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Optimization of the Retinal Vein Occlusion Mouse Model to Limit Variability

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Please provide any comments to the journal here.	This work should be of broad interest as it provided a detailed explanation of the techniques we have developed to optimize a mouse retinal vein occlusion model, allowing wider use of this model.
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TITLE:

Optimization of the Retinal Vein Occlusion Mouse Model to Limit Variability

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SUMMARY:

Here, we describe an optimized protocol for retinal vein occlusion using rose bengal and a laser-guided retinal imaging microscope system with recommendations to maximize its reproducibility in genetically modified strains.

ABSTRACT:

Mouse models of retinal vein occlusion (RVO) are often used in ophthalmology to study hypoxic-ischemic injury in the neural retina. In this report, a detailed method pointing out critical steps is provided with recommendations for optimization to achieve consistently successful occlusion rates across different genetically modified mouse strains. The RVO mouse model consists primarily of the intravenous administration of a photosensitizer dye followed by laser photocoagulation using a retinal imaging microscope attached to an ophthalmic guided laser. Three variables were identified as determinants of occlusion consistency. By adjusting the wait time after rose bengal administration and balancing the baseline and experimental laser output, the variability across experiments can be limited and a higher success rate of occlusions achieved. This method can be used to study retinal diseases that are characterized by retinal edema and hypoxic-ischemic injury. Additionally, as this model induces vascular injury, it can also be applied to study the neurovasculature, neuronal death, and inflammation.

INTRODUCTION:

Retinal vein occlusion (RVO) is a common retinal vascular disease that affected approximately 28 million people worldwide in 2015[1]. RVO leads to vision decline and loss in working aged adults and elders, representing an ongoing sight-threatening disease estimated to increase over the proximate decade. Some of the distinct pathologies of RVO include hypoxic-ischemic injury, retinal edema, inflammation, and neuronal loss[2]. Currently, the first line of treatment for this disorder is through the administration of vascular endothelial growth factor (VEGF) inhibitors. While anti-VEGF treatment has helped ameliorate retinal edema, many patients still face vision decline[3]. To further understand the pathophysiology of this disease and to test potential new lines of treatment, there is a need to constitute a functional and detailed RVO mouse model protocol for different mouse strains.

Mouse models have been developed implementing the same laser device used in human patients, paired with an imaging system scaled to the correct size for a mouse. This mouse model of RVO was first reported in 2007[4] and further established by Ebneter and others[4,5]. Eventually, the model was optimized by Fuma et al. to replicate key clinical manifestations of RVO such as retinal edema[6]. Since the model was first reported, many studies have employed it using the administration of a photosensitizer dye followed by photocoagulation of major retinal veins with a laser. However, the amount and type of the dye that is administered, laser power, and time of exposure vary significantly across studies that have used this method. These differences can often lead to increased variability in the model, making it difficult to replicate. To date, there are no published studies with specific details about potential avenues for its optimization.

This report presents a detailed methodology of the RVO mouse model in the C57BL/6J strain and a tamoxifen-inducible endothelial caspase-9 knockout (iEC Casp9KO) strain with a C57BL/6J background and of relevance to RVO pathology as a reference strain for a genetically modified mouse. A previous study had shown that non-apoptotic activation of endothelial caspase-9 instigates retinal edema and promotes neuronal death[8]. Experience using this strain helped determine and provide insight towards potential modifications to tailor the RVO mouse model, which can be applicable to other genetically modified strains.

PROTOCOL:

This protocol follows the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research. Rodent experiments were approved and monitored by the Institutional Animal Care and Use Committee (IACUC) of Columbia University.

NOTE: All experiments used two-month-old male mice that weighed approximately 20 g.

1. Preparation and administration of tamoxifen for inducible genetic ablation of floxed genes

NOTE: Retinal vessel diameter can be affected by the weight of the animal. Make sure that all animals used for an experiment are of similar weights.

1.1. Dilute tamoxifen in corn oil to a concentration of 20 mg/mL.

NOTE: Tamoxifen is a toxicant and is light-sensitive. Protect from light, e.g., with aluminum foil.

1.2. Vortex the solution for a couple of seconds.

1.3. Leave in the oven at 55 °C for 15 min.

NOTE: Make sure that the tamoxifen has dissolved completely. Additional vortexing may be necessary.

1.4. Store the solution at 4 °C until usage.

1.5. Administer 2 mg of tamoxifen (100 µL of 20 mg/mL) intraperitoneally (IP) for the established time according to the specific inducible Cre line.

1.6. Allow two days of rest for the animals before starting the experiments.

2. Preparation of reagents for laser photocoagulation

2.1. Rose bengal

NOTE: Rose bengal is light-sensitive. Store in the dark until usage and prepare fresh for best results.

2.1.1. Prepare rose bengal by diluting it to 5 mg/mL in sterile saline and filter it through a 0.2 µm syringe filter.

2.1.2. Prepare a 1 mL syringe fitted with a 26 G needle with rose bengal.

2.2. Ketamine/xylazine

2.2.1. Dilute ketamine and xylazine in sterile saline accordingly for the following concentrations: ketamine (80–100 mg/kg) and xylazine (5–10 mg/kg).

2.3. Carprofen

2.3.1. Dilute carprofen to 1 mg/mL in sterile saline.

2.3.2. Prepare a 1 mL syringe fitted with a 26 G needle with carprofen.

2.4. Sterile saline

2.4.1. Prepare a 5 mL syringe fitted with a 26 G needle with sterile saline.

3. Laser setup

3.1. Gently handle the fiber optic cable and connect it to the laser control box and the laser adapter of the retinal imaging microscope.

3.2. Turn the retinal imaging microscope lamp box on.

3.3. Turn the computer on and open the imaging program.

3.4. Adjust the white balance by using a piece of white paper and putting it in front of the mouse eye piece and clicking on **Adjust** in the imaging program.

3.5. Turn the laser control box on by turning the key and following the instructions on the screen of the laser control box.

NOTE: The laser used in this experiment is Class 3B and can cause eye damage. Wear protective goggles when operating the laser.

3.6. Verify the baseline laser power.

3.6.1. Use a laser power meter.

3.6.2. Adjust the screen of the laser control box to the following parameters: 50 mW and 2,000 ms.

3.6.3. Turn the laser on and place the power meter in front of the eyepiece.

3.6.4. Press the footswitch pedal to activate the laser.

3.6.5. Aim for the laser power readout to be 13–15 mW.

NOTE: The laser power readout will determine the success rate for retinal vein occlusions. If the laser power readout is too low, adjustments can be made to the power and time of laser exposure. See **Table 1** for recommendations.

3.7. Adjust the experimental laser power by setting up the **screen of the laser control box** for the following parameters: 100 mW, 1,000 ms.

3.8. Turn off the laser.

NOTE: For safety and to prevent overheating, it is best to keep the laser off between mice.

4. Mouse tail vein injection of rose bengal

4.1. Pour 300 mL of water into a 500 mL beaker.

4.2. Warm the beaker in a microwave oven for 1 min.

4.3. Put gauze in the warm water in the beaker.

4.4. Put the mouse in a restrainer.

4.5. Press the gauze into the mouse tail gently and look for the dilated veins.

4.6. Using the needle, inject the mouse tail vein, administering the correct amount according to the weight of the animal (37.5 mg/kg).

4.7. Release the mouse from the restrainer and return it to the cage.

4.8. Allow 8 min for the rose bengal to circulate before the injection of anesthetics.

NOTE: This will provide a total of 10 min between the rose bengal injection and laser irradiation.

5. Occlusion of major veins

5.1. Turn on the heated mouse platform.

5.2. Add one drop of phenylephrine and tropicamide in each eye.

5.3. Inject 150 µL of the anesthetics, ketamine (80–100 mg/kg) and xylazine (5–10 mg/kg) IP.

5.4. Toe-pinch the animal to determine the depth of anesthesia and wait until it is unresponsive.

5.5. Add one drop of proparacaine hydrochloride per eye (analgesic).

5.6. Add eye ointment to both eyes.

5.7. Inject 150 µL of carprofen subcutaneously between the ears.

5.8. Accommodate the mouse on the platform.

5.9. Adjust the platform until the view of the retinal fundus is clear and focused.

5.10. Count the retinal veins and take an image of the fundus.

NOTE: Retinal veins are darker and broader than arteries. Veins and arteries alternate; however, sometimes there can be a branched artery close to the optic nerve, and therefore, two adjacent arteries.

