

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

I. 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

I. 151: you have to turn on the lamp again.

This has been resolved

I. 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.

I. 188: the experimental voltage should not be given in mW.

This has been resolved, voltage changed to power.

I. 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 photoactivatable 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