not cause significant side effects[37].

4.6. Move the optic nerve head to the center of the image acquisition area and take the first image of the central retina. Then, move the lens of the imaging device horizontally to the nasal side of the eye until the optic nerve head is located at the midpoint of one side of the image acquisition area and take the second image. Continue to take images of the temporal, superior and inferior retina, respectively using this method (**Figure 3C**).

NOTE: Take "Five-orientation" images within 12 min as the regression phase occurs. The position of the optic nerve head in the inferior image is allowed not to fall on the sideline due to the limited angle adjustment of the lens.

4.7. Save the images and use an image processing program for stitching.

5. Image processing of the fluorescein fundus angiography (FFA)

5.1. Open the imaging processing program and click on **New** in **File** to create a new canvas with a black background (**Figure 4A**).

5.2. Open an image of the central retina first in the background layer. Click on **File** and add the second image. Adjust the opacity of the second image to 60%, move and resize the second image until the same parts of the two images highly overlap. Click on the **Switch Between Free Transform and Warp Modes** button and make subtle adjustments to the vessels if necessary. Then, turn the opacity of the second image back to 100% (**Figure 4A,B**).

5.3. Select two images at the same time and click on **Auto-Blend Layers**. Check **Panorama** as the blend method as well as select the following two sentences. Click on **OK** and finish the image stitching of the first two images (**Figure 4C,D**).

5.4. Take the first two stitched images as a whole, add the third image, and continue to blend. Repeat the methods above to complete the stitching of five images (**Figure 4E**).

5.5. Use the **Crop Tool** to cut images of FFA at different time points to a uniform size and observe dynamic changes of retinal vasculature from P15 to P25 in both normal and OIR pups.

6. Statistical analysis

6.1. Present values as mean ± standard deviation (s.d.).

6.2. Use the Student's *t*-test to compare two independent samples. Use One-Way ANOVA to compare multiple sets of data and combine with Dunnett or Tukey's test, which is a commonly

used multiple comparison test.

6.3. For non-normally distributed data, use Mann-Whitney U test or Kruskal Wallis test. Consider significant statistical differences when $P < 0.05$.

REPRESENTATIVE RESULTS:

In the OIR mouse model, the most important and basic result is the quantification of the VO and NV area. After living in the hyperoxia environment for 5 days from P7, the central retina of the pups showed the largest non-perfusion area. Under the stimulation of hypoxia in another 5 days, retinal neovascularization was gradually produced which fluoresced more intensely than surrounding normal vessels. After P17, the fluorescence signal of pathological neovascularization regressed rapidly as the remodeling of the retina (**Figure 5A**). By controlling the litter size and the postnatal weight gain of the pups, the area of the VO and NV of the OIR mouse model showed good repeatability and stability and the peak of retinal neovascularization occurred at P17, which was in line with the previous studies (**Figure 5B,C**).

FFA is an ideal tool for studying retinal vasculature. Given the application of FFA *in vivo*, it shows a great reduction in the waste of experimental animals as well as displays the dynamic changes of the retinal vessels with time. In previous studies, FFA was not often used in mice pups and was presented in a single-view image, which was difficult for further study. In this protocol, the "Five-orientation" images of the retina vasculature were stitched together using an image processing software to display a wider field of the retina at one time, which was helpful for subsequent analysis, if needed (**Figure 4**). Besides, the OIR mouse pups showed a prolonged eye opening so the FFA images were taken from P15 to meet the requirements of animal ethics. In the retina of the OIR mouse model, the diameter of blood vessels increased evidently and became highly tortuous when comparing to normal mice. Besides, the FFA showed a similar trend of dynamic changes of retinal vasculature with immunofluorescence staining with isolectin B4-594 from P15–P25 without the death of the pups (**Figure 6**).

FIGURE AND TABLE LEGENDS:

Figure 1: Cartoon schematic of OIR mouse model. OIR mouse model was induced by keeping pups and their nursing mothers in a room for some time (P0–P7). At P7, both of them were exposed to 75% oxygen for 5 days, which inhibited retinal vessel growth and caused significant vessel loss in the central retina. Mice were then brought back to room air at P12 and the avascular retina started becoming relatively hypoxic, triggering both normal vessel regrowth and a pathological response around the mid-peripheral retina. The maximum neovascularization (NV) was seen at P17. Then, pathological neovascularization underwent a process of spontaneous regression. The retinal vascular system was back to normal again at around P25.

Figure 2: Measurement of vaso-obliteration (VO) and neovascularization (NV) in the mouse

retina. (**A**) Image of 10x P12 OIR retinal whole-mount stained for endothelial cells with isolectin B4-594. (**B**) Screenshot of a retina with the avascular area selected. Tools necessary to make this measurement is highlighted with white arrows: **Magic Wand Tool** and **Lasso Tool**. (**C**) Highlight the avascular area of the retina and save the image as a copy. (**D**) Image of 10x P17 OIR retinal whole-mount stained for endothelial cells with isolectin B4-594. (**E**) Screenshot of a retina with neovascular tufts selected. Use **Magic Wand Tool** and set an optimal Tolerance to highlight NV. Set the tolerance to 3–5 and check the anti-alias and contiguous boxes. (**F**) Save the neovascularization area only as a copy. Scale bars represent 1,000 μm.

Figure 3: Acquisition of the "Five-orientation" images in the mouse retina. (**A**) The normal mouse pupil. (**B**) Mouse pupil in mydriasis. (**C**) The "Five-orientation" images of the central, nasal, temporal, superior, and inferior area of the retina were collected, respectively (P17 pups in room air). Scale bars represent 500 μm.

Figure 4: General workflow of stitching the "Five-orientation" images from fluorescein fundus angiography (FFA). (**A**) Create a new canvas with a black background and open the FFA image of the central retina. (**B**) Open an FFA image of the temporal retina and adjust the opacity of the second image to 60%; move and resize the image until the same parts of the two images highly overlap. Click on **Switch Between Free Transform** and **Warp Modes** to make subtle adjustments if necessary. Turn the opacity of the second image back to 100%. (**C**) Select two images at the same time and click on **Auto-Blend Layers**. (**D**) Use **Panorama** as the blend method to finish the image stitching of the first two images. (**E**) Continue to stitch images by repeating the methods above to complete the stitching of all the images.

Figure 5: Quantification of vaso-obliteration (VO) and neovascularization (NV) in the retina of the OIR mouse model. (**A**) Image of 10x OIR retinal whole-mounts stained for endothelial cells with isolectin B4-594 from P12 to P25. After being exposed to 75% oxygen for 5 days, pups and their nursing mothers were brought back to the room air at P12 at which the area of vaso-obliteration reached the maximum. The relative hypoxia in the central retina led to vessel regrowth in this area as well as pathological angiogenesis in the mid-peripheral retina. At P17, pre-retinal neovascular tufts reached the maximum and then shrank quickly. NV regressed completely and the retina seemed to be normal at around P25. (**B**) Quantification of the area of VO showed a peak at P12 and disappearance at around P25. (**C**) Quantification of the area of NV showed a peak at P17 and regression at around P25. Scale bars represent 1,000 μm in A. (One-Way ANOVA, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$).

Figure 6: *In vivo* imaging of fluorescein fundus angiography (FFA) in the OIR mouse model. In the retina of the OIR mouse model, the diameter of blood vessels increased evidently and became highly tortuous when comparing to normal mice. Besides, the FFA showed a similar trend of dynamic changes of retinal vasculature with immunofluorescence staining with isolectin B4-594 from P15–P25 without the death of mice pups. Scale bars represent 500 μm.

DISCUSSION:

The susceptibility of mice to OIR is affected by many factors. The pups of different genetic background and strains cannot be compared. In BALB/c albino mice, vessels regrow into the VO area rapidly with significant reduced neovascular tufts[38], which bring some difficulties to the research. In C57BL/6 mice, there is increased photoreceptor damage when compared to BALB/cJ mouse strain[39,40]. The same goes for different types of transgenic mice[41–43]. Besides, C57BL/6 mice display a lower level of angiogenesis when compared to 129S3/SvIM mice[44].

