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Rat Model of Photochemically-Induced Posterior Ischemic Optic Neuropathy

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Corresponding Author:	Jeffrey L. Goldberg Shiley Eye Center La Jolla, CA UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	jlgoldberg@ucsd.edu
Corresponding Author's Institution:	Shiley Eye Center
Corresponding Author's Secondary Institution:	
First Author:	Yan Wang
First Author Secondary Information:	
Other Authors:	Yan Wang Dale P. Brown Brant D. Watson
Order of Authors Secondary Information:	
Abstract:	<p>Posterior Ischemic optic neuropathy (PION) is a sight devastating disease in clinical practice. However its pathogenesis and natural history has remained poorly understood. Recently, we developed a reliable, reproducible animal model of PION and tested the treatment effect of some neurotrophic factors on this model. The purpose of this video is to demonstrate our photochemically induced model of posterior ischemic optic neuropathy, and to evaluate its effects with retrograde labeling of retinal ganglion cells. Following surgical exposure of the posterior optic nerve, a photosensitizing dye, erythrosin B, is intravenously injected and a laser beam is focused onto the optic nerve surface. Photochemical interaction of erythrosin B and the laser during irradiation damages the vascular endothelium, prompting microvascular occlusion mediated by platelet thrombosis and edematous compression. The resulting ischemic injury yields a gradual but pronounced retinal ganglion cell dieback, owing to a loss of axonal input- a remote, injury-induced and clinically relevant outcome. Thus, this model provides a novel platform to study the pathophysiologic course of PION; and can be further optimized for testing therapeutic approaches for optic neuropathies as well as other CNS ischemic disease.</p>
Author Comments:	<p>Posterior Ischemic optic neuropathy (PION) is a sight devastating disease in clinical practice. However its pathogenesis and natural history has remained poorly understood. Recently, we developed a reliable, reproducible animal model of PION and tested the treatment effect of some neurotrophic factors on this model. This paper and video demonstrate the entire procedure and all the materials and devices for our photochemically induced model of posterior ischemic optic neuropathy. We think JoVE is a great platform to show our video. We are sure it will be very helpful for other scientists to apply this novel model in future researches.</p>
Additional Information:	

Question	Response
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TITLE:
Rat Model of Photochemically-Induced Posterior Ischemic Optic Neuropathy

AUTHORS:
Wang, Yan
Shiley Eye Center, University of California, San Diego, CA, USA
Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, Miami, FL, USA
Department of Ophthalmology and Vision Science,
Eye & ENT Hospital,
Shanghai Medical College,
Fudan University,
Shanghai, China
yaw059@ucsd.edu

Brown, Dale P. Jr.
Bascom Palmer Eye Institute,
University of Miami Miller School of Medicine,
Miami, FL, USA
mtntop89@gmail.com

Watson, Brant D.
Departments of Neurology and Biomedical Engineering,
University of Miami Miller School of Medicine,
Miami, FL, USA
BWatson@med.miami.edu

Goldberg, Jeffrey L.
Shiley Eye Center,
University of California,
San Diego, CA USA

Bascom Palmer Eye Institute,
University of Miami Miller School of Medicine,
Miami, FL, USA
jlgoldberg@ucsd.edu

CORRESPONDING AUTHOR:
Goldberg, Jeffrey L.
9415 Campus Point Dr. #0946
Shiley Eye Center, UC San Diego
La Jolla, CA 92093
E-mail: jlgoldberg@ucsd.edu
Office: 858-534-9794

KEYWORDS:
Optic nerve, Ischemic, Animal model, Photo-chemical, Retinal ganglion cell, Survival

SHORT ABSTRACT:

The goal of this protocol is to photochemically induce ischemic injury to the posterior optic nerve in rat. This model is critical to study the pathophysiology of posterior ischemic optic neuropathy, and therapeutic approaches for this and other optic neuropathies, as well as of other CNS ischemic diseases.