5.11. Turn the laser on and aim towards a retinal vein at approximately 375 µm from the optic disc.

5.12. Irradiate the vessel by pressing the footswitch and slightly moving the laser beam up to 100 µm. Repeat this step three times and move the laser beam after each pulse so that the irradiation is not focused in one spot.

5.13. Repeat irradiation on other major vessels to achieve 2–3 occlusions.

6. Establishing the number of veins occluded at day 0

6.1. Turn off the lamp after irradiating the vessels and wait for 10 min.

NOTE: Light exposure can cause retinal damage and inflammation; turn off the lamp during the waiting time to minimize exposure[7].

6.2. Turn the lamp back on and count the number of veins occluded.

6.3. Take an image of the fundus.

7. Aftercare

7.1. Inject 1 mL of sterile saline IP.

7.2. Add lubricant eye drops to both eyes.

7.3. Add gel ointment to both eyes.

7.3. Watch the mouse as it recovers from anesthesia and do not return it to the cage with the other animals until fully recovered.

NOTE: Do not leave the animals unattended until they fully recover from anesthesia.

8. Assessment of retinal edema by optical coherence tomography (OCT)

NOTE: This step can be done at the investigator's time point of interest. The peak of retinal edema for a C57BL/6J mouse is 1 day after the RVO procedure. This time point might vary depending on the background of the mouse.

8.1. Turn on the retinal imaging microscope light box, the OCT machine, and the heated mouse platform.

8.2. The day after the occlusion, follow steps 5.2 to 5.7 to prepare the animal.

8.3. Open the imaging and OCT software programs.

8.4. In the OCT program, adjust the nudge to 5.

8.5. Take OCT at 75 µm distal from the burn or 4 clicks.

8.6. Take OCT images at four quadrants of the retina.

8.7. Analyze the OCT images using tracing software.

8.8. Compare the retinal thickness of pre-irradiated measures to 1 day post RVO or at the time point of interest.

NOTE: When analyzing the data, take into consideration the number of veins irradiated as this can influence the development of retinal edema. Animals are then euthanized by administering anesthetic followed by perfusion non-survival surgery.

REPRESENTATIVE RESULTS:

The RVO mouse model aims to successfully achieve occlusions in the retinal veins, leading to hypoxic-ischemic injury, breakdown of the blood retinal barrier, neuronal death, and retinal edema[8]. **Figure 1** shows a timeline of steps to ensure reproducibility, a schematic of the experimental design, and outlines steps that can be further optimized depending on the experimental questions. The three main steps that can be modified are the waiting time after rose bengal administration, the baseline laser power, and the experimental laser output. In this report, C57BL/6J mice, as well as WT and KO littermates from an inducible endothelial cell caspase-9 knockout mouse line (iEC Casp9), were used to determine the optimal settings across different strains.

The wait time from rose bengal injection to laser irradiation can alter the success of photocoagulation in the veins. Too short a wait time may result in low rose bengal concentration within the veins, whereas too long a wait time can lead to rose bengal being cleared from the retinal circulation. Both situations can lead to poor photocoagulation and unsuccessful occlusions. When testing the number of occlusions obtained immediately after laser irradiation, comparing animals that were lasered 10 and 20 min after rose bengal administration revealed that there were no differences in the number of occlusions achieved (**Figure 2B**). However, the number of occlusions sustained up to 1 day after RVO significantly decreased in animals that were lasered 20 min after rose bengal administration independently of genotype. This result suggests that when studying acute RVO-induced injury, the waiting time after rose bengal administration can impact occlusion stability. Early reperfusion of veins (prior to 24 h after injury) could impact

the development of retinal edema and therefore, should be controlled by determining the correct waiting time from rose bengal administration to laser irradiation.

In principle, successful photocoagulation leading to occlusion is driven by laser power. Although this is such an important part of the process, it is also one of the greatest sources of variability in the model and should be optimized for consistency. To accomplish this, it is recommended to measure the laser output during setup before the mice are injected with rose bengal. The recommended output for the baseline laser power is between 13.0 and 15.0 mW. Low baseline laser power, such as 11.5 mW, without modifying the experimental power (100 mW), resulted in no occlusions, as shown in **Figure 3**. In contrast, a baseline laser output of 13.5 mW with an experimental power of 100 mW resulted in successful occlusions. In cases where the laser output was below 13.0 mW, the experimental power was increased to 110 mW to achieve the same successful occlusions as with higher baseline laser output. Typically, 100 mW is the standard experimental power; however, if the laser output is below 13.0 mW, it can be compensated by modifying the experimental power with the recommended ranges in **Table 1**.

Four main types of occlusions have been noted to occur after laser photocoagulation of the veins. These types of occlusions are summarized in **Figure 4A** and have been classified according to the amount of blood flow; fully occluded vessels (no blood flow), partially occluded vessels (mostly blocked with occasional flow), partially reperfused (uninterrupted steady blood flow with hindrance), and fully reperfused vessels (no obvious obstruction whatsoever). To investigate if the types of occlusions change according to the genotype and determine the time spent per occlusion state, 10 min videos after laser irradiation were evaluated. This assessment helped determine that the irradiated vasculature of iEC Casp9 mice spend more time in partially reperfused and partially occluded states than C57BL6/J, which spend more time in fully occluded states (**Figure 4B**).

Figure 5 demonstrates how the occlusion state of the vessels changes rapidly within the first 10 min after laser photocoagulation. Once these initial 10 min have passed, the occlusions stabilize and are maintained up to a 24 h time point. Thus, to assess the accurate initial number of occlusions per eye, it is recommended to wait for 10 min after irradiation. A previous assessment of this model determined that most occlusions reperfuse by 8 days after irradiation[8]; however, the rate of occlusions that reperfuse per day can vary by strain and must be determined in each experimental model. The model presented here is of acute injury and intended to be used to understand pathways that lead to edema, which develops within 24 h after occlusions. Another characteristic of RVO is flame-shaped hemorrhages, which can be observed at 24 h post injury, as depicted in **Figure 5**.

Follow-up at 24 h post RVO may reveal other ophthalmic pathologies that can occur as a result of the RVO method. Some include but are not limited to subretinal hemorrhage (characterized by continuous blood patch), retinal detachment, fully ischemic retina (no blood flow in veins and arteries), and cataracts. **Figure 6** shows fundus images with corresponding OCT as examples of these except in eyes where cataracts form (**Figure 6F**), as OCT cannot be performed in the presence of a cataract. **Figure 6A** shows examples of what a fundus image and OCT of an

uninjured eye look like for reference.

The main morphologic pathology of RVO in this model is retinal edema. To assess the level of retinal edema, it is recommended to take OCT images before the day of the RVO procedure for a baseline reading and at the time point of interest. **Figure 7** shows OCT quantification of retinal edema in injured eyes. Another measure used to determine the state of neuronal layers is to assess the disorganization of retinal inner layers (DRIL). This is a measure used in the clinical setting that represents capillary nonperfusion, another hallmark of RVO[5,9,10]. An example of this assessment can be found in **Figure 7B**. **Figure 7C** shows an example of an OCT image with the corresponding labels for each layer.

FIGURE AND TABLE LEGENDS:

Figure 1: Timeline and schematic of the RVO mouse model. (**A**) Timeline of events from rose bengal administration to the imaging of occluded veins. (**B**) Summarized representation of the method to achieve successful retinal vein photocoagulation. Red boxes represent important steps in the process that are highly variable and that can be optimized per mouse model and question of interest. (**C**) Retinal major veins (V) are wider and darker compared to arteries (**A**). Each major vein will be irradiated with a guided laser of 532 nm, spot size 50 µm, power 100 mW, duration 1 s, total energy 0.3 J, and radiant exposure 15278.87 J/cm^2, at an average distance of 375 µm from the optic nerve. (**D**) Laser application causes a vaporization bubble visible on fundus imaging of approximately 150 µm and covers <4% of the total retinal area. Numbers represent the suggested location and direction of movement (arrows) of the laser beam when irradiating vessels. Abbreviations: A = artery; V = vein; ON = optic nerve.