Postnatal weight gain (PWG) is also important to consider[45] and is one of the indicators to evaluate the nutritional status of newborns. It has also become a reliable method to predict ROP, which attracts the attention of many animal modelers[46]. PWG affects the response of mice to hyperoxia and hypoxia. At P7, pups with increased body weight (>5 g) show an insufficient vaso-obliteration and retinal neovascularization, while pups with decreased body weight (<5 g) show obvious response to hyperoxia and hypoxia. Besides, at P17, pups with poor (<5 g) and extensive (>7.5 g) weight gain show a decreased NV. However, pups with poor weight gain (<5 g) have significantly prolonged vaso-obliteration (VO) and neovascularization (NV) stage with a delay in the occurrence of NV peak[45]. Therefore, it's necessary to record and control the PWG of pups at P7 and P17 and eliminate pups with low PWG (< 6 g at P17) to ensure the repeatability and comparability of the experiment.

The litter size has a greater impact on PWG, and some researchers suggest it should be limited to 6–8 pups/dam to meet the requirements for PWG[22,31]. The state of the nursing mother needs a consideration as well. Nursing mothers are more likely to die from lung damage in a hyperoxic environment[47]. If nursing mothers die or neglect their pups during and after the induction of OIR, pups will easily lose weight or even die due to the lack of nutrition[32]. Therefore, it is necessary to ensure that there are enough surrogate mothers to replace them. However, these surrogate mothers are suggested to be used only when the mother expires, which usually happens during the period of hyperoxia exposure or return to the room air[22]. Providing adequate food for surrogates is also helpful to improve the nutritional status of their pups.

A useful note to prepare the retinal flat mounts is that an optimal time of fixation is usually necessary for further long-time staining. As mice of P12–P25, a 15 min + 45 min fixation at room temperature is recommended[29]. Fixing the retina at 4 °C overnight is an alternative if time is limited. Besides, the permeable and blocking buffer with a higher concentration of 1% Triton X-100 and 5% normal donkey serum effectively reduce the background of immunofluorescence staining according to our experience.

Isolectin B4 staining and FITC-dextran perfusion are commonly used methods to visualize and quantify the neovascular[48,49]. A major limitation of these two methods is that the mice must be sacrificed. So, the methods for *in vivo* imaging and quantification of NV are needed[29].

Paques et al. developed a technique named topical endoscopy fundus imaging (TEFI), which provides high-resolution digital photographs of the retina in live mice[50]. The TEFI can detect retinal vascular changes as early as P15 and the images obtained are in accordance with the conventional methods of assessment. Mezu-Ndubuisi et al. then provided the methods for *in vivo* retinal vascular oxygen tension (PO_2) measurements and fluorescein angiography (FA), improving the understanding of retinal vascular changes and oxygenation alterations due to ROP and other ischemic retinal diseases[37]. Although neither TEFI nor FA is as accurate as conventional methods, they reduce the death of experimental animals and can be performed repeatedly. Besides, they allow each mouse to serve as its own control, thus making the OIR data more comparable. In this paper, an improved method of FFA imaging and image stitching is provided. Performing FFA on pups within 1 month is not easy because excessive anesthesia and hypothermia directly cause the death of the pups. Thus, try to use the minimum dose of anesthesia and pay special attention to maintaining the body temperature of pups throughout and after the process by using a small heating pad. Always moisten the ocular surface with saline and Hypromellose in case of failure of the following observation.

In summary, the OIR mouse model is a very common and widely used model of retinal ischemia and pathological neovascularization. One of the major problems of this model is that the neonatal mice pups are essentially healthy and do not have metabolic instability or respiratory problems when compared to prematurely born infants. Another difference between the OIR mouse model and humans is that there is always fibrovascular proliferation in human retinal neovascularization whereas the retinal neovascular is not associated with fibrosis in the OIR mouse model[51]. To make better use of this model and acquire more information, a detailed description of using FFA to monitor the dynamic changes of OIR retinal vasculature is provided, including the methods of taking “Five-orientation” images and image processing. It is believed that FFA will become an effective method partially or fully to replace the immunofluorescence staining to observe and evaluate the morphology and function of retinal vasculature[49]. Although the OIR mouse model doesn’t fully resemble the microenvironment and pathogenesis of various ischemic retinopathy in humans, it provides us with an opportunity to conduct drug and transgenic experiments as well as to explore the mechanism of pathological angiogenesis on the ischemic retina[51].

ACKNOWLEDGMENTS:

We thank all the members from our lab and Ophthalmic Animal Laboratory of Zhongshan Ophthalmic Center for their technical assistance. This work was supported by grants from the National Natural Science Foundation of China (NSFC: 81670872; Beijing, China), the Natural Science Foundation of Guangdong Province, China (Grant No.2019A1515011347), and High-level hospital construction project from State Key Laboratory of Ophthalmology at Zhongshan Ophthalmic Center (Grant No. 303020103; Guangzhou, Guangdong Province, China).

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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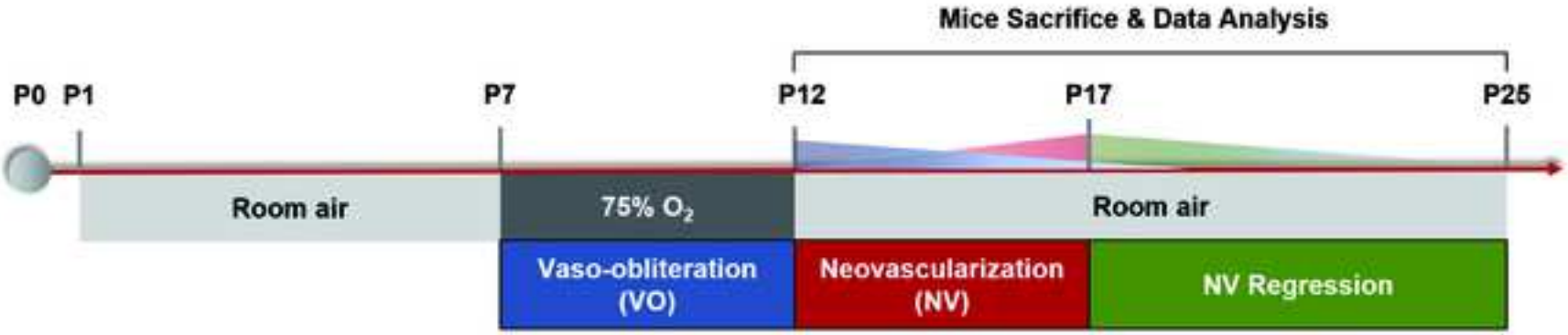