LONG ABSTRACT:

Posterior Ischemic optic neuropathy (PION) is a sight devastating disease in clinical practice. However its pathogenesis and natural history has remained poorly understood. Recently, we developed a reliable, reproducible animal model of PION and tested the treatment effect of some neurotrophic factors on this model¹. The purpose of this video is to demonstrate our photochemically induced model of posterior ischemic optic neuropathy, and to evaluate its effects with retrograde labeling of retinal ganglion cells. Following surgical exposure of the posterior optic nerve, a photosensitizing dye, erythrosin B, is intravenously injected and a laser beam is focused onto the optic nerve surface. Photochemical interaction of erythrosin B and the laser during irradiation damages the vascular endothelium, prompting microvascular occlusion mediated by platelet thrombosis and edematous compression. The resulting ischemic injury yields a gradual but pronounced retinal ganglion cell dieback, owing to a loss of axonal input- a remote, injury-induced and clinically relevant outcome. Thus, this model provides a novel platform to study the pathophysiologic course of PION; and can be further optimized for testing therapeutic approaches for optic neuropathies as well as other CNS ischemic diseases.

INTRODUCTION:

In patients over 50 years old, ischemic optic neuropathy (ION) is the most prevalent type of acute optic neuropathy². The condition can present as one of two subtypes according to the source of specific affected blood supplies and clinical presentation: anterior (AION) or posterior (PION)³. While the pathogenesis and course of AION has been studied extensively⁴⁻⁷, PION has remained poorly understood due to its low prevalence, variable presentation, ill-defined diagnostic criteria and lack of an animal model. Furthermore, no treatments have been proven to effectively prevent or reverse vision loss from AION or PION. Therefore, a reproducible and reliable animal model of PION is of great value to study the disease process in vivo and test new therapeutic regimens for neuroprotection and axon regeneration.

Photochemically induced ischemic injury to the microvasculature resulting in vasogenic edema and thrombosis effectively creates regional tissue ischemia⁸⁻¹². After injection into the vascular circulation, the photosensitive dye erythrosin B produces reactive singlet molecular oxygen upon activation by laser irradiation on target vessels. The singlet oxygen directly peroxidizes the vascular endothelium, stimulating platelet adherence/ aggregation and leading to occlusive thrombus formation. Ischemic damage is spread and further exacerbated to neighboring areas by microvascular compression due to vasogenic edema. The overall goal of this protocol is to photochemically induce ischemia to the retrobulbar optic nerve to mirror the damage caused by PION.

To our knowledge, this is the first model of ischemic injury in the posterior optic nerve¹. As this model produces ischemia while avoiding physical trauma, the physiological processes of posterior ischemic optic neuropathy are better mimicked and studied. Also, this model offers a

novel platform for screening of candidate therapeutics for optic neuropathies and other CNS ischemic disease. Here, a detailed protocol for femoral vein catheterization, optic nerve exposure, intravenous injection of Erythrosin B and laser irradiation in a rat PION model are described.

PROTOCOL:

All animal procedures were approved by the University of California San Diego and University of Miami institutional animal care and use committees (IACUC) and performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. All reagents and instruments used in surgical procedures are sterile.

1. Anesthetize and prepare the rat for surgery

1.1) Prior to procedure, rats are anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (8 mg/kg) according to body weight. Adequate depth of anesthesia should be determined by a negative response to toe pinch stimulus.

1.2) Once anesthetized, pull the tongue forward to prevent asphyxia and apply lubricating ointment to both eyes to prevent drying of the corneas during surgery.

1.3) Shave the surgical sites using a hair clipper and wipe the area with 10% providone-iodine detergent solution and 70% ethanol for three times.

1.4) Drape animal with a sterile field. Sterile gloves and surgical instruments are used during survival surgery. Re-sterilize the tips of instruments using a hot bead sterilizer between animals.