Figure 2: The time of photo-occlusion relative to rose bengal administration is critical for successful photocoagulation. (**A**) Fundus retinal images of iEC Casp9 WT and iEC Casp9 KO photo-occluded 10 and 20 min after rose bengal administration. White circles represent veins that had successful occlusions. (**B**) Number of occlusions immediately after irradiation (0 h) and 1 day post irradiation for 10 min and 20 min post rose bengal injection of combined genotypes. Welch's *t*-test, error bars indicate SEM. (**C**) Number of occlusions separated by genotype. Two-way ANOVA and Fisher's LSD test; error bars indicate SEM. Abbreviations: WT = wild-type; KO = knockout; SEM = standard error of the mean; ANOVA = analysis of variance; LSD = least significant difference; ns = not significant; P-RVO = post retinal vein occlusion.

Figure 3: Measuring baseline and experimental laser output are critical steps for successful photocoagulation. Fundus retinal images of iEC Casp9 WT and iEC Casp9 KO 10 min after photocoagulation; photo-occluded with different baseline and experimental laser output levels. Low baseline laser output can be compensated with experimental laser output (12.8 mW, 110 mW). White circles represent veins that had successful occlusions. Abbreviations: WT = wild-type; KO = knockout.

Figure 4: The RVO method results in different types of occlusions. (**A**) Fundus retinal images of C57BL/6J, iEC Casp9 WT, and iEC Casp9 KO 10 min after photocoagulation with different types of occlusions: fully occluded, partially occluded, partially reperfused, and fully reperfused. Insets

show a focused view of a vein that resulted in a specific type of occlusion after photocoagulation. (**B**) Quantification of the percentage of veins occluded in the different occlusion states for each genotype during the first 10 min post irradiation. Ten-minute videos were evaluated by two investigators, blinded to genotypes, who assigned numbers to the different occlusion states (fully occluded (-2), partially occluded (-1), fully reperfused (2), and partially reperfused (1)) per duration. Abbreviations: RVO = retinal vein occlusion; WT = wild-type; KO = knockout.

Figure 5: Timeline of occlusions after RVO. Fundus retinal images of C57BL/6J, iEC Casp9 WT, and iEC Casp9 KO 0, 5 min, 10 min, and 24 h after laser irradiation. The first 10 min are critical for the state of the occlusions and may change rapidly. After the initial 10 min, occlusions are stable up to at least 24 h. White circles represent veins that had successful occlusions, and yellow arrowheads depict flame-shaped hemorrhages. Abbreviations: RVO = retinal vein occlusion; WT = wild-type; KO = knockout.

Figure 6: Different ophthalmic pathologies may occur after RVO. (**A–E**) show fundus retinal images and corresponding OCT. (**A**) An example of an uninjured eye that did not undergo the RVO process. (**B**) Subretinal hemorrhage showing blood leaking out of the vessels in the fundus image. (**C**) Retinal detachment seen by the blurry fold in the fundus and the lifting of the retina in the OCT. (**D**) Excessive edema shown by a large amount of swelling in the OCT. (**E**) A fully ischemic eye with completely impaired blood flow resulting in a white retina. (**F**) Two different examples of a cataracted eye where a clear fundus image and OCT could not be obtained. OCT scale bars: 100 µm. Abbreviations: RVO = retinal vein occlusion; OCT = optical coherence tomography.

Figure 7: Quantification of OCT images. (**A**) Examining the layer thickness and the DRIL in unlasered control eyes and eyes that went through the RVO procedure. GCL, IPL, INL, OPL, ONL, Outer segment, and Whole Retina measurements. Statistics, Mann-Whitney test p-values: GCL: 0.0070, IPL: 0.0205, INL: <0.0001, OPL: 0.0014, ONL: 0.5582, Outer Segment: 0.44852, Whole Retina:0.0019. Error bars show SEM. (**B**) DRIL quantification measured from the OCT images from unlasered controls and RVO WT and KO iEC Casp9 mice as well as c57/BL6J mice that had RVO. Error bars show SEM. (**C**) Example OCT with the labels of each retinal layer. Abbreviations: DRIL = Disorganization of the inner retinal layers; RVO = retinal vein occlusion; WT = wild-type; KO = knockout; GCL = Ganglion Cell Layer; IPL = Inner Plexiform Layer; INL = Inner Nuclear Layer; OPL = Outer Plexiform Layer; ONL = Outer Nuclear Layer; SEM = standard error of the mean; OCT = optical coherence tomography; ns = not significant.

Table 1: Low baseline laser output can be compensated with higher experimental laser output. Variations in baseline laser output and recommended experimental laser output and exposure time.

DISCUSSION:

The mouse RVO model provides an avenue to further understand RVO pathology and to test potential therapeutics. While the mouse RVO model is widely used in the field, there is a need for a current detailed protocol of the model that addresses its variability and describes the

optimization of the model. Here, we provide a guide with examples from experience on what can be altered to get the most consistent results across a cohort of experimental animals and provide reliable data.

The two most essential elements of the RVO mouse model are the laser output and successful intravenous injection of the photosensitizer dye. To produce the power necessary to induce coagulation when the laser is aimed at a particular vein, the laser output has to be properly adjusted. While this can be achieved using the techniques suggested in the method, it is important to consider the differences in each laboratory's system setup. Variations of the fiber-optic cable and how it is accommodated in relation to the equipment and room temperature are some of the variables that can account for low laser output. Independent tunings to the system setup are recommended to increase the laser output.

However, this effort is inoperable without an apt tail vein technique to deliver the photosensitizer dye. Tail vein injections can be difficult to achieve, and it is a skill that takes time to develop. Poor injections can result in no occlusions; in this case, rose bengal can be administered via IP. Rose bengal administration via IP has been used to model RVO but with a longer laser irradiation time (3 s) and higher rose bengal concentration (40 mg/mL)[11]. To limit prolonged laser irradiation and specifically target the vasculature, tail veining is the preferred mode of administration.

This model could also be accomplished using other photoactivatable dyes such as Y eosin and sodium fluorescein[12-14], although rose bengal is the most used photoactivatable dye[4-6,8]. All dyes have been shown to produce early features of clinical disease such as retinal hemorrhage and retinal edema[15]. Photoactivatable dyes have been shown to have no adverse effects on the animals, thus showing insignificant system toxicity[15,16]. It should also be noted that the dye chosen should have an absorption maximum compatible with the wavelength of the laser being used. Rose Bengal has an excitation at 525 nm[17], sodium fluorescein at 475-490 nm[18], and Y eosin at 490 nm[19].

The main sources of variability in this model are the time of photo-occlusion relative to rose bengal administration and the baseline and experimental laser output. While **Figure 2** shows 10- and 20-min time points for photo-occlusion, a small number of 5- and 15-min experiments were also performed (data not shown), yielding occlusions that were not as consistent as the 10 min time point. Therefore, 10 min was chosen to be the optimal wait time between rose bengal administration and photo-occlusion for this method. However, studies have reported that RVO can also be induced as early as 3 min after the administration of rose bengal[5]. Another way to determine the mouse strain-specific optimal waiting time from rose bengal to photo-occlusion is to monitor the relative rose bengal concentration using the fluorescence imaging mode with a tetramethylrhodamine (TRITC) filter. However, the protocol described here can be performed using retinal imaging microscopes that do not have a TRITC filter.

The other source of high variability in this model is the baseline laser output. As day-to-day levels of baseline laser output can be vastly different, it is important to assess the levels before each experiment. Standardizing the baseline laser output across studies can help potentiate and

expand the use of the RVO mouse model. Readjusting the fiber cable can be enough to modify the baseline levels; however, if a 13.0 mW measurement cannot be reached, **Table 1** provides a guide for compensation using the experimental power. It is important to note that because the retina is a closed system, determining the fraction of veins occluded (the number of veins occluded divided by the number of veins irradiated) is essential for understanding, controlling, and predicting the severity of the damage in the RVO model. Previous analysis of damaged readouts (DRIL and retinal thickness) correlated to the fraction of veins occluded and predicted retinal atrophy at 8 days post RVO[8]. Thus, the fraction of veins occluded should be considered and evaluated. It is still unclear how other intermediate occlusion states, such as partially reperfused, partially occluded, or veins that were once occluded and reperfused by 10 min, contribute to the development of retinal edema and atrophy.