Figure 2

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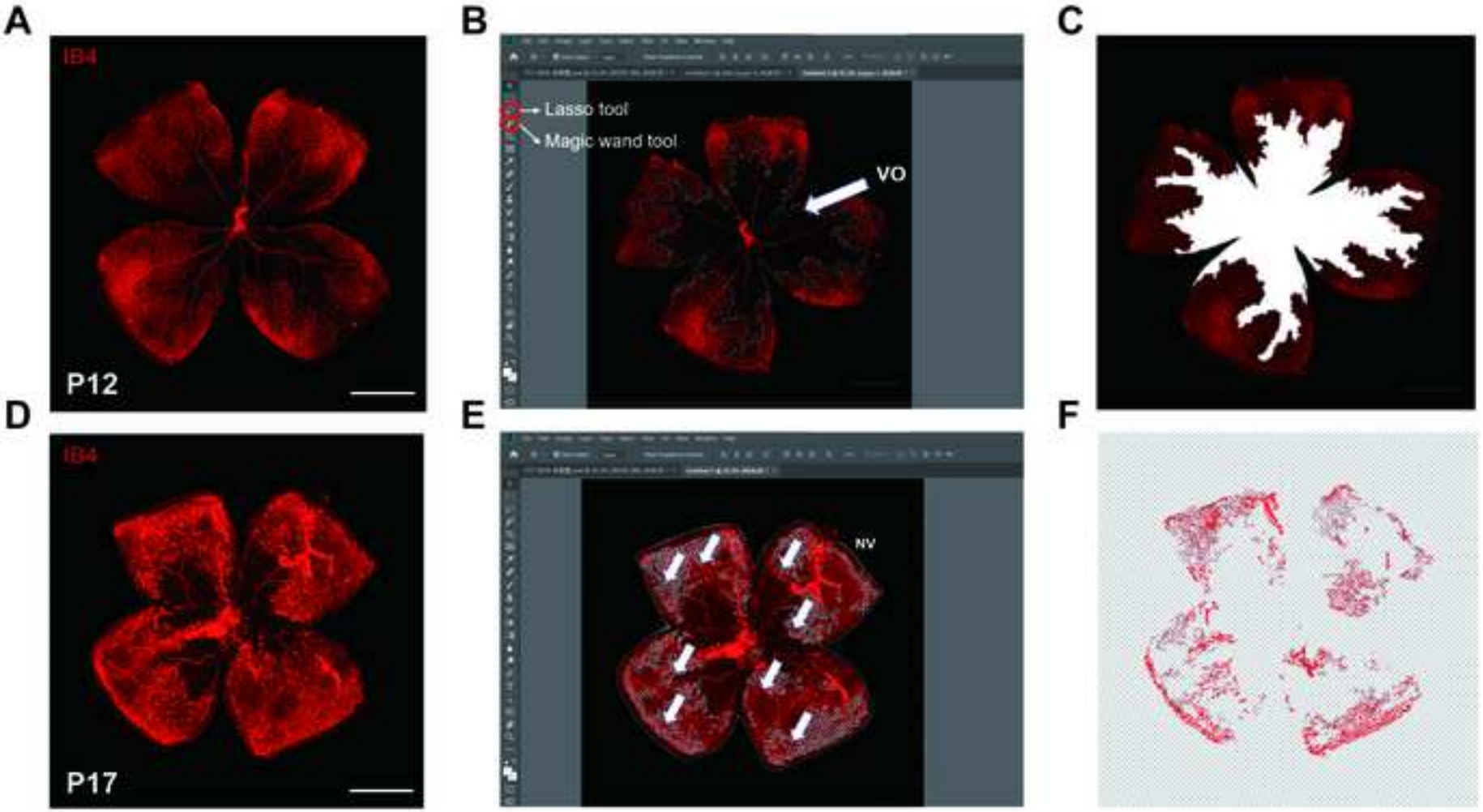