2. Surgical Approach

2.1) PION induction

2.1.1) Femoral vein catheterization

2.1.1.1) Prepare and clean the surgical site. Shave the right inner thigh using a hair clipper and wipe the area three times each with 10% providone-iodine detergent solution and 70% ethanol.

2.1.1.2) Prepare the tubing. Cut a 40cm length of polyethylene tubing (PE 10) sterilized in 70% ethanol. Flush the tubing with saline and connect it to a 1 ml syringe containing a pre-measured solution of 2% erythrosine B dye (1 μ L/mg, yielding a dose of 20 mg/kg body weight). Mount the syringe into a foot-switch controlled infusion pump set to a rate of 600 μ L/min.

2.1.1.3) Using a No.15 blade, make a small horizontal incision at the base of the right thigh. Cut and spread the membrane inside and clean the area with sterile cotton swabs.

2.1.1.4) Separate the muscle with forceps until the branch of the femoral vein is visible. A sheath surrounds the artery, vein and the nerve. Pinch and pull this sheath upwards with forceps (fine tip Dumont forceps), and cut a small incision (2-4 mm is usually adequate) near the base of the triangular shaped wedge with Vannas spring scissors. Expand the cut as necessary.

2.1.1.5) Separate the vein and artery with a blunt micro-surgical hook parallel to the direction of the vein. Be careful not to damage the delicate membrane and vein branches. Then, gently lift the vein and separate it from the underlying connective tissue.

2.1.1.6) Obtain a needleless nylon suture and place it next to the femoral vein. Using the micro-surgical hook, elevate the vein and pass fine tip forceps beneath the distal region. Grab one end of the suture and pull it underneath the vein. Ligate the distal vein tightly. Pass a second suture in a similar manner under the proximal vein and make a loose knot.

2.1.1.7) Make a small cut in the vein near the distal ligation with Vannas spring scissors. Expand the hole as necessary with fine tip forceps. Some blood may leak through the cut. Clean the surgical area with cold, sterile BSS and sterile cotton swabs.

2.1.1.8) Holding the vein wall at the edge of the cut, catheterize the vessel with the prepared saline-flushed tubing using a needle holder. Tighten the proximal knot around the vein and tubing. Then, anchor the tubing by tying it to the distal suture.

2.1.1.9) Check the quality of the catheterization by pressing the foot-switch to inject saline, <1 ml is adequate. Make sure the tubing is unobstructed and has no leaks. Temporarily close the incision with sutures to protect the catheterization and tissue.

2.1.2) Exposure of the optic nerve

2.1.2.1) Prepare the surgical site by wiping the pre-shaved area above the left eye three times each with 10% providone-iodine detergent solution and 70% ethanol.

2.1.2.2) Make an incision along the skin 2-3 mm behind the eye with a No.15 blade. Pinch and lift the connective tissue with serrated forceps, and make a small incision with Vannas spring scissors. This small incision is generally about 5 mm in length, but can be longer to provide greater exposure for the starting surgeon. Continue to bluntly dissect through the connective tissue along the superior rim of the orbital bone, taking care to avoid disrupting blood vessels. Clean the surgical area with cotton swabs.

2.1.2.3) Dissect downward through the conjunctiva until the superior rectus muscle is visible. Pinch and dissect through the muscle; the muscle will be liberated from deep within the orbit. Now, surrounding tissues can be utilized to aid in the retraction and elevation of the eye for ease of visualization.

2.1.2.4) Retract the flap of skin and connective tissue laterally and downward, and hold in place with a suture and hemostat. This will rotate the eye forward and outward in order to reveal the fat containing sheath that surrounds the optic nerve.

2.1.2.5) Carefully insert a pair of sharp forceps and expand parallel to the optic nerve to separate the connective tissue surrounding the sheath. Do not touch the optic nerve with the sharp tips of the forceps.

2.1.2.6) A 5 mm length of the optic nerve and surrounding sheath should now be visible. A network of microvessels on the sheath surface encircles the optic nerve. These will be targeted during laser irradiation.