Further studies of the eyes with these types of occlusions can help investigate whether a sustained occlusion is necessary for substantial damage or if even transient occlusions are important in this model. Depending on the experimental question being asked, different occlusion rates will be optimal. A 40–50% occlusion rate is ideal in most cases, meaning two or three occlusions in an eye with six veins. This ensures substantial injury, but the retina is intact and can be dissected for immunohistochemical and biochemical analyses.

To determine this, distinguishing the pathological view of a successful and representative occlusion of the RVO signature is relevant. The natural occlusion presented by RVO includes flame-shaped hemorrhages[20] (not to be confused with subretinal hemorrhage), which can be observed in this model 24 h after injury. The RVO model can also lead to unwanted retinal injuries (not distinctive of RVO pathology) such as the ones shown in **Figure 6B**, if its parameters are not cautiously controlled. An approach that can be taken to avoid these and regulate the concentration of rose bengal and the experimental laser power, is to stop irradiating the vessels that have a clear formation of a thrombus after the first or second irradiation.

Other factors to consider when employing this model and deciding which veins to occlude are the mouse strain and the vessel diameter. Some mouse strains, such as BALB/c, most commonly known as albino, are susceptible to light damage[21]. Additionally, they have retinal developmental deficits that lead to defects in the decussation at the optic chiasm and visual acuity[22,23]. It is recommended to fully evaluate basal retinal and vascular integrity of uninjured controls for the mouse strain chosen for RVO studies. Studies have shown that vein width can interfere with the development of retinal edema and disease pathology[24]. Thus, animals of similar weight should be used to avoid further variability. The exclusion factors of which eyes to use in a study will also depend on the experimental question. It may be wise to include any eye that was once occluded, even if it is reperfused at the follow-up time point for signaling. However, if stable occlusions are desired, these eyes would be excluded. **Figure 6** shows examples of possible exclusion criteria or eyes that could be used for pathological analysis.

To show that these laser settings were not causing damage and the effects seen were truly driven by the occlusions themselves, sham controls were done in previous studies[8]. The sham mice still received a tail vein injection of rose bengal but were irradiated in the parenchymal space

between the major vessels instead of being irradiated on the vein. This was an important step in ensuring that the model replicated RVO injuries seen in patients instead of simply injuring tissue with a laser. These shams show no activation of caspase-9 or -7 or any of the edema seen in the mice who received normal laser irradiation to the vessels, indicating that the laser did not have any adverse effects. Having these controls is essential, especially if higher laser settings will be used to ensure that the injury being modeled is an accurate representation of the desired damage[8].

The RVO mouse model can be applied to study other diseases that result from hypoxic-ischemic injuries in the retina and brain such as diabetic retinopathy, retinopathy of prematurity, and stroke. Additionally, it can serve as a model in which to study signaling pathways relevant for vascular injury development and test potential treatments that ameliorate neurodegeneration in the central nervous system. The optimization tailored in this report can limit variability in the mouse RVO model and shed light on the pathophysiology of RVO.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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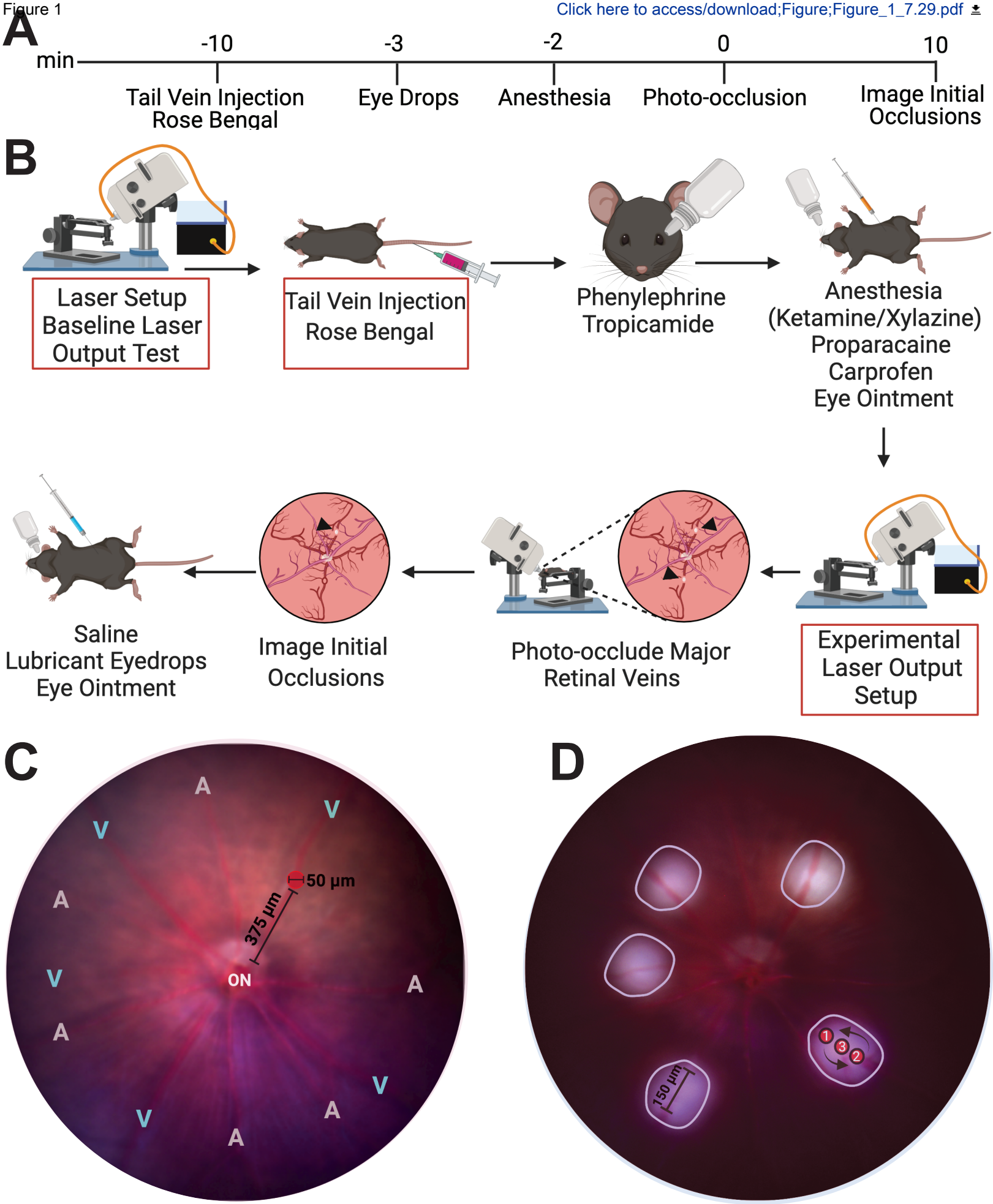
Figure 1