Figure 3

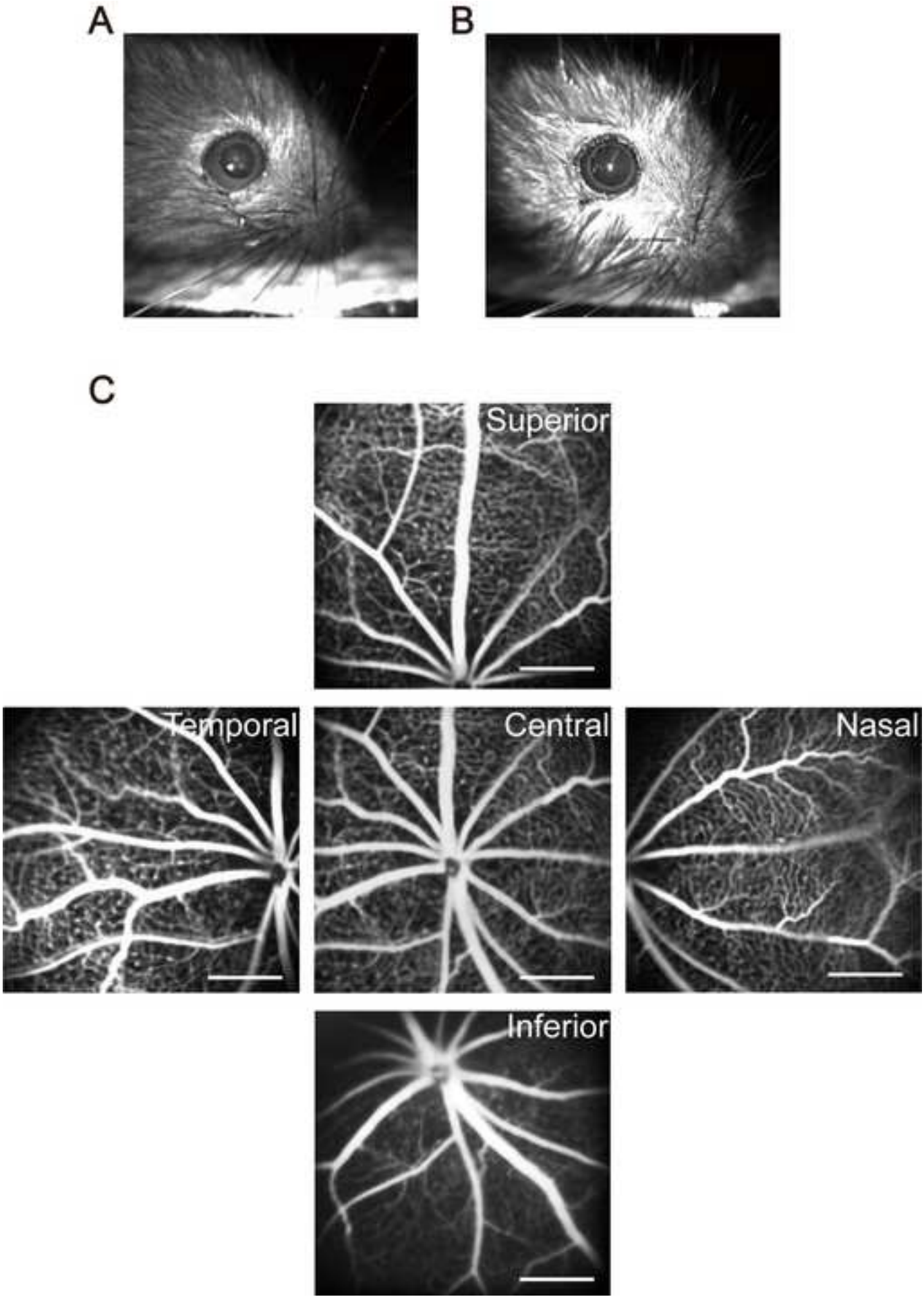

Figure 4

Click here to access/download;Figure;Figure 4 New-FFA stitching method.tif

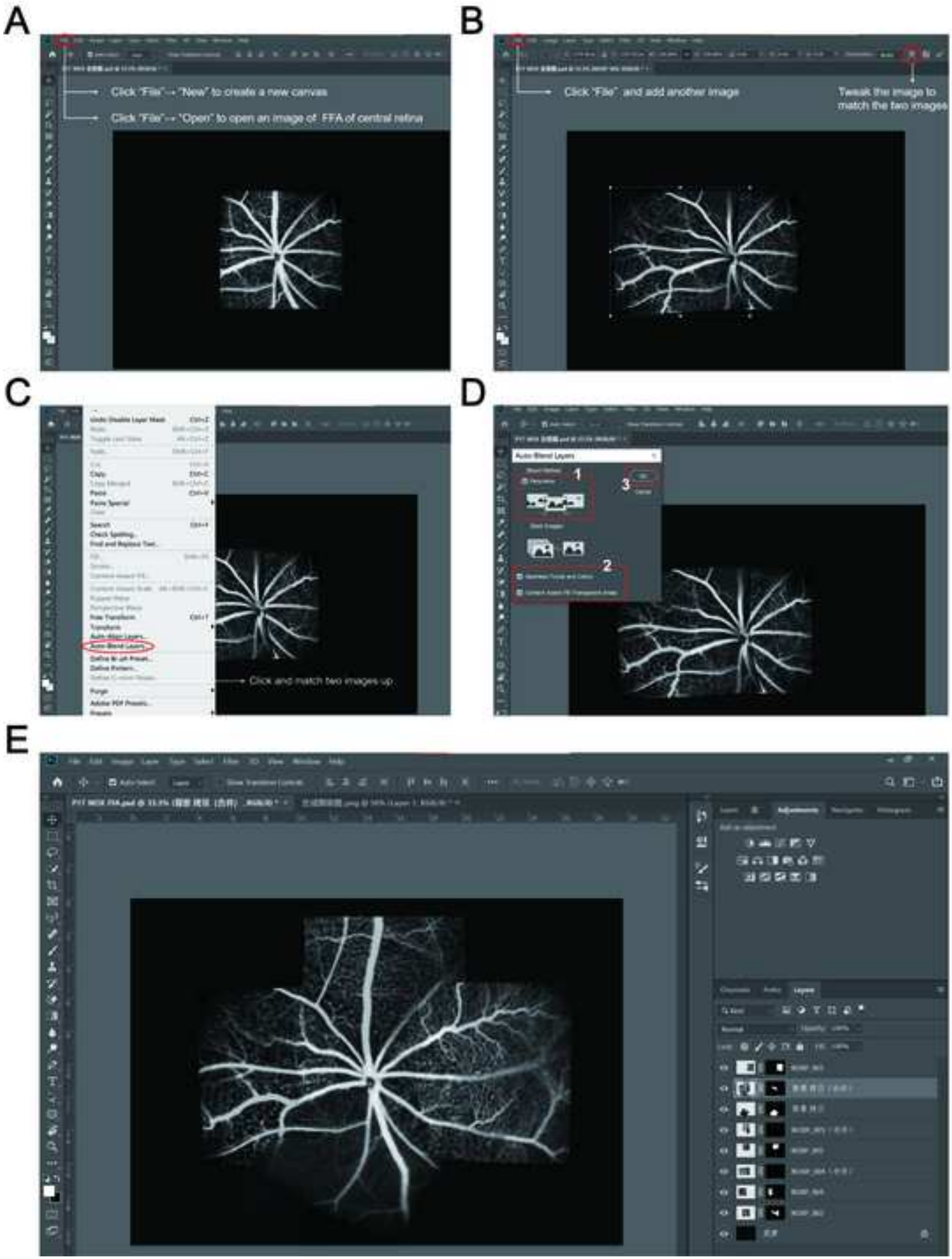

Figure 5

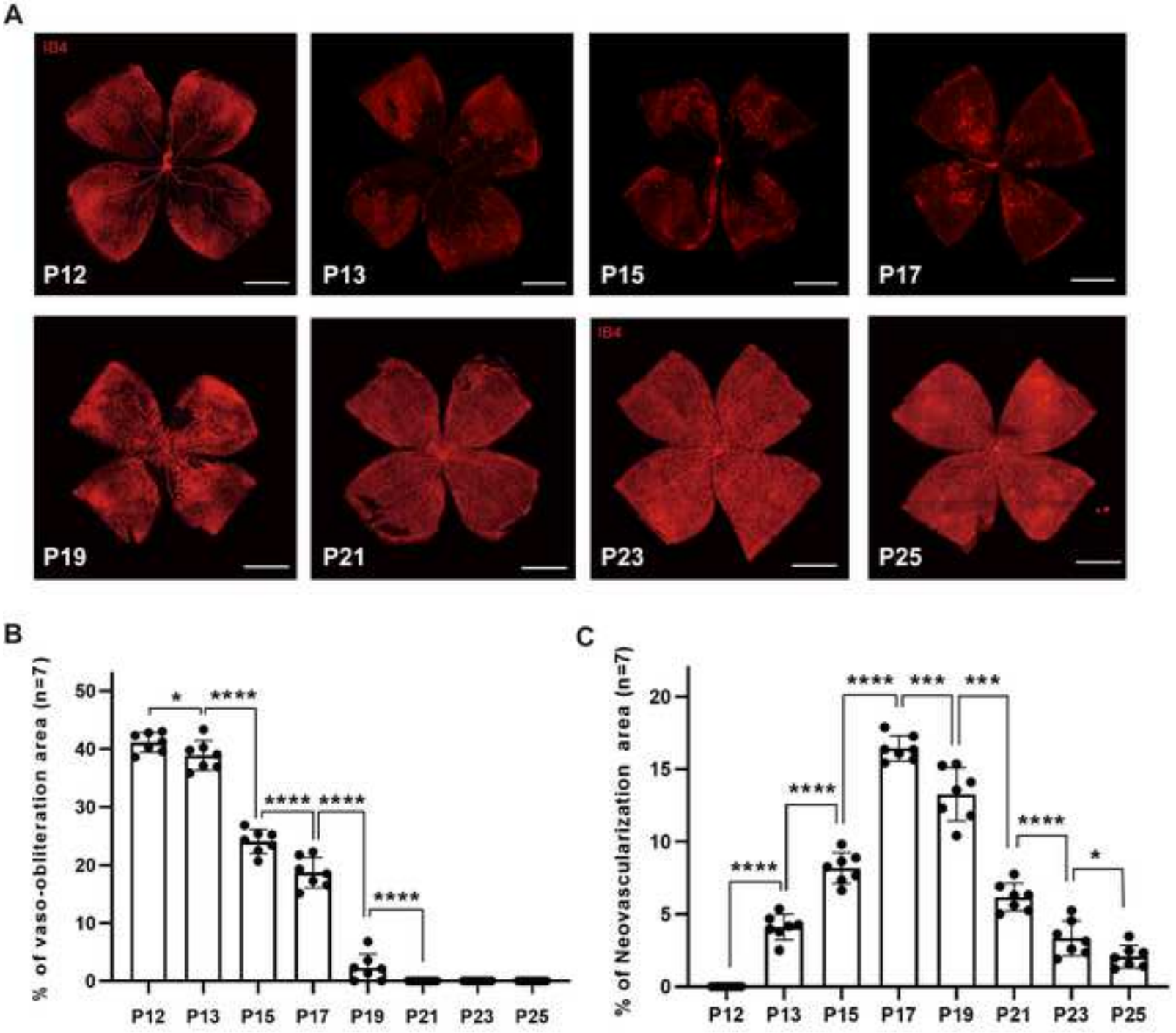

Figure 6

Click here to access/download;Figure;Figure 6-Scale Bars.tif

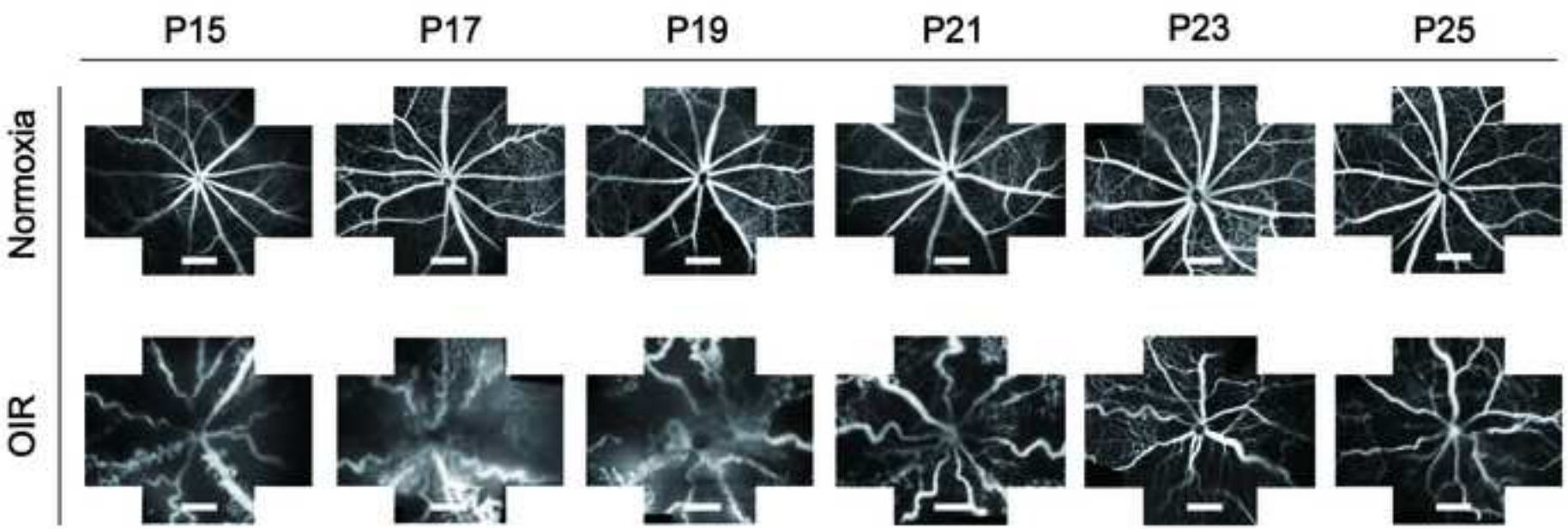

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials (Revision by Tao Li).xls