2.1.3) Intravenous injection of Erythrosin B and laser irradiation

2.1.3.1) Wear orange-colored safety glasses at all times while operating the laser irradiation apparatus to shield yourself from the laser light. Turn on the laser, open the shutter and adjust the peak and average powers of the laser as necessary. Close the shutter.

2.1.3.2) Position the rat in the laser irradiation apparatus. To ensure proper beam placement, a weak aiming beam is produced by spatially filtering the laser through a 100µm diameter hole drilled in the closed shutter blade. Re-expose the optic nerve with a pair of fine tip forceps, and position the aiming beam onto the intraorbital optic nerve between 3 mm and 4 mm behind the optic nerve head.

2.1.3.3) Inject the solution of 2% erythrosine B via activation of the infusion pump. It may circulate for a few seconds while the surgeon adds a small drop of BSS to moisten the surface of the optic nerve.

2.1.3.4) Verify the position of the aiming beam and then click the foot-switch to initiate irradiation. An orange-colored safety filter, which is added into the optical path of the microscope, will be immediately triggered followed by the opening of the shutter after a one second delay.

2.1.3.5) Irradiate the optic nerve for 90 seconds with a peak power of 150mW and average power of 18mW. Yellow fluorescence, visualized as bright orange through the safety filter, will emit from the superior surface of the optic nerve and is sufficient to ensure that the beam irradiated the nerve symmetrically.

2.1.3.6) After the irradiation, the orange-colored safety filter will open automatically. Microhemorrhage can be observed in some cases.

2.1.3.7) Relieve the traction on the extra ocular muscles and return the eye to a neutral position. Close the incision with interrupted sutures. Then withdraw the catheterization and tie off the femoral vein tightly to prevent leakage; close with interrupted sutures. Apply antibiotic ointment to both incisions. Check the fundus to verify the vascular integrity of the central retinal vein and artery.

2.2) Retrograde Labeling of Retinal Ganglion Cells (RGCs)

NOTE: In order to evaluate RGCs survival, retrograde labeling with fluorogold (FG) should be completed one week before PION. The method is described in detail in *JoVE* protocol 819¹³.

2.2.1) In brief: anesthetize the animal with ketamine (60 mg/kg) and xylazine (8 mg/kg) and shave the head.

2.2.2) Surgically scrub the incision site and make a midline incision across the head to expose the skull.

2.2.3) Drill bilateral holes through the skull (\varnothing 2x2 mm) 0.5 mm from both the sagittal and transverse sutures.

2.2.4) Carefully aspirate the cerebral content that lies over the dorsal surface of the superior colliculus (SC) using a vacuum pump. Then, place a small piece of gelfoam soaked with 4% FG onto the surface of the SC.

2.2.5) Close the incision with sutures and care for the animal using standard postoperative care.

3. Post-operative care and Euthanasia

3.1) After surgery, place animal in a separate clean cage on top of a recirculating heated water pad until the animal is recovered.

3.2) Post-surgical analgesics (buprenorphine HCl, 0.01 mg/kg) should be administered twice per day for three consecutive days to minimize discomfort.

3.3) Rat should be kept separately and observed until they are able to maintain sternal recumbency and regain sufficient consciousness.

3.4) Signs of recovery and good health are monitored daily for at least 5 days after the surgery, or until suture removal and adequate healing of the surgical site, whichever occurs latest.

3.5) Euthanize the animals by perfusion with 4% PFA at scientifically appropriate time points after the surgery according to investigative interest.

REPRESENTATIVE RESULTS:

The resulting ischemic injury induced by this technique yields a gradual but pronounced death of retinal ganglion cells after ischemic axon injury. This is a clinically relevant outcome similar to that observed in the human disease. FG retrograde labeling is used to quantify RGC survival after PION. The same method is employed to validate a successful model creation as well as to assess the effects of different therapeutic regimens. Figure 1 shows representative confocal images of FG positive cells in retinal flat mounts from control (Fig. 1A), sham-treated (laser only/no erythrosin B Fig. 1B), and 2 weeks post-PION-treated (Fig. 1C) animals. Compared to control animals, fewer FG positive cells are present in animals 2 weeks after PION induction. No significant difference between the number of RGCs in control and sham-treated (laser only/no erythrosin B) animals is observed. This indicates that the PION-induced RGC loss is elicited by the combination of erythrosin B and laser irradiation, instead of thermal energy from the laser alone.