Figure

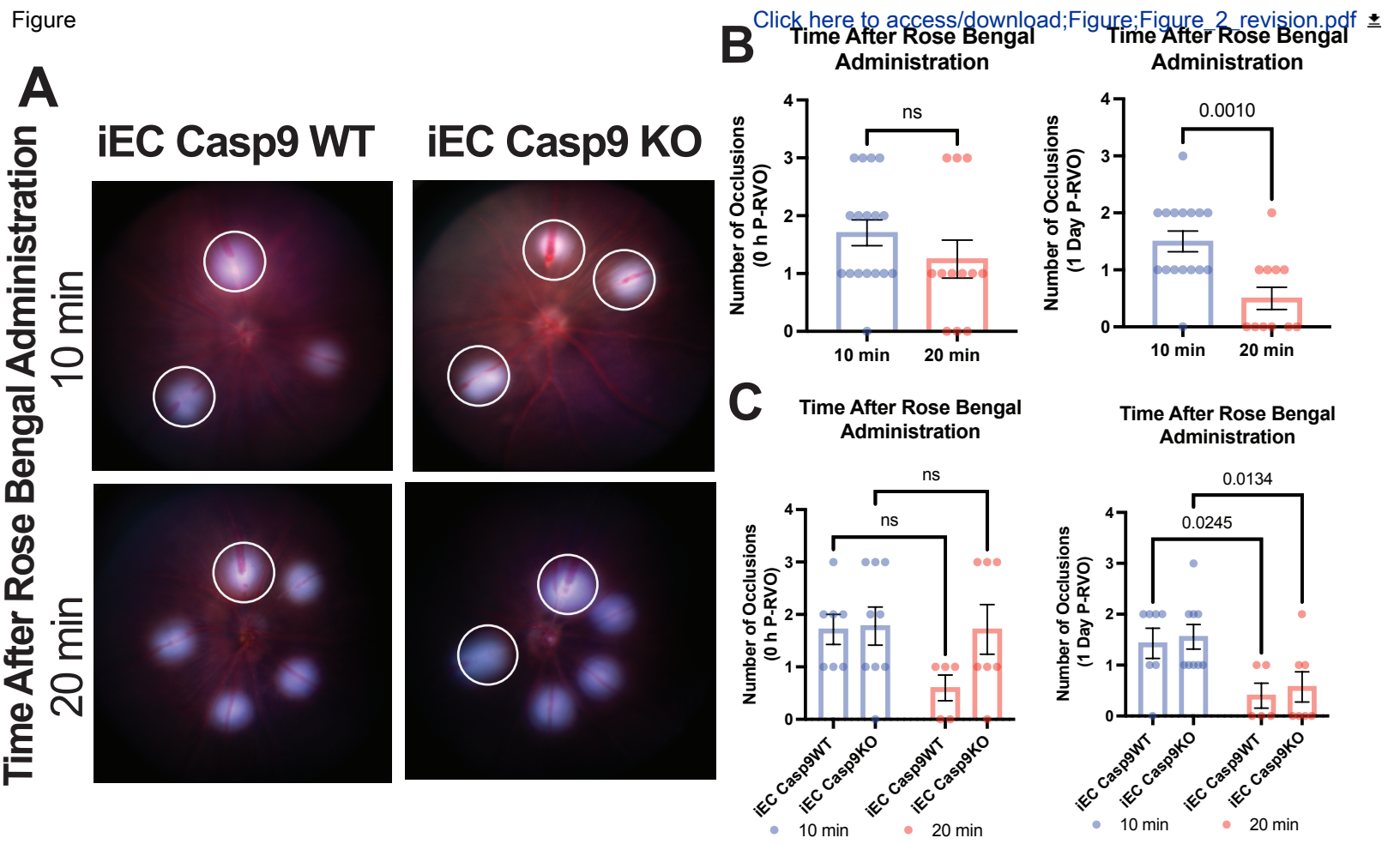


Figure 3

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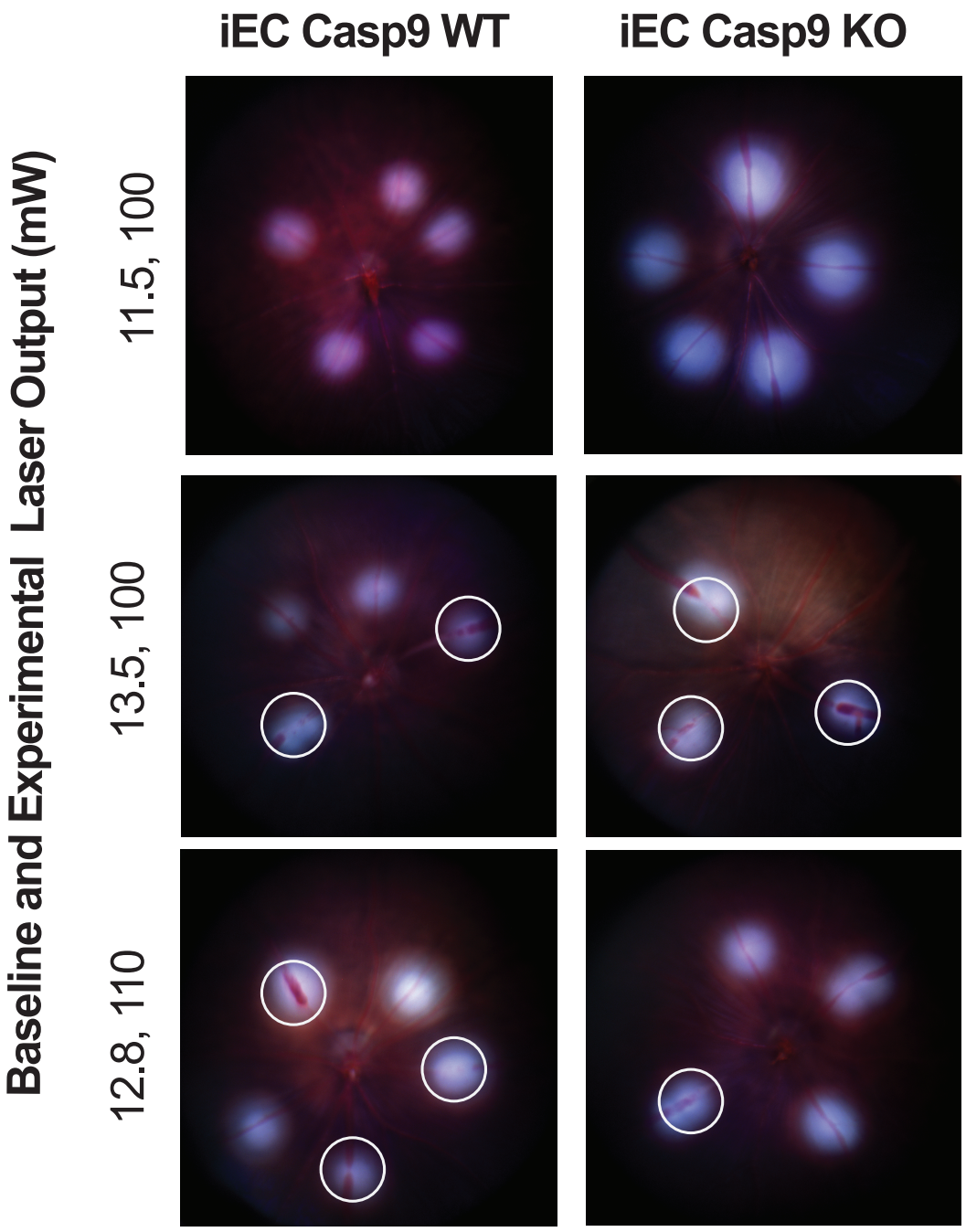

