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Research article

# Real-time imaging of RGC death with a cell-impermeable nucleic acid dyeing compound after optic nerve crush in a murine model

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ABSTRACT

The retinal ganglion cells (RGCs) are the main source of therapeutic targets for neuroprotective glaucoma treatment, and evaluating RGCs is key for effective glaucoma care. Thus, we developed a minimally invasive, quick, real-time method to evaluate RGC death in mice. In this article we describe the details of our method, report new results obtained from C57BL/6J mice, and report that our method was usable in wild type (WT) and knockout (KO) mice lacking an RGC-death-suppressing gene. It used a non-invasive confocal scanning laser ophthalmoscope (cSLO) and a low molecular weight, photo-switching, cell-impermeant, fluorescent nucleic acid dyeing compound, SYTOX orange (SO). The RGCs were retrogradely labeled with Fluorogold (FG), the optic nerve was crushed (ONC), and SO was injected into the vitreous. After ten minutes, RGC death was visualized with cSLO *in vivo*. The retinas were then extracted and flat mounted for histological observation. SO-labeled RGCs were counted *in vivo* and FG-labeled RGCs were counted in retinal flat mounts. The time course of RGC death was examined in *Calpastatin* KO mice and wild type (WT) mice. Our *in vivo* imaging method revealed that SO-positive dead RGCs were mainly present from 4 to 6 days after ONC, and the peak of RGC death was after 5 days. Moreover, the number of SO-positive dead RGCs after 5 days differed significantly in the *Calpastatin* KO mice and the WT mice. Counting FG-labeled RGCs in isolated retinas confirmed these results. Thus, real-time imaging with SO was able to quickly quantify ONC-induced RGC death. This technique may aid research into RGC death and the development of new neuroprotective therapies for glaucoma.



## 1. Introduction

Glaucoma, a leading cause of blindness worldwide (Kwon et al., 2001), causes slow but progressive and irreversible visual field (VF) loss that is associated with retinal thinning and retinal ganglion cell (RGC) death (Weinreb and Khaw, 2004). The early diagnosis of glaucoma is challenging, even for experienced ophthalmologists, and progressive RGC death is difficult to evaluate with current methods. Thus, the current situation calls for new techniques to diagnose glaucoma and evaluate its progression.

VF loss and retinal thinning are secondary effects of glaucoma that are respectively measured with standard automated perimetry (SAP) and optical coherence tomography (OCT). However, VF loss and retinal thinning both progress very slowly, and patients may require monitoring for several years to confirm a diagnosis of glaucoma and to effectively evaluate its progression (Chauhan et al., 2008). The ultimate cause of VF loss is believed to be RGC death, and the RGCs are thus the main source of therapeutic targets for neuroprotective glaucoma treatments. Therefore, the evaluation of RGC death is a key part of glaucoma care. Conventionally, RGC death is measured by examining extracted retinas, a technique that can only be used in research based on animal models (Ryu et al., 2012; Allcutt et al., 1984; Shanab et al., 2012; McKernan et al., 2007). There is thus a need for a minimally invasive, clinically usable method to directly evaluate RGC death, as this would provide precise, detailed information that could improve the early diagnosis of glaucoma and aid the development of novel glaucoma

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treatments.

One promising method of evaluating RGC death is based on real-time imaging of the fundus after the intravitreal injection of a fluorescent dye. This technique takes advantage of the transparency of the eye, which enables the direct observation of the retina. Previously, dyes that target cells undergoing apoptosis have been used to visualize dying RGCs (Cordeiro et al., 2004; Barnett et al., 2009). However, the usability of these dyes is limited by their relatively high molecular weight and their lack of a photo-switching mechanism. Furthermore, they need many hours to detect dying RGCs (Cordeiro et al., 2004; Barnett et al., 2009). This slow action may be related to problems with the diffusivity of these dyes in the vitreous cavity, and a resulting low signal-to-noise ratio. As an alternative to these previously used dyes, cell-impermeant nucleic acid dyes have significant advantages: they are low molecular weight compounds that fluoresce highly only upon binding to double-stranded DNA and they are impermeant to healthy cell membranes, but can penetrate the unstable cell membranes of dead cells (Lossi et al., 2009; Yan et al., 2000). Cell-impermeant nucleic acid dyes have previously been used to discriminate dead cells, including apoptotic cells, from living cells using fluorescence-activated cell sorting and fluorescence microscopy (Lossi et al., 2009). Cell-impermeant nucleic acid dyes may therefore be able to quickly and easily visualize RGC death *in vivo*. Previously, we published a review article in Japanese (Nakazawa, 2015) in which we summarized a special presentation by one of the authors at the annual meeting of the Japanese Ophthalmology Society in 2014. In that presentation, we reported that the intravitreal injection of SYTOX orange (SO), a cell-impermeant nucleic acid dye, could clearly reveal dying RGCs in C57BL/6N mice after the mice had undergone optic nerve crush (ONC), that this method took only ten minutes, that the number of dead RGCs was significantly increased 4–6 days after ONC, and that RGC death peaked on day 5 after ONC. However, the Japanese review article provided only a brief summary of results from a small number of mice and did not include any details of our results or experimental method, such as the volume and concentration of the reagents, or device settings, such as the selection of the laser and the band-pass filter. Moreover, it has recently been reported that C57BL/6N mice have disadvantages for studies such as the present one, due to the presence of the rd8 mutation in the Crb1 gene (Mattapallil et al., 2012).