FIGURE LEGENDS:

Figure 1. Retinal ganglion cell (RGC) survival after posterior ischemic optic neuropathy (PION). Retinal ganglion cells retrogradely labeled with Fluorogold were imaged in retinal flat mounts (A-C). Two weeks after PION, a similar number of RGCs is observed in control (A) and sham-

treated (B, laser only/no erythrosin B) eyes. However, the number of fluorogold-labeled RGCs is markedly reduced in the setting of PION (C). Scale bar=100 μ m.

DISCUSSION:

Here we describe in detail a method for inducing PION in a rat model. The most critical part of the protocol is the exposure and irradiation of the optic nerve – to expose the nerve as long as possible while avoiding damage caused by the sharp fine tip forceps or from stretching. In rats, the ophthalmic artery enters the optic nerve ≤ 1 mm from the optic nerve head. Therefore, irradiation of the optic nerve 3-4 mm away from the optic nerve head should only result in ischemia of the capillaries feeding the nerve, rather than the artery supplying the inner retina. Fundoscopic examination is necessary to ensure the vascular integrity of central retinal artery and vein. With practice, the full surgical procedure can be accomplished in half an hour. And with set parameters for laser irradiation, this model has a reproducible time course of cell death.

As peak intensity and irradiation duration are two determinants of ischemic damage severity, modification of these parameters can be made to adjust the degree of damage for different study aims.

As the first and currently only available animal model for PION, this technique provides a novel platform for research on pathogenesis and molecular changes of PION, and can be used to screen treatment drugs for this and other CNS ischemic diseases.

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DISCLOSURES:

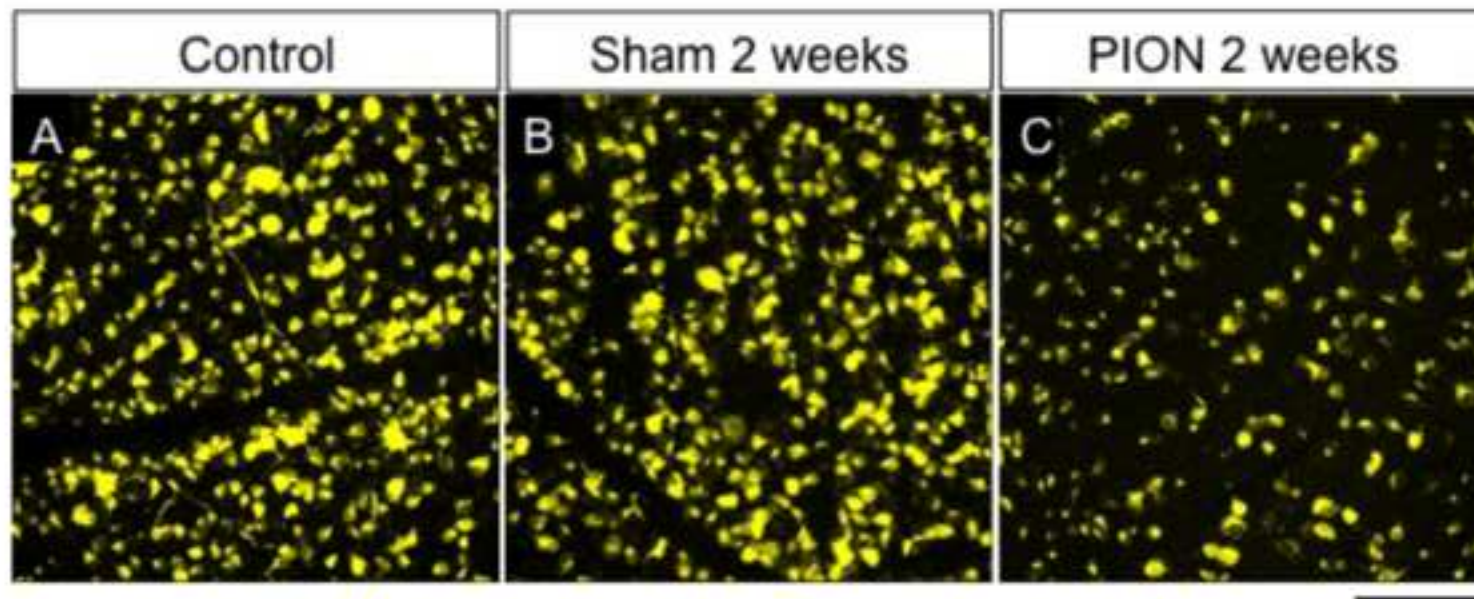
The authors have nothing to disclose

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Figure 1 Retinal ganglion cell (RGC) survival after PION



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
532-nm Nd:YAG laser Beam chopper	Laser glow custom-made	LRS-532-KM-200-3 custom-made	
Mechanical shutter and corresponding shutter drive timer	AAM Vincent Associates	SD-10	
25-cm focal length spherical lens	CVI/Mellles-Griot	01 LPX 293	plano-convexBK7 glass lens with HEBBARTM antireflect
Erythrosin B	MP Biomedicals	190449	
Fluorogold	Fluorochrome,LLC		
Gelfoam	Cardinal Health	CAH1203421	
Polyethylene tubing (PE10)	BD Intramedic	427400	
No. 10 Blade	Miltex	4-110	
Fine Forceps	F.S.T.	91150-20	DUMONT #5 RUSTLESS NON-MAGNETIC
Forceps with Teeth	F.S.T.	11153-10	Germany stainless
Forceps	F.S.T.	18025-10	Germany stainless
Vannas spring scissors	F.S.T.	2-220	JJECK Stainless
Polyglactin suture	Ethicon	J488G	7-0 suture
hemostat	F.S.T.	12075-12	Germany stainless

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1 Alewife Center #200
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Yan Wang, Dale P. Brown Jr., Brant D. Watson, Jeffrey L. Goldberg

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CORRESPONDING AUTHOR:

Name:

Jeffrey L. Goldberg

Department:

Ophthalmology

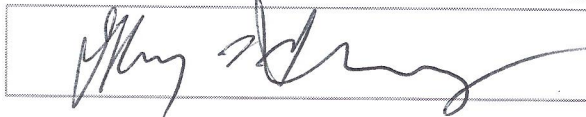
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Shiley Eye Center, UC San Diego

Article Title:

Rat Model of Photochemically-Induced Posterior Ischemic Optic Neuropathy

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This piece of the submission is being sent via mail.

March 19, 2015

Dear Editor,

We thank you for your careful assessment of our recent resubmission “Rat Model of Photochemically-Induced Posterior Ischemic Optic Neuropathy” (**JoVE52402**). We have now addressed all of the issues raised for our manuscript that were suggested by the editors. We feel that the manuscript is greatly improved through the suggestions from editors, and hope the editors find the revised manuscript appropriate for JoVE.

Responses to comments:

Editorial comments:

1) Video comments:

a) Frame size/proportions issues

i) The thin green lines seen on the left and top of frame are an artifact generated in the conversion to our web player's size and are not present in the original video. The artifacts were likely created because we had to scale the video up to fit our web player's size. Future submissions should be at least 512x384.

RESPONSE: Thank you, we have exported the video size at 720x540 and hopefully this will solve the problem. Note that we had downsized it because the submission required an initial size of <50MB. We will certainly comply with that on all future submissions. The recent proliferation of higher pixel density cameras will facilitate this for our future work.

b) Audio issues

i) The audio levels need to be normalized to between -6 and -12 dB below peaking. In particular, Yan Wang's narration is lower than most of the rest of the narration.