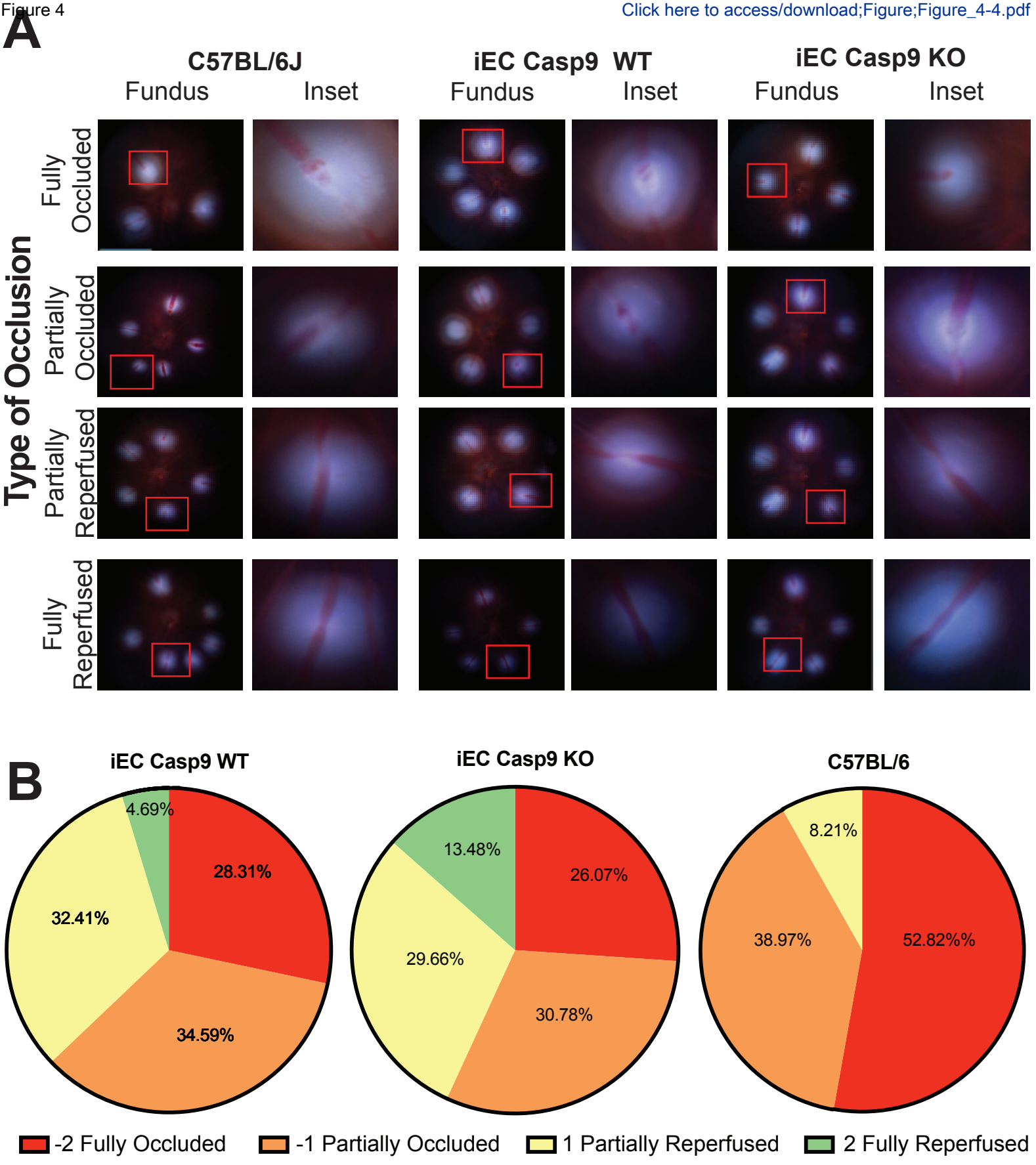

Figure

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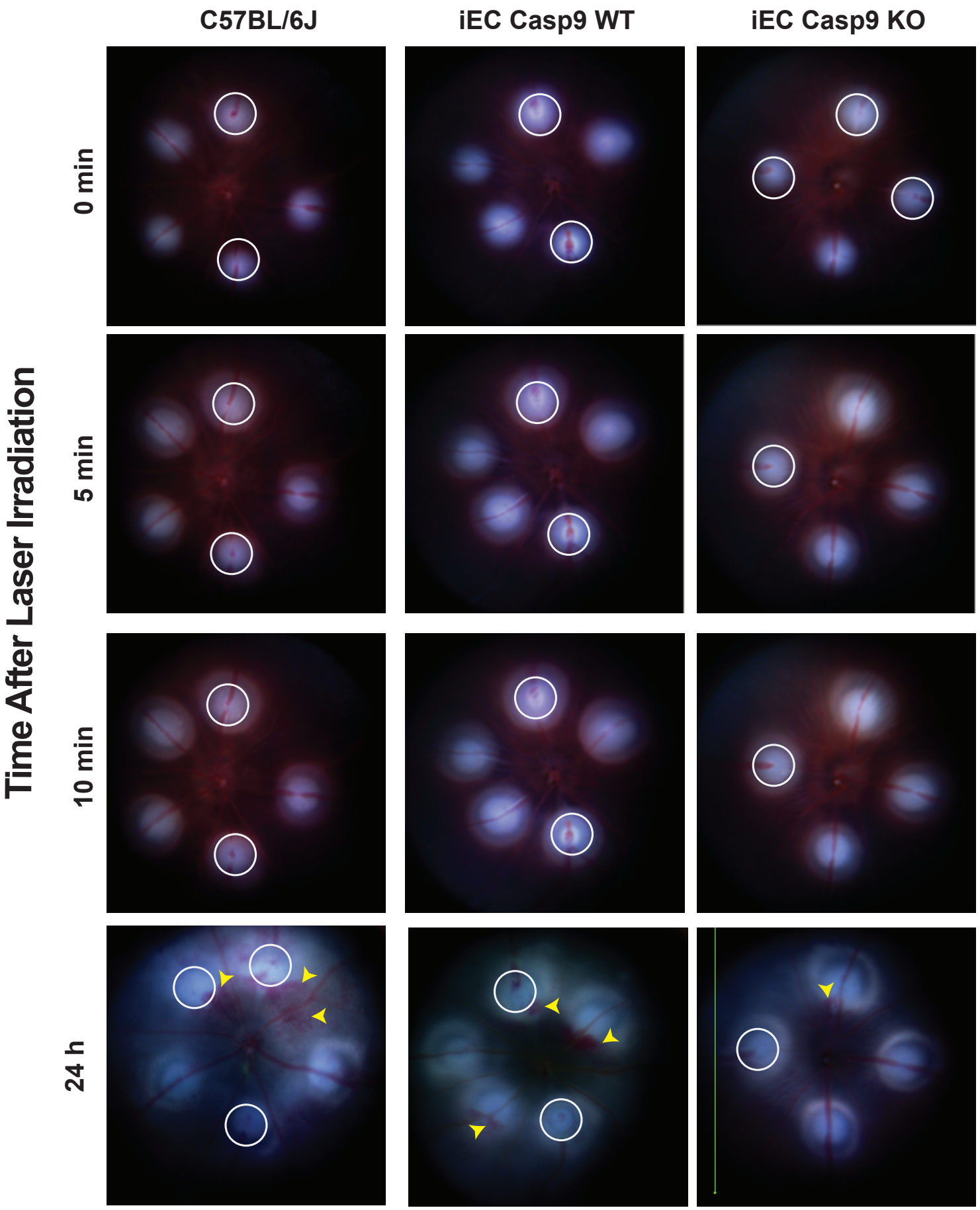
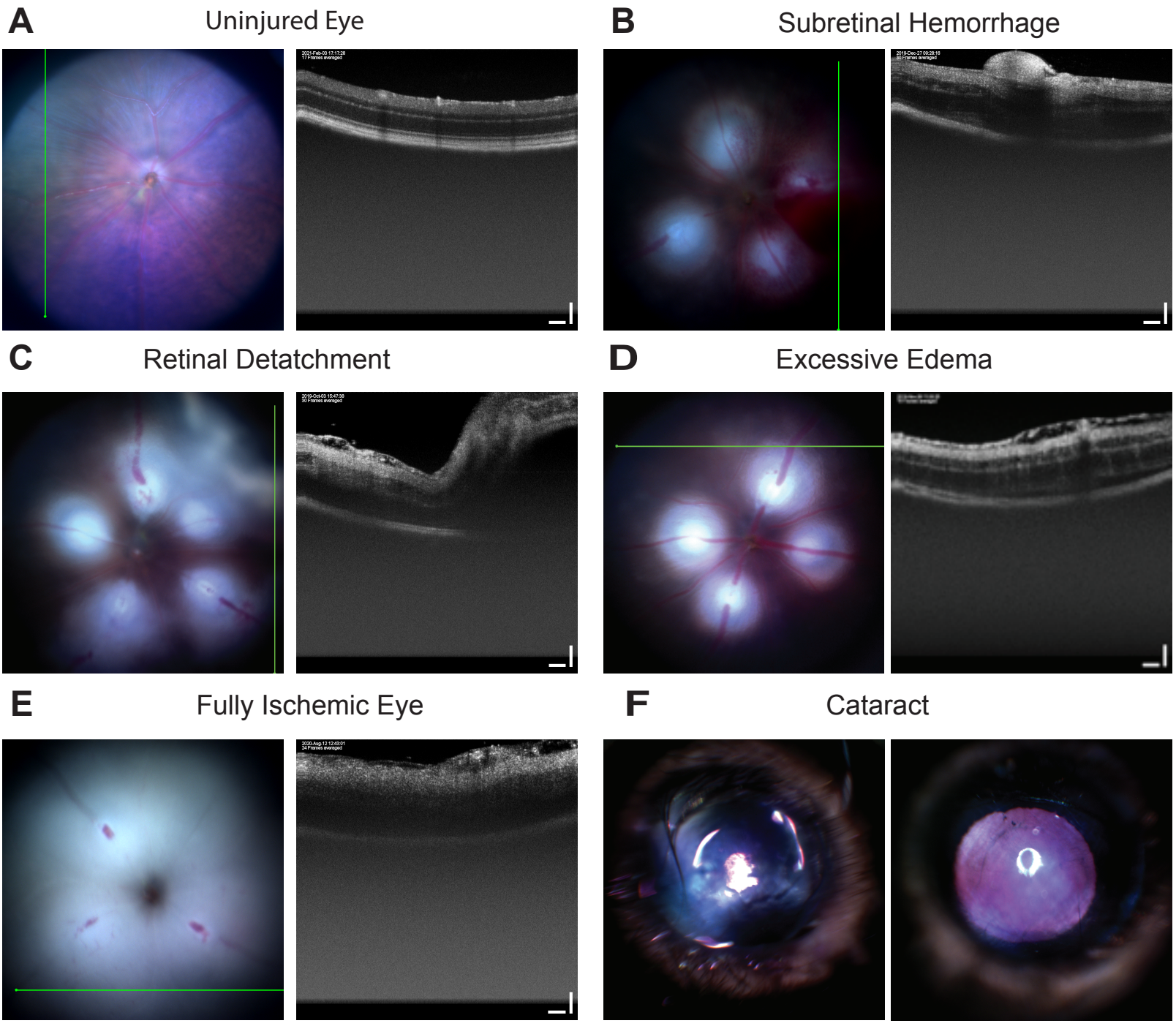