Name of Material/ Equipment	Company	Catalog Number
1 mL sterile syringe	Solarbio	YA0550
1× Phosphate buffered saline (PBS)	Transgen Biotech	FG701-01
2 ml Microcentrifuge Tube	Corning	MCT-200-C
48 Well Clear TC-Treated Multiple Well Plates	Corning	3548
Adhesive microscope slides	Various	
Adobe Photoshop CC 2019	Adobe Inc.	
Carbon dioxide gas	Various	
Cover slide	Various	
Curved forceps	World Precision Instruments	14127
DAPI staining solution	Abcam	ab228549
Dissecting microscope	Olmpus	SZ61
Fluorescein sodium	Sigma-Aldrich	F6377
Fluorescent Microscope	Zeiss	AxioImager.Z2
Fluoromount-G Mounting media	SouthernBiotech	0100-01
Hydroxypropyl Methylcellulose	Maya	89161
Isolectin B4 594 antibody	Invitrogen	I21413
Mice C57/BL6J	GemPharmatech of Jiangsu Province	
Micro dissecting scissors-straight blade	World Precision Instruments	503242
No.4 straight forceps	World Precision Instruments	501978-6
Normal donkey serum	Abcam	ab7475
O_2 sensor	Various	
OxyCycler	Biospherix	A84XOV
Paraformaldehyde (PFA)	Sigma	P6148-1KG
Pentobarbital sodium	Various	
Soda lime	Various	
SPECTRALIS HRA+OCT	Heidelberg	HC00500002
SPSS Statistics 22.0	IBM	
Tansference decloring shaker	Kylin-Bell	ZD-2008
Tissue culture dish (Low attachment)	Corning	3261-20EA
Transfer pipettes	Various	
Triton X-100	Sigma-Aldrich	SLBW6818

Tropicamide	Various
ZEN Imaging Software	ZEISS

Comments/Description
For preparation of retinal flat mounts and intraperitoneal injection
For preparation of retinal flat mounts
For preparation of retinal flat mounts
For preparation of retinal flat mounts
For preparation of retinal flat mounts
For image analysis
For sacrifice
For preparation of retinal flat mounts
For preparation of retinal flat mounts
For labeling nucleus on retinal flat mounts
For preparation of retinal flat mounts
For *in vivo* imaging
For acquisition of fluorescence images of retinal flat mounts
For preparation of retinal flat mounts
For *in vivo* imaging
For labeling retinal vasculature on retinal flat mounts
For OIR model induction
For preparation of retinal flat mounts
For preparation of retinal flat mounts
For preparation of retinal flat mounts
For monitoring the level of O_2
For OIR model induction
For tissue fixation
For anesthesia
For absorbing excess CO_2 in the oxygen chamber
For *in vivo* imaging
For statistical analysis
For preparation of retinal flat mounts
For preparation of retinal flat mounts
For preparation of retinal flat mounts
For preparation of retinal flat mounts

For *in vivo* imaging
For image acquisition and export

Dear Editor and Reviewers,

Thank you for your letter and the reviewer's comments concerning our manuscript. Those constructive comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our manuscript. We have studied the comments carefully and have modified the manuscript appropriately which we hope to meet with approval. We believe that the quality and the depth of the subject matter have been enhanced based on your comments and it will be of the interest of readers of ***Journal of Visualized Experiments***.

What is appended to this letter is our point-by-point response to the comments raised by the editor and reviewers. To distinguish my answers and reviewers' easily, I highlighted all of my answers in red in our revised manuscript while keeping your letter and reviewers' questions/comments in black.

Thank you very much for your time and consideration.

I am looking forward to hearing from you.

Sincerely,

Tao Li M.D., PhD on behalf of all authors

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E-mail: litao2@mail.sysu.edu.cn

List of Responses

Editorial comments:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. E.g. line 191: "from the periphery to the centre" instead of "peripheral to central...", etc.

Response:
Thank you for your comment. We re-checked the manuscript to avoid spelling and grammar errors and highlighted all of the words and sentences after spelling or grammar correction with red in our revised manuscript.

2. Please revise the following lines to avoid previously published work: 65-74, 78-79, 382-388.

Response:
Thank you for making this valuable suggestion. As you suggested, we changed the expression from **Line 65-74** to **Line 88-96**, from **Line 78-79** to **Line 110-111** and from **Line 382-388** to **Line 481-496** in our revised manuscript. Besides, we added the description of the relationship between oxygen and retinal neovascular diseases in **Line 52-61**, then compared the differences briefly in retinal vascular development between human and rodents in **Line 63-65** and gave a brief account of the development history of the OIR mouse model point out some advantages of the mouse model in **Line 71-80** to make a more logical content of the introduction section. Hope it can meet your request.

3. Please define all abbreviations before use. E.g., PBS, PBST, etc.

Response:
Thanks for your comment. We checked all the abbreviations e.g., PBS, PBST, etc. we used and attached a brief introduction when we used them for the first time. At the same time, we marked the font of these abbreviations in red in our revised manuscript.

4. Use "mL" instead of "ml", "µL" instead of "uL", etc.

Response:
Thank you for your comment. We are very sorry for our improper use of "ml" and "uL", etc. We have corrected them in our revised manuscript (highlighted in red). Hope it can meet your request.

5. Line 95: Please provide details about the gender, breed of mice.

Response:
Thank you very much for your comment. It has been changed to "Use mice with a

lower rate of congenital malformation of the eyes, e.g., C57BL/6J mice, and mate them at a ratio of male/female = 1:2. Get the pups born on the same day and start to induce the OIR model at P7. Record the bodyweight of mouse pups strictly before modelling." in our revised manuscript **(P4, Line 131-134)**.

6. Line 103: Specify what "suitable environment" means.

Response:
Thank you very much for your suggestion. As you suggested, we have explained the definition of a "suitable environment" according to previous studies and our own experience **(P4, Line 141-145)**.

7. Line 109: What behaviours should be checked? How?

Response:
Thanks for your valuable comment. We are very sorry for our omission of a detailed description. As the survival and postnatal weight gain of the pups depend on the lactation of their nursing mothers, we pay special attention to the state and behaviors of nursing mothers before model induction. If nursing mothers refuse to feed their pups or even bites them, the probability of malnutrition or death of mice will be greatly increased, which does not meet the requirements of the model. We have re-written this part according to your suggestion **(P4, Line 157-159)**. Hope it can meet your request.

8. Line 129-130, 247-248: Please do not highlight steps involving euthanasia or anesthesia.

Response:
Thank you for your comment. We have removed the highlighting of these steps according to the requirements which could be found in **Line 179-180, 314-315** in our revised manuscript.

9. Line 138, 151: What is the speed of the shaker?

Response:
Thank you for your comment. We have added the description of the speed of the shaker which was "at a speed of 12-15 r/m" displayed in red font in our revised manuscript.

10. Line 197: What mounting medium is used?

Response:

Thank you for your comment. The mounting medium we used was Fluoromount-G® (Southern Biotech, USA) and the catalog number was 0100-01, as we showed in the "**Table of Materials"**.

11. Line 257-258: Dosage?

Response:
Thank you for your comment. We have added this detail in **P8, Line 323-324** in our revised manuscript.

12. Line 260: Please provide details about the "imaging device".

Response:
Thank you for your comment. We have added the make and model of the "imaging device" in our revised "**Table of Materials"**. Hope it can meet your request.

13. Avoid the use of personal pronouns in the protocol. E.g., "we", "our", etc.

Response:
Thank you for your comment. We have removed the personal pronouns in the protocol and discussion section. Hope it can meet your request.

14. Please use "Calibri (body), size 12"font consistently throughout the manuscript.

Response:
Thank you very much for your comment. We went through the whole paper and adjusted the font to "Calibri (body), size 12" uniformly according to the requirements of the article format.

15. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. E.g., Line 95: "Control the starting time…" instead of "The starting time was controlled…", etc.