RESPONSE: Done as requested.

c) Text/formatting issues

i) 6:25 – They numbered this chapter "II. I" and the next chapter (at 8:42) "II. II". Either this chapter should just be "II" and the next one "III", or this should be "II. A" and the next "II. B"

RESPONSE: Done as requested.

2) Vet Review Report:

6:46-6:51 - Surgical scrub only once performed. Change in the appropriate sections in the text, line 137 and line 177 for both surgical sites, that “the skin needs to be scrubbed at least twice alternating skin disinfectant and alcohol”.

In line 246, it states "clean the surgical area...". It should rather state "surgically scrub" the incision site.

RESPONSE: All edited as requested, see lines 114-115, 128, 169-170, and 239.

3) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

RESPONSE: We have carefully given the manuscript an additional proofreading.

4) If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as "Re-print with permission from (reference#)" or "Modified from..." etc. And please send a copy of the re-print permission for JoVE's record keeping purposes.

RESPONSE: All figures/tables are original.

Reviewer #1:

Major points: None

Minor points:

Line 75, Should disease be diseases?

Line 83: of "an" animal model?

LINE 171: How much saline approximately?

RESPONSE: All corrected/edited as suggested. Please see lines 67, 75 and 164.

On lines 145 and 180 of the text says to make a "small incision". The length of the incision can be estimated when watching the video, but perhaps replace "small" with an exact length, e.g. "a ~1cm incision". The same applies to line 179 where the text says to make an incision "along the skin behind the eye". Since the location of the ischemic injury is specific (3-4mm behind the ONH) it would be help to define the exact surgery location.

RESPONSE: All details added as suggested. Please see lines 142-143, 174-175 and 172.

Erythrosin B is stated to be circulated for "a few seconds". Since irradiation is started right after erythrosin B injection, an accurate time estimate or a range would benefit to prevent any misinterpretation of a "few seconds". For instance, how long after injection is the dye still sensitive to laser irradiation? On line 209, the word "week" is used. Is this meant to be "weak"? In the video you mention anesthetizing the rat via intraperitoneal injection, however on line 116 it references an intraparietal injection.

RESPONSE: All details corrected as suggested. Please see lines 209, 203 and 107.

Overall this is a nice study. Congratulations!

RESPONSE: We thank the reviewer for the supportive comments.

Reviewer #2:

Manuscript Summary: The authors of this video clearly describe their technique for producing posterior ischemic optic neuropathy in the rat.

Major Concerns: None; the procedure is beautifully described and illustrated.

Minor Concerns: It would have been nice for the authors to introduce themselves at the beginning of the video so that the viewer knows who is speaking.

RESPONSE: We have included visual name labels rather than adding verbal introductions.

There is a fair amount of background noise when Dr. Yang and Mr. Brown are speaking. In addition, the volume must be changed (increased) when Dr. Yang is speaking and decreased when Drs. Watson and Goldberg are speaking. This is a particular issue when Dr. Yang is speaking because of her asian accent.

RESPONSE: Corrected as noted in the editorial suggestion above.

Additional Comments to Authors: One would think that you could purchase (or fashion) blunt- or fine-tipped forceps that could lock in place so that you did not have to hold them at a precise length (or as precise as possible) during radiation of the nerve.

RESPONSE: True, and we even tried to fashion some ourselves! But the space was too tight to manage a locking, opening forceps, so we hold them after all.

Reviewer #3:

The video and its accompanying manuscript are extremely clear and provide detailed step-by-step procedures to implement this model in virtually any laboratory with an interest in ischemic optic neuropathies. This model provides a valuable in vivo platform to study the pathophysiology of posterior ischemic optic neuropathy and potentially other CNS ischemic diseases.

RESPONSE: Thank you!