Figure 6



Figure

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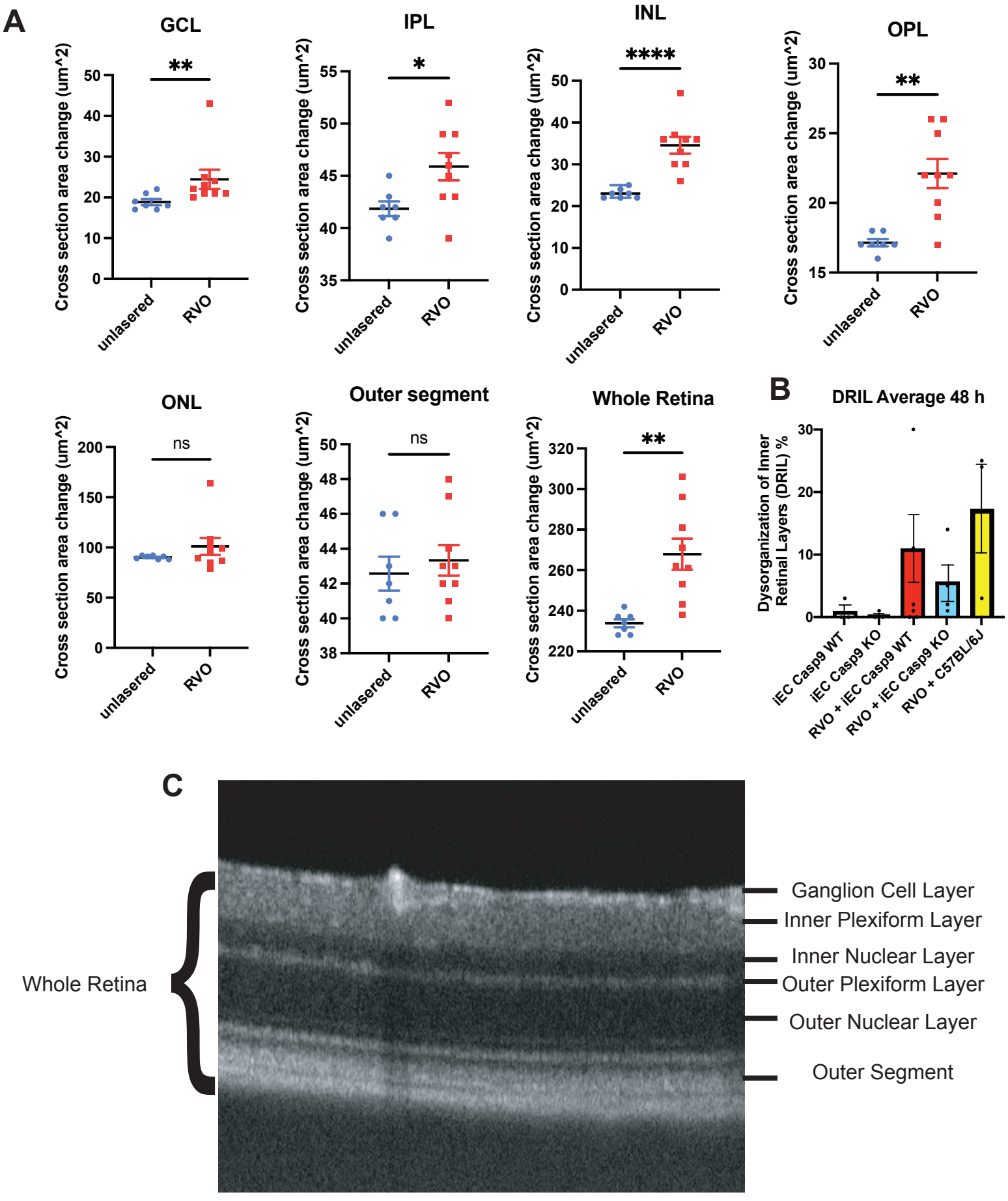

Table 1

Baseline Laser Output (mW)	Recommended Experimental Laser Output (mW)
<11.0 or >15.0	Turn off the laser and adjust the fiber at the enc box. Unscrew it and slightly move it towards th again, until it reaches a high
11.0-12.0	120
12.0-13.0	110
13.0-14.0	100
14.0-15.0	100

Recommended Time Exposure (ms)
d that is connected to the laser control ne right or left. Measure the outcome ner or lower value.
1,000
1,000
1,000
1,000

Response to reviewers JoVE62980

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns and all the editorial comments. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Responses are in red.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Addressed.

2. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Addressed.

3. 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. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Addressed.

4. 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 and Reagents.

For example: Micron IV laser adapter; Meridian laser box (?); StreamPix 6 program; Meridian screen; GenTeal; Refresh Tears; OCT software and machine; InSight software etc Are OCT software and OCT machine proprietary names? If so, please replace with more generic terms. If not, please see comment about Table of Mat Being a video based journal,

This has been addressed and all commercial language taken out of the manuscript.

5. JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:

a) Please include an ethics statement before all of the numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.

Added.

b) What happened to the mice after the study? Please specify the euthanasia method without highlighting the steps.

Non-survival perfusion surgery – added in lines 295-296.

c) How was proper anesthetization confirmed?

Addressed in line 219
d) For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.
There are no survival surgeries
e) Discuss maintenance of sterile conditions during survival surgery.
There are no survival surgeries
f) Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.
Addressed in line 270
g) Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.
There is no surgery. Please see lines 267-270 for recovery from anesthesia.
6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
Addressed.
7. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.
Addressed.
8. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable).
Addressed.
9. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.
Addressed.
10. Please add Meridian laser box (?); StreamPix 6 program; Meridian screen; GenTeal; Refresh Tears; OCT software and machine; InSight software etc to your Table of Materials and then sort the Materials Table alphabetically by the name of the material.
These have been added to the Table of Materials.

Reviewers' comments:

Reviewer #1:

The authors describe the mouse model of laser-induced retinal vein occlusion pointing to some important parameters that must be standardized to get reproducible results. This is

an important subject, and the manuscript is well-written. Nevertheless, the following issues should be carefully revised.