Response:
Thank you very much for your comment. We revised the description in the protocol section and re-written this part in the imperative tense. Besides, we removed the usage of "could be," "should be," and "would be" throughout the Protocol. The details can be found in our revised manuscript.

16. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an

instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. E.g. Zeiss AXIOVISION, Adobe Photoshop, etc.

Response:
Thank you very much for your comment. We revised the description in the protocol and used generic terms instead. All the equipment, reagent materials and analysis software involved in the protocol were recorded in the revised "**Table of Materials**".

17. Figure 6: Please include appropriate scale bars.

Response:
Thank you very much for your comment. We added the appropriate scale bars in Figure 6 (Scale bars: 500 μm) according to your suggestion. Hope it can meet your request.

18. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not use "&/and" in the author list for references. Do not abbreviate the journal names.

Response:
Thank you very much for your comment. We have revised the format of the references as required. Hope it can meet your request.

19. Please sort the Materials Table alphabetically by the name of the material.

Response:
Thank you for your kind suggestion. We have reorganized the "**Table of Materials**" alphabetically according to the name of the materials.

Reviewer 1:
General Comments:
The article titled "Monitoring Dynamic Growth of Retinal Vessels in Oxygen-Induced Retinopathy Mouse Model" by Ma and Li provides a detailed description of the OIR model and a novel method to visualize dynamic retinal vascular changes in vivo. The article is well written and the introduction provides important detail about the model. There are major concerns regarding the details of the repeated fluorescein injection method. The authors do not mention the imaging equipment used and a discussion on the how often fluorescein can be injected without sickening the animal is lacking. There are also concerns regarding swapping out surrogate mothers every 2 days during the hyperoxygen phase, as this is uncommon practice. There are several previous detailed descriptions of the OIR model and how to quantify it; 2 examples are PMIDs: 19816419 and 29263301. The uniqueness of this article is in the repeated in vivo imaging protocol, which requires additional detail for users to faithfully follow.

Response:
We thank the reviewer for these generous comments and we will address these comments below point by point.

Major Concerns:
Induction:
1. I am hesitant of the recommendation to replace nursing mothers every 2 days during hyperoxia for a few reasons. (1) Typically, surrogates are introduced on an as need basis because there is a decent probability that the surrogate will reject the pups. (2) Disturbing the pups and mother is stressful for the animals and increases the probability of maternal aggression. (3) For potential users that do not have a hyperbaric oxygenation chamber with glove attachments and a transfer chamber (many use a homemade chamber), opening the chamber to remove animals causes a rapid drop in oxygen levels. The temporary (1-2h) drop in O_2 induces transient retinal hypoxia in pups and acutely exacerbates respiratory problems in mothers. Therefore, to avoid these potential confounders, our lab and others [PMID: 19816419] use surrogate mothers on an as need basis specifically in the hyperoxygen phase.

Response:
We appreciate the reviewer's great points regarding replacing nursing mothers. Although the situation of rejecting pups has not happened in our practice so far, we followed the great suggestions by the reviewer—we carefully read the paper the reviewer mentioned above and switched to the methods used in the paper. Accordingly, the previous description regarding nursing mothers has been changed to "Avoid opening the oxygen chamber during the period of model induction. Ensure that there are extra surrogate mothers for replacement in case the nursing mothers die due to lung injury while in hyperoxia." in **P4-5, Line 162-171**.

In vivo imaging:
1. Can the authors expand on the imaging equipment used in the in vivo experiment? Make, model, etc

Response:
We thank the reviewer's reminding for adding the detailed information of the imaging equipment used in the *in vivo* experiment. However, we have put the information in our previous "**Table of Materials"**. The imaging equipment we used was SPECTRALIS® HRA+OCT device (Heidelberg, Germany) and the catalog number was HC00500002.

2. The authors mention "Click the mode of "infrared fundus imaging (IR)" to adjust…" without stating the name of the software.

Response:

We thank the reviewer's comment regarding the software we used in this step. However, we need to clarify that the operation in this step was not done in the software, but a module button on the machine's touch panel, which can be clicked to switch the imaging options such as OCT, multiple scanning laser fundus imaging modalities, widefield and ultra-widefield, scanning laser angiography and OCT angiography and set different imaging parameters. An image of the device is attached below.

(Picture were collected from the internet: http://www.mingwangmedical.com/En/productd-246.html)

3. Can the authors remark on any toxicity they observe following multiple anesthesia and fluorescein injections in these pups? How often can fluorescein be administered to OIR pups?

Response:
We thank the reviewer for this very insightful comment. In our experiments, we did not find any significant toxicity of repeated anesthesia and intraperitoneal injection of the fluorescein sodium. After intraperitoneal injection of 0.15 mL 0.5% fluorescein sodium salt solution in each pup, the skin, mucosa and urine of the pups will show obvious yellowish-green, which means that fluorescein enters the blood circulation. However, we observed that most of the fluorescein will be excreted by the pups within one day. Thus, we injected the fluorescein sodium intraperitoneally every other day without significant side effects.

Representative results:
1. Figure 5, showing whole mount staining of other cell types, is out of place. While the images are nice, the data deviates from the central purpose of the article, which is the quantification and visualization of the retinal vasculature in OIR. I would suggest to remove it and the discussion on astrocytes and microglia, which are out of scope.

Response:
We thank the reviewer for making this valuable suggestion which is of great help

to improve the quality of our protocol. We added this section initially to show the distribution of other types of cells involved in normal retinal vascular development and pathological neovascularization as more and more researches revealed that retinal cells such as microglia and astrocytes are involved in the occurrence and development of pathological neovascularization as well as the vessel remodeling (PMID:21623369; PMID:26513504; PMID:29891713). However, as you mentioned, these data were not our focus and even deviated from the central purpose of this protocol. Accordingly, we removed the display of other cell types in Fig5 and deleted the corresponding content from the "Representative Results" section in our manuscript.

Minor Concerns:

Induction:

1. The authors mention that pups >5g at P7 should be excluded. Body weight is an important confounding factor in OIR, especially at later timepoints during the hypoxic phase, where low weight and high weight pups have significantly less NV at P17 [PMID: 21056995]. The authors should consider extending their body weight recommendations to low weight pups and to body weight in the hypoxic phase.

Response:

We thank the reviewer for making this valuable suggestion. According to the paper the reviewer recommended, both poor (< 5 g at P17) and extensive (> 7.5 g at P17) weight gain pups have less NV area at P17 when compared with medium weight gain pups (5 -7.5 g at P17). Also, poor weight gain pups show a delayed onset of NV and a prolonged NV peak. Therefore, to ensure the repeatability and comparability of the experiment, we studied the standards in the paper and paid attention to the body weight of the pups in the later stage of hypoxia at P17 as well. Accordingly, we describe the weight control in detail in the discussion section in our revised manuscript (**P12, Line 481-493**).

Preparation of whole mounts:

1.An alternative to cutting the sclera with scissors to release the retina is to instead hold the posterior cup by the optic nerve with forceps and then use the curved end of another forceps to press down on the sclera at the optic nerve head and gently massage out the retina in a forward sweeping motion.

Response:

We thank the reviewer for providing us with another effective method to get the retina. We tried this method in our experiment and it worked well. Thus, we added this method to the "Note" section, which can be found in **P5-6, Line 209-211** in our revised manuscript and hoped to provide an optional way for researchers.

2. Further detail on the removal of hyaloid vessels would be helpful. This requires precise dissection.

Response:
We thank the reviewer for the comment. We described a method of removing hyaloid vessels according to your suggestion, hoping to provide a reference for other researchers. The details can be seen in **P6, Line 213-215** in our revised manuscript.

3. Users may also block and permeabilize retinas quicker at room temperature. 1h at room temp is common.

Response:
We thank the reviewer for this valuable suggestion. We took the reviewer's advice and put "1h at room temperature" as an alternative in the NOTE section. The details can be seen in **P6, Line 224** in our revised manuscript.

4. Users may also need to change serum depending on the source of the secondary antibody used.

Response:
We appreciate the reviewer's suggestion for choosing the blocking serum. Generally, the blocking serum is from the same source as the second antibody, which can reduce the nonspecific binding. Accordingly, we revised this part in **P6, Line 224-225** to make our statement more accurate.

