

Video Article

# Rat Model of Photochemically-Induced Posterior Ischemic Optic Neuropathy

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## Abstract

Posterior Ischemic optic neuropathy (PION) is a sight-devastating disease in clinical practice. However, its pathogenesis and natural history have remained poorly understood. Recently, we developed a reliable, reproducible animal model of PION and tested the treatment effect of some neurotrophic factors in this model<sup>1</sup>. 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.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/52402/>

## Introduction

In patients over 50 years old, ischemic optic neuropathy (ION) is the most prevalent type of acute optic neuropathy<sup>2</sup>. The condition can present as one of two subtypes according to the source of specific affected blood supply and clinical presentation: anterior (AION) or posterior (PION)<sup>3</sup>. While the pathogenesis and course of AION have been studied extensively<sup>4-7</sup>, 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 ischemia<sup>8-12</sup>. After injection into the vascular circulation, the photosensitive dye erythrosin B produces reactive singlet molecular oxygen upon activation by laser irradiation of target vessels. The singlet oxygen directly peroxidizes the vascular endothelium, stimulating platelet adherence/aggregation and leading to occlusive thrombus formation. Ischemic damage is spread to neighboring areas and further exacerbated by microvascular compression due to vasogenic edema. The overall goal of this protocol is to photochemically induce ischemia in 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 nerve<sup>1</sup>. 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. 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.
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.
3. Shave the surgical sites using a hair clipper and wipe the area three times with 10% providone-iodine detergent solution and 70% ethanol.
4. Drape the animal within 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

### 1. PION induction

#### 1. Femoral vein catheterization

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. 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% erythrosin B dye (1 µl/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 µl/min.
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.
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 upward 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.
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.
6. Obtain a needleless nylon suture and place it next to the femoral vein. Using the micro-surgical hook, elevate the vein and pass the 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.
7. Make a small cut in the vein near the distal ligation with Vannas spring scissors. Expand the hole as necessary with the fine-tip forceps. Some blood may leak through the cut. Clean the surgical area with cold, sterile BSS and sterile cotton swabs.
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.
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. Exposure of the optic nerve

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. 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 a surgeon in training. 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.
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.
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.
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.
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.

#### 3. Intravenous injection of Erythrosin B and laser irradiation

1. Wear orange-colored safety glasses at all times while operating the laser irradiation apparatus to shield yourself from the green 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. 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 100 µm 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.
3. Inject the solution of 2% erythrosin 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.
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 1 sec delay.
5. Irradiate the optic nerve for 90 sec with a peak power of 135mW and average power of 18mW produced by a beam chopper rotating at 250 Hz with a duty cycle of 15% (this minimizes thermal effects). Yellow fluorescence, visualized as bright orange

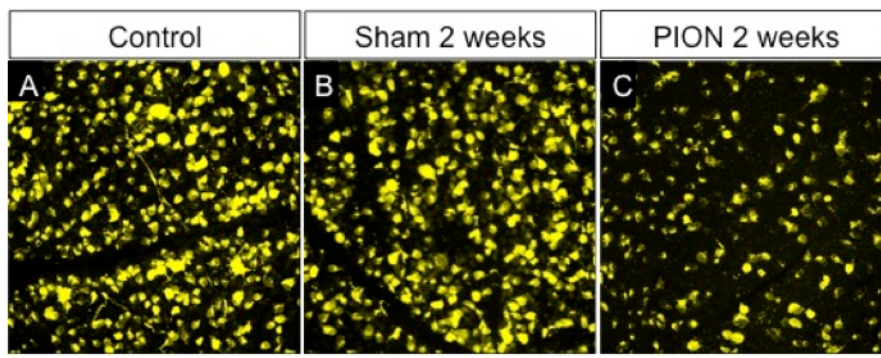
through the safety filter, will be emitted from the superior surface of the optic nerve and is sufficient to ensure that the beam irradiates the nerve symmetrically.

6. After the irradiation, the orange-colored safety filter will open automatically. Microhemorrhage can be observed in some cases.
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 catheter 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. **Retrograde Labeling of Retinal Ganglion Cells (RGCs).** NOTE: In order to evaluate RGC survival, retrograde labeling with fluorogold (FG) should be completed one week before PION. The method is described in detail in JoVE protocol 819<sup>13</sup>.
  1. In brief: anesthetize the animal with ketamine (60 mg/kg) and xylazine (8 mg/kg) and shave the head.
  2. Surgically scrub the incision site and make a midline incision across the head to expose the skull.
  3. Drill bilateral holes through the skull ( $\varnothing$  2x2 mm) 0.5 mm from both the sagittal and transverse sutures.
  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.
  5. Close the incision with sutures and care for the animal using standard postoperative care.
3. **Post-operative care and Euthanasia**
  1. After surgery, place animal in a separate clean cage on top of a recirculating heated water pad until the animal is recovered.
  2. Post-surgical analgesics (buprenorphine HCl, 0.01 mg/kg) should be administered twice per day for three consecutive days to minimize discomfort.
  3. Rat should be kept separately and observed until they are able to maintain sternal recumbency and regain sufficient consciousness.
  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.
  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 (**Figure 1A**), sham-treated (laser only/no erythrosin B, **Figure 1B**), and 2 weeks post-PION-treated (**Figure 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 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  $\mu$ m. [Please click here to view a larger version of this figure.](#)

## 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  $\leq 1$  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.

## Disclosures

The authors have nothing to disclose.

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