In this study, we report details on a quick, *in vivo* imaging method to evaluate RGC death in mice and present new data from both C57BL/6J wild type (WT) mice, which have no rd8 mutation, and knockout (KO) mice lacking an RGC-death-suppressing gene. Our method is based on real-time imaging with a low molecular weight, photo-switching, cell-impermeant nucleic acid dyeing compound and a non-invasive, confocal scanning laser ophthalmoscope (cSLO). This is thus the first study to investigate our method by using it to compare RGC viability in WT mice and KO mice.

## 2. Materials and methods

### 2.1. Animals

This study used 42 male, 11–13-week old C57BL/6J mice (Nippon CLEA, Shizuoka, Japan) and 6 male *Calpastatin* (*Cast*) KO mice that were backcrossed with C57BL/6J mice (Higuchi et al., 2005; Takano et al., 2005). All surgical procedures were performed with the animals under deep anesthesia, induced with the intramuscular administration of a mixture of ketamine (100 mg/kg) and xylazine (9 mg/kg). All animals were maintained and handled in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines from the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals. All experimental procedures described in this study were approved by the Ethics Committee for Animal Experiments at Tohoku University Graduate School of Medicine, and were performed according to the National Institutes of Health guidelines for the care and use of laboratory animals.

### 2.2. Surgical procedure

#### 2.2.1. Retrograde labeling of the RGCs

Seven days before ONC surgery, retrograde labeling was performed in all WT and *Cast* KO mice, as described previously (Nakazawa et al., 2006; Nakazawa et al., 2007). Briefly, a 2% saline solution of the neuronal retrograde tracer Fluorogold (FG) (Fluorochrome, LLC, Denver, CO) was prepared in a saline solution also containing 1% dimethylsulfoxide (DMSO). The animals were anesthetized and the skin over the cranium was incised to expose the scalp. A 1-mm diameter hole was made on each side of the skull with a drill, one located 4 mm posterior to the bregma and the other 1 mm lateral to the midline. In each hole, 1 μL of the 2% FG solution was slowly injected at a 2-mm depth from the surface of the skull with a Hamilton syringe equipped with a 32-gauge needle. The overlying skin was then sutured with 6-0 nylon, and antibiotic ointment was applied externally.

#### 2.2.2. Induction of axonal injury

Optic nerve crush (ONC) was used to induce axonal injury. Axonal injury has been reported to be a contributor to the progression of RGC death in such disorders as traumatic optic nerve injury and glaucoma (Ryu et al., 2012; Allcutt et al., 1984; Shanab et al., 2012). An incision was made in the superior conjunctiva to allow gentle outward retraction of the globe using fine forceps. The optic nerve was exposed and crushed approximately 1 mm posterior to the globe with forceps for 5 s and released. A fundus examination was used to confirm the appearance of normal blood circulation, and antibiotic ointment was applied. The operation was similar in the sham group, but after exposure, the optic nerve was not crushed. A single surgeon performed all ONC procedures.

### 2.3. Real-time imaging of RGC death

To identify dead retinal cells, the dead-cell stain SYTOX orange (SO) (Lossi et al., 2009; Yan et al., 2000) (1 μl, 2.5 μM dissolved in DPBS; Molecular Probes; Fig. 1A), was injected into both eyes (Fig. 1B). After 10 min, imaging was performed with a cSLO device (F10, Nidek, Gamagori, Japan) (Tanaka et al., 2010). The band-pass filter of the device was modified for use in animal experiments (Fig. 1C), which was also equipped with a photodetector. The animal was positioned in front of the cSLO after the pupil was dilated with one drop of 0.4% tropicamide (Mydrin M; Santen Pharmaceutical). A 532-nm diode laser was scanned across the retina to excite the injected SO and cause it to fluoresce. This fluorescence was optically focused onto a confocal aperture in order to exclude unwanted fluorescence signals from depth planes above or below the plane of interest. After passing back through a scanning mirror, the fluorescence was detected by a solid-state photodetector with a band-pass filter (short wavelength cutoff: 555 nm, no long wavelength cutoff). The signal was then digitized by a frame grabber and stored on the computer. For each eye, a retinal montage was constructed from the captured images. Baseline images were also captured before the intravitreal injection of SO.

### 2.4. Analysis of real-time imaging

The analysis of RGC death used a 640 × 480 pixel image from the