5. Secondary antibodies bind with high-affinity and can be incubated for shorter periods of time. 1h at room temp is common.

Response:
We appreciate the reviewer's suggestion. We have tried two different incubation times (4°C overnight and room temperature 1h) in the past and found that the staining of retinas was clearer at 4°C overnight without the increasing of the background caused by non-specific staining when compared with room temperature for 1h. Even so, we will add your suggestion to the protocol as it is a common practice and encourage researchers to try more according to their experimental conditions. At the same time, we separated the staining of DAPI from "incubation with secondary antibodies" because it had high affinity and only took 20-25 min at room temperature. The details can be seen in **P6, Line 243-244** in our revised manuscript.

Analysis and quantification:
1. There is also now a free machine learning based OIR quantification pipeline that records both vasobliteration and neovascularization [PMID: 29263301; http://oirseg.org/]

Response:
We thank the reviewer for providing us with a novel method of quantification. We have

tried this machine and found it very convenient and efficient. We are very willing to attach this link to our protocol in **P8, Line 297-300** and recommend this method to more researchers.

In vivo imaging:
1. Can the authors provide further clarification on why users should wait 3mins to record images?

Response:
We thank the reviewer for the comment. The fluorescein sodium injected intraperitoneally will go through four stages in the retina, which are the arterial phase, arteriovenous phase, venous phase and regression phase. Indeed, the retinal neovascular originates from the venules. According to our observation and previous research (PMID: 30324449), it begins to enter the venous phase 3 min after intraperitoneally injecting the fluorescein sodium. Thus, we regard 3 min as a starting point for recording the images of FFA.

2. Can the authors provide further detail on how one can capture temporal, nasal, superior, and inferior FA images?

Response:
We appreciate the reviewer's suggestion on providing further details on how to capture temporal, nasal, superior and inferior FFA images. We added the detailed workflow of capturing "Five-orientation" images in both normal and OIR pups **(P9, Line 344-352)**. Hope it can meet the reviewer's request.

3. Do the authors experience "haziness" in fundus images from leaked residual fluorescein in the vitreous during follow up injections?

Response:
We thank the reviewer for raising the point. Yes, we indeed experienced the haziness of the image due to vitreous leakage as shown in the OIR mouse model in Fig.5 in our revised figures. However, the haziness was very mild and did not compromise our observation of OIR vessels, and the leakage only lasted for a couple of days and became less concerned once the vessel remodeling occurs.

Representative results:
1. Can the authors provide further detail on the photoshop steps taken to tile the images? This may be of use to new users.

Response:
We thank the reviewer for providing this valuable suggestion. We added the detailed description of stitching the FFA images in our revised manuscript **(P9, Line 356-375)**, hoping to provide a reference for researchers.

Discussion:
1. The authors should clarify their statement about body weight citing Stahl et al 2010. At P17, NV in low and high body weight animals is decreased. However, NV peaks later in low body weight pups. Vasobliteration is also increase at all ages in low weight pups.

Response:
We thank the reviewer for underlining this deficiency. We read this paper carefully and added the statement about the impact of the postnatal weight gain on the key value of the OIR mouse model **(P12, Line 484-493)**. We believe that this is of great help in ensuring the stability of the experiment and in helping researchers to understand the relation between nutritional status and retinal ischemic diseases such as ROP.

Reviewer 2:
General Comments:
The manuscript describes methods used in mouse model of oxygen induced retinopathy (OIR) induction and analysis using flat mount retinas and fluorescein angiography. The OIR model is widely used model for ischemic retinopathies and ischemic angiogenesis. However, this model has already been described and published in the JOVE by Vahatupa et al. The manuscript by Yuan Ma and Tao Li does not provide new information.

Response:
We thank the reviewer for these generous comments and we have read this paper carefully. Although there are some similarities between the two papers, we think our detailed description of the FFA used in mice pups is the highlight of our manuscript which provides an alternative method to observe the dynamic changes of retinal vessels during and after the model induction. We thank the reviewer again and address these comments below point by point.

Major Concerns:
1. In introduction authors write: "Retinal neovascularization, which is defined as a state where new pathologic vessels originate from existing retinal vessels." Authors should discuss whether neovessels originate from veins or from arteries

Response:
We thank the reviewer for this great question. As written in the fifth edition of *Retina*, “Neovascularization is defined as a state where new pathologic vessels originate from the existing retinal veins and extend along the inner surface of the retina”, retinal neovascularization is caused by hypoxia in the retina after vascular occlusion, increasing the level of angiogenic factors, which stimulate the proliferation and migration of endothelial cells and the formation of pathological tufts. These occluded vessels include veins, venules and capillaries and neovascular usually occur around them. Therefore, we change “vessels” to “veins” to make a more accurate expression

(P1, Line 35-37).

2. Authors should describe which mice strains should be used in OIR model since no all mice strains develop neovascularization. Most commonly used mice strain in OIR model is C57Bl/6J. Differences between mice strains should be described.

Response:
We thank the reviewer for the valuable suggestion. We carefully reviewed the papers and added this part to the "Discussion" section in **P12, Line 473-479** in our revised manuscript.

3. How CO2 is controlled and accumulation of excess CO2 is avoided during hyperoxia?

Response:
We thank the reviewer for the comment. We are very sorry for our omission of a detailed description. Previous studies showed that CO_2 levels might get elevated within the hyperoxia chamber, which harmed vascular development, leading to vaso-attenuation. Therefore, we followed the instructions of Connor et al. and put 50 g of soda lime on the bottom of the chamber to absorb excessive CO_2 and maintain CO_2 values below 3% (usually below 1%). We have re-written this part according to your suggestion **(P4, Line 154-155)**. Hope it can meet the reviewer's request.

4. When using OIR model, pups should be grouped based their weight so that each experimental group has similar weight distribution.

Response:
We thank the reviewer for the insightful suggestion. We feel sorry that we did not include this detail information in our protocol. Ensuring the comparability between groups is the premise of experimental analysis. Therefore, we have added this part in **P5, Line 174-175** in our revised manuscript according to your suggestion.

5. Dilution of antibodies should be tested for all antibodies used separately and dilution depends on the antibody used.

Response:
We thank the reviewer for the kind suggestion. This is a detail that cannot be ignored. Antibodies from different companies have different ratios of dilution, which need to be adjusted by researchers after testing. The dilution ratio we provided should be used as a reference only, not as an absolute value. Therefore, we have added your suggestion in "Note" to prevent misleading other researchers. The details can be found in **P6, Line 249-250** in our revised manuscript.

6. Authors state that nursing mothers should be replaced using surrogate moms every 2 days. Based on our experience, mothers may do well with hyperoxia and surrogate

mothers do not always accept pups. Thus, surrogate moms should not be used routinely, they should be used only when necessary.

Response:
We appreciate the reviewer for the valuable suggestion. We have realized the irrationality of this replacement. Although there is no phenomenon of rejecting pups in our surrogates so far, it is still inappropriate to replace them frequently. In our previous experiments, we did not carefully consider the potential impact of this substitution on the experiments. To prevent our incorrect experience from misleading the researchers, we changed the description of this part from the previous **P3, Line 117-123** to **P4-5, Line 162-171** in our revised manuscript according to your suggestion and previous studies. We will also pay more attention to this point in the later experiment.

7. Figure 2. The quality of pictures should be better. It is difficult to see areas of neovascularization. Using black and white images should be considered.

Response:
We appreciate the reviewer for the valuable suggestion. We processed the images and re-uploaded them in our revised Figure 5. We also tried to convert the retina flat mounts into the black-and-white image according to the reviewer's suggestion. To some extent, it increases the contrast between pathological and normal retinal vessels.

8. 2F: It looks like at the periphery the edge of retina is rolled and thus sample is thicker. Thus areas shown at the edge of retina are not neovessels in Fig 2F.

Response:
We appreciate the reviewer for the kind suggestion. Indeed, we checked the image of the retinal whole-mount and found the edge of the retina was flat. We also used Edu to label the retina and found there were lots of Edu+ cells co-labelled with CD31, which reflected the proliferation of endothelial cells. Thus, we thought the area of strong fluorescence was also part of the neovascularization. We will continue to explain this phenomenon in further experiments.