What is the background of your genetically modified mouse line? C57BL/6J?
Addressed in lines 68-70
Using the fluorescence imaging mode (TRITC) of the mouse camera it is possible to directly monitor the time course of increase and decrease of the rose bengal concentration in the retinal vessels. It may be quantified by evaluation of an image series. The optimal waiting time is expected to be constant for all mouse lines.
We do not have a TRITC filter in our apparatus so are unable to perform this experiment.
As an ophthalmic laser device for human patients is commonly used, please, shortly describe the differences in handling in comparison to the Phoenix system.
Addressed in lines 56-58
What is the spot size or diameter of the laser? This information is important when using devices where the spot size can be changed. The most important photochemical term in the context of laser treatment is radiant exposure that is radiant energy received by a surface per unit area, or equivalently irradiance of a surface integrated over time of irradiation. It includes power, area and time.
Addressed in Figure 1 legend lines 400-405
Please, recommend and discuss control groups that should be included in every experiment.
Addressed in lines 534-543
l. 130: what is the wait time between injection of rose bengal and laser treatment? You only mention 10 min between injection and anesthesia.
Addressed in time line added to Figure 1A
l. 151: you have to turn on the lamp again.
This has been resolved
l. 179: is rose bengal really metabolized? Most of it may be removed by the kidneys.
Addressed in line 318-319, changed the wording to 'being cleared from the retinal circulation.' as it is a more accurate description.
l. 188: the experimental voltage should not be given in mW.
This has been resolved, voltage changed to power.
l. 273: it may be mentioned that the absorption maximum of the dye has to fit to the wavelength of the laser.
Addressed in lines 481-484
Fig. 2c: Test of statistical differences is only necessary between different time points of wt or different time points of ko. The abbreviation for minutes is min.
The stats in figure 2C have been fixed to represent this and mins has been changed to min

Reviewer #2:
Manuscript Summary:
The manuscript addressed the standardized protocol to induce RVO mouse model with limiting variability.

Minor Concerns:
1) In line 144, it would be better to address the approximate interval distance between 3 laser spots in occlude the major veins.

Figure 1D has been added to represent this
2) It seems to have a possibility of vessel rupture with hemorrhage trying to fully occlude the veins. It would be better to share the knowhow to avoid this unwanted complication.
Flame hemorrhages which can occur after RVO are now identified in Figure 5. Also addressed in lines 511-519.

Reviewer #3:
Manuscript Summary:
Manuscript Number : JoVE62980
Title : Optimization of the Retinal Vein Occlusion (RVO) Mouse Model to Limit Variability.
The authors showed an optimized protocol of the mouse model of Retinal Vein Occlusion (RVO) . The manuscript submitted by Troy et al. is interesting, but there are some problems that authors should indicate and need additional experiments.

Major Concerns:
1. inducible endothelial caspase-9 knock out (iEC Casp9 KO) strain
The author should describe why caspase-9 in endothelial cells was targeted to determine the optimal setting across different strains, and the information about targeted gene in detail. These information can ensure the credibility of this study.
Addressed in lines 70-73
2. RVO mouse model
The author should unify the number of vessels which were laser-irradiated. It is assumed that the numbers of laser-irradiated vessels can influence the degree of retinal edema.
Addressed in lines 498-509

3. Figure 2
The author should show the data of other time points such as 5 and 15 minutes after rose bengal injections. It can be important data to show the irradiation at 10 minutes after injection is ideal for optimized protocol.
Addressed in lines 486-490
4. Figure 2A
The representative image should be replaced (iEC Casp9 WT, 10 minutes after rose bengal administration). Comparing other occluded vessels in this study, the vessel in this image seems not to have successful occlusion.
Image corrected, a misplaced circle was around a vessel that was not occluded and is now only around the two that are.
5. Figure 2B and C
The table legend should be described about the methods of Figure 2B and C in detail. In this manuscript, the description in table legend for Figure 2 lacks the explanation about Figure 2B and C. Furthermore, the author should also describe the statistical method for Figure 2B and C.
Added in lines 411-413
6. Figure 4
The author should describe the meaning of classification in types of occlusions. It is unclear how the grades correlate with the damage in RVO model and the pathology of RVO.

Addressed with the addition of figure 4B and description in lines 346-354.
7. Figure 6A-D
The authors should show fundus and OCT images of unlasered mouse. They are essential data to show whether the pathology of RVO certainly forms by the protocol.
An unlasered control fundus image and OCT have been added to figure 6.
In addition, the authors should describe the names of the retinal layers in representative images.
An example OCT with the layers labeled has been added to figure 7.
8. Discussion
The authors should compare the effects of rose bengal with other photoactivatable dyes such as Y eosin and sodium fluorescein. It can be important to show the difference between photoactivable dyes to recommend optimized protocol for RVO model.
We have optimized our model using rose bengal and it is the most commonly used photoactivatable dye, however we did want to point out that there were other dyes available as options. We have not personally done experiments with the other dyes and have cited the studies that use them.

Minor Concerns:
1. Figure 2B, C and Figure 7
The author should describe what error bars indicate in table legends. Do they indicate standard Errors (SE)? In addition, the sample numbers for experiments should be described in each table legend.
Added into figure legends.
2. Figure 5
The author should describe what the white circles in images represent.
Added into figure legends.
3. Figure 6A-D
The author should describe how long the scale bars indicate in OCT images.
Added into figure legend.
4. Figure 7A
In every graph, error bars should be added.
Added into figure 7.
5. Discussion
The author should insert references about the description on page 8, lines 302-304.
This is something we have noticed in our measurement of the laser before each mouse, other studies don't address this

Reviewer #4:
Manuscript Summary:
The authors have shown detailed and specific instructions about laser output conditions and ingenuity in how to use photo sensitizer in order to create a mouse retinal vein occlusion (RVO) model. This manuscript is well documented.
I think this article would be useful for peoples who wants to investigate retinal ischemic changes by RVO.
Researchers may want to know the condition of laser burn to obtain perfect and sustained occlusion of the retinal vessels. Thus, I think the search for ideal condition to sustainable

vein occlusion is indispensably important for this manuscript.
The details of improvement suggestions are as follows.

Major Concerns:

・Although the authors only referred to C57black mice, albino mice would be preferred for vascular occlusion, because laser burns develop uncontrollably to the surrounding tissues in colored mice, in contrast, albino mice develop minimal damage by laser photocoagulation if accompanied with Rose Bengal dye.

While this is an interesting suggestion albino mice are susceptible to light damage adding another variable to the model and that does not recapitulate RVOs population. https://doi.org/10.1177/0192623312469308 Additionally, they have retinal developmental issues that lead to defects in optic chiasm and visual acuity. https://www.sciencedirect.com/science/article/pii/S0166223696100801

Our previous publication https://www.nature.com/articles/s41467-020-16902-5 shows that laser burns that don't lead to occlusion don't cause pathological effects in the retina.

I think the authors should explain why they used only colored mice, and the reason why they used iEC casp9 KO mice.

Addressed in lines 70-73

And they should perform laser against albino or brown mice to compare the condition of laser among them.

See answer above regarding albino mice.

・I know that Rose Bengal administration method is widely used for laser-induced RVO model. According to the literature, intraperitoneal injection is sufficient and easier method compared to tail vein administration. The authors should explain why they selected tail vein injection other than intraperitoneal injection.

As for intraperitoneal injection, 3 minutes after injection is best for laser coagulation. I think longer wait after injection around 10 minutes may weaken the capability of photosensitizing.

Addressed in lines 470-475

・As a researcher, we sometimes experience reperfusion of the vessels after laser. Therefore, perfect occlusion at the first day does not always mean full occlusion at later time. It may not last longer. The authors should show the percentage of successful occlusion rate for certain time point like 1, 3, 7, 14 days after laser coagulation.

Addressed in lines 356-363.

・However, the authors showed many photos of various condition of laser photocoagulation.

None of them are quantitative.

To show the efficacy of laser photocoagulation, the authors should illustrate quantitative difference.

Addressed with the addition of figure 4B