9. Figure 5 is very unclear. Authors should consider using 3D images. Higher magnification should be used to show the morphology of microglia.

Response:
We appreciate the reviewer for the valuable suggestion. However, we thought the images of microglia and astrocytes were not our focus and may deviate from the central purpose of this protocol after careful consideration. Accordingly, we removed the display of previous Fig. 5 and deleted the corresponding content from the "Representative Results" section in our

manuscript. We will use 3D images with higher magnification in later experiments according to the reviewer's suggestion.

Reviewer 3:
General Comments:
Overall, the manuscript presents a timely video article on the mice model of OIR. It can be accepted for publication following some revision.

Response:
We appreciate the reviewer for the kind comment. We addressed all the points raised by the reviewer as summarized below.

Major Concerns:
1. Animal protocol number needs to be mentioned.

Response:
We thank the reviewer for the comment. We feel sorry for our negligence of the animal protocol number and we have added the number in **P3, Line 124-125**. Hope it can meet the reviewer's request.

2. How many mice pups from each mother were put in a chamber? Since the number of mice greatly determines body weight, the number of mice pups put in chamber needs to be consistent throughout different experimental groups.

Response:
We thank the reviewer for the insightful suggestion. The number of the pups is closely related with body weight, which affects the results. To ensure the comparability of the experiment, we restricted the number to 6-8 pups for each mother. The details can be found in **P4, Line 166-167** in our revised manuscript. Hope it can meet the reviewer's request.

3. Authors need to briefly mention the implication of injected fluorescein as it tends to stay in the system for sometime.

Response:
We thank the reviewer for the insightful suggestion. In our experiments, we did not find any significant toxicity of repeated anesthesia and intraperitoneal injection of the fluorescein sodium. After intraperitoneal injection of 0.15 mL 0.5% fluorescein sodium salt solution in each pup, the skin, mucosa and urine of the pups will show obvious yellowish-green, which means that fluorescein enters the blood circulation. However, we observed that most of the fluorescein will be excreted by the pups within one day. Thus, we injected the fluorescein sodium intraperitoneally every other day without significant side effects. We added a brief description of the changes after injecting fluorescein **(P9, Line 339-342)** according to

the reviewer's suggestion.

Minor Concerns:
1. "The font types are different throughout the text."

Response:
We thank the reviewer for the comment. We went through the whole paper and adjusted the font to "Calibri (body), size 12" uniformly according to the requirements of the article format.

2. Authors should provide colored images for figure.6.

Response:
We thank the reviewer for the kind suggestion. On the one hand, the imaging equipment we used in the *in vivo* experiment is the Heidelberg SPECTRALIS® HRA+OCT, of which the images displayed and finally exported are all black-and-white. We are very sorry that we are unable to provide colored images as you requested. On the other hand, we think that the color of the images is not the focus of our attention and we are mainly concerned with the dynamic changes of vascular morphology during a period. Thank the reviewer again and we will try our best to improve it in our future experiments.

3. First two paragraphs of the introduction section needs more references.

Response:
We thank the reviewer for the valuable suggestion. We added more citations to the introduction section to support our description according to the reviewer's suggestion.

Dear Editor Vineeta Bajaj,

Thank you for your comments concerning our manuscript and for helping us to solve our questions in a timely manner. Your suggestion is of great help to improve the quality of our manuscript. We have studied the comments carefully and have modified the manuscript appropriately which we hope to meet with approval.

What is appended to this letter is our point-by-point response to the comments. To distinguish my answers and your comments easily, I highlighted all of my responses in red in our revised manuscript while keeping your comments in black.

Thank you very much for your time and consideration.

I am looking forward to hearing from you.

Sincerely,

Tao Li M.D., PhD on behalf of all authors

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List of Responses

Editorial comments:
1. Some of the paragraphs have different formatting, please check and edit. Please proofread the manuscript well before submitting.

Response:
Thank you for your comment. We re-checked and proofread the manuscript to correct the different formatting in some paragraphs. Hope it can meet your request.

2. Please reword for clarity. How is hyperoxia chamber related to ROP? ***(P2, Line 43-44)***

Response:
Thank you for your comment. We have re-written this part according to your comment in our revised manuscript **(P1-2, Line 42-45)**. Hope the description this time is clear.

3. "ROP is a kind of vascular proliferative retinopathy in preterm infants, which is characterized by retinal hypoxia, retinal neovascularization and fibrous hyperplasia." ***(P2, Line 52-54)*** *Citation?*

Response:
Thank you for your comment. We added some citations to this sentence in our revised manuscript which can be found in the **reference section (8-12)**.

4. "In the 1950s, a large number of clinical studies found that high concentration of oxygen can significantly improve the respiratory symptoms of premature infants…" ***(P2, Line 54-56)*** *Citation?*

Response:
Thank you for your comment. We added some citations to this sentence in our revised manuscript which can be found in the **reference section (13,14)**.

5. "As for premature infants, the peripheral retina is not completely vascularized when exposed to hyperoxia. So vascular occlusion and neovascularization mainly appears in the peripheral retina." ***(P3, Line 94-96)*** *Citation?*

Response:
Thank you for your comment. We added some citations to these sentences in our revised manuscript which can be found in the **reference section (25-28)**.

6. So before hypoxia there is a hyperoxia condition? Or this is related to normal oxygen levels.

Response:
Thank you for your insightful comment. The animal models of OIR usually undergo different levels of hyperoxia to inhibit the development of retinal vasculature. The hyperoxia here is a condition in which the O_2 level is higher than that in room air. We also added a citation here which can be found in the **reference section (29)**.

7. "retinal vascular development is arrested or retarded with occlusion and regression of blood vessels." Please include why this happens in hyperoxic phase?

Response:
Thank you for your comment. We read the paper carefully and added some citations to this sentence to make our statement more valid. We have re-written this part according to your comment in our revised manuscript **(P3, Line 100-102).** The citations can be found in the **reference section (24,30)**.

8. Added to bring out clarity- why they lack normalvasculature due to hypoxia?

Response:
Thank you for your valuable comment. We read our manuscript and thought that there was a deviation in our description of phase 2. So, we have re-written this part in our revised manuscript **(P3, Line 103-105).** The citations can be found in the **reference section (19,29,31)**.

*9. "At present, there are few methods to observe dynamic changes of retinal vasculature continuously during the process of vascular regression and pathologic angiogenesis." **(P3, Line 114-116)** Citation?*

Response:
Thank you for your valuable comment. We added the citation in our revised manuscript which can be found in the **reference section (32)**.

10. Please adjust the highlight to fit to 3 pages limit including headings and spacings. Presently it is around 4 pages after excluding notes. Please remove the highlights from notes. Notes cannot be filmed. If this needs filming, please make it an action step in imperative tense. Some of the shorter steps can be combined to have 2-3 sentences in one step.

Response:
Thank you for your kind suggestion. We have removed the highlights from notes and adjusted the highlight to fit 3-page limiting.

11. "maintain CO2 values below 3%" How is this monitored?

Response:
Thank you for your valuable comment. The oxygen chamber we use can automatically monitor and record oxygen and carbon dioxide levels for 24 hours. Therefore, we didn't use additional carbon dioxide sensors to monitor the level of carbon dioxide.

12. "Expose pups (male and female) and their nursing mothers to 75% oxygen from postnatal day P7 for 5 days to P12" How is this done?

Response:
Thank you for your valuable comment. We adjusted the description about exposure to hyperoxia to make it clearer which can be found in our revised manuscript in **P4, Line 161-162**.

*13. "Previous studies have shown **(Line 278)**" Citation?*

Response:
Thank you for your valuable comment. We added some citations to support the statement which can be found in our revised manuscript in the **reference section (22,29)**.

14. "Most of the fluorescein is excreted by the pups within one day. Injecting the fluorescein sodium intraperitoneally every other day for 6 times do not cause significant side effects." Citation?

Response:
Thank you for your valuable comment. We added the citation to support the statement which can be found in our revised manuscript in the **reference section (37)**.

15. Please include more citations in the discussion section.

Response:
Thank you for your insightful comment. We added some citations to support the statements we written in the discussion part which can be found in our revised **reference section**.