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Methods in eye research

A freely available semi-automated method for quantifying retinal ganglion cells in entire retinal flatmounts

E. Geeraerts [a], E. Dekeyster [a], D. Gaublomme [b, c], M. Salinas-Navarro [a], L. De Groef [a], L. Moons [a, *]

[a] Laboratory of Neural Circuit Development and Regeneration, Animal Physiology and Neurobiology Section, Department of Biology, KU Leuven, Leuven, Belgium
[b] Unit for Molecular Immunology and Inflammation, VIB Inflammation Research Centre, Ghent University, Ghent, Belgium
[c] Laboratory for Molecular Immunology and Inflammation, Department of Rheumatology, Ghent University Hospital, Ghent, Belgium

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ABSTRACT

Glaucomatous optic neuropathies are characterized by progressive loss of retinal ganglion cells (RGCs), the neurons that connect the eye to the brain. Quantification of these RGCs is a cornerstone in experimental optic neuropathy research and commonly performed via manually quantifying parts of the retina. However, this is a time-consuming process subject to inter- and intra-observer variability. Here we present a freely available ImageJ script to semi-automatically quantify RGCs in entire retinal flatmounts after immunostaining for the RGC-specific transcription factor Brn3a. The blob-like signal of Brn3a-immunopositive RGCs is enhanced via eigenvalues of the Hessian matrix and the resulting local maxima are counted as RGCs. After the user has outlined the retinal flatmount area, the total RGC number and retinal area are reported and an isodensity map, showing the RGC density distribution across the retina, is created. The semi-automated quantification shows a very strong correlation (Pearson's $r \geq 0.99$) with manual counts for both widefield and confocal images, thereby validating the data generated via the developed script. Moreover, application of this method in established glaucomatous optic neuropathy models such as N-methyl-D-aspartate-induced excitotoxicity, optic nerve crush and laser-induced ocular hypertension revealed RGC loss conform with literature. Compared to manual counting, the described automated quantification method is faster and shows user-independent consistency. Furthermore, as the script detects the RGC number in entire retinal flatmounts, the method allows detection of regional differences in RGC density. As such, it can help advance research investigating the degenerative mechanisms of glaucomatous optic neuropathies and the effectiveness of new neuroprotective treatments. Because the script is flexible and easy to optimize due to a low number of critical parameters, it can potentially be applied in combination with other tissues or alternative labeling protocols.

1. Introduction

Glaucomatous optic neuropathies (GONs) are a group of neurodegenerative diseases characterized by a progressive loss of retinal ganglion cells (RGCs) and put 64 million patients worldwide at risk for vision loss (Tham et al., 2014). An elevated intraocular pressure (IOP) is one of the most important known risk factors for GONs, together with age, myopathy, central corneal thickness and certain genetic predispositions (Coleman and Miglior, 2008; De Moraes et al., 2013; Wang and Wiggs, 2014). No real curative therapy is available to date and the current clinical treatment strategy mainly involves controlling IOP (Kolko, 2015). Because patients can continue to lose vision, even with a well-controlled eye pressure, there is an urgent need for new neuroprotective treatments directly stimulating RGC survival and functionality (De Moraes et al., 2013; Heijl et al., 2013; Tian et al., 2015).

Most of the current research investigating the pathological mechanisms underlying RGC death or evaluating novel

Abbreviations: GONs, Glaucomatous optic neuropathies; RGCs, Retinal ganglion cells; IOP, Intraocular pressure; Brn3a+, Brn3a-immunopositive; PBST, Phosphate buffered saline containing 0.5% Triton X-100; NMDA, N-methyl-D-aspartate.

* Corresponding author. Research Group Neural Circuit Development and Regeneration, Animal Physiology and Neurobiology Section, Department of Biology, KU Leuven, Naamsestraat 61, Box 2464, B-3000 Leuven, Belgium.
E-mail address: lieve.moons@bio.kuleuven.be (L. Moons).

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therapeutics is conducted in genetic or experimental mouse models of GONs (Almasieh et al., 2012; Bouhenni et al., 2012). All these models share their dependence on RGC quantification to follow the course of glaucomatous degeneration. The multiple methods that can be used to visualize RGCs in naive and experimentally manipulated rodent retinas were recently reviewed (Balendra et al., 2015; Nuschke et al., 2015). Briefly summarizing, several basic histological (e.g. H&E (Hedberg-Buenz et al., 2015) or Nissl staining (Liu et al., 2014)) or neuronal immunostainings (e.g. for NeuN (Buckingham et al., 2008) or β-tubulin (Mead et al., 2014b)) can be used to visualize RGCs. However, these methods also label displaced amacrine cells, estimated to make up around 50% of all cells present in the RGC layer of the rodent retina and thus hindering an exact count of RGC number (Mead et al., 2014b; Schlamp et al., 2013). Alternatively, taking advantage of the fact that RGCs are the only retinal cells extending projections to the brain, they can be labeled by injecting or applying retrograde tracers (e.g. Fluoro-gold/hydroxystilbamidin, DiI, Cholera toxin B, dextran tetramethylrhodamine) in/on the superior colliculus or optic nerve stump (Chiu et al., 2008; Nuschke et al., 2015; Salinas-Navarro et al., 2009b; Vidal-Sanz et al., 1988). These fluorescent molecules are taken up by the RGCs, labeling them retrogradely. Unfortunately, this method requires laborious and damaging surgeries and is hampered by the fact that retinal microglia take up the cellular debris after experimental injury and become labeled as well (Peinado-Ramon et al., 1996). Finally, RGCs can be visualized via immunostaining for specific RGC markers, such as Brn3a, RNA-binding protein with multiple splicing (RBPMS), and gamma-synuclein (Kwong et al., 2011; Nadal-Nicolas et al., 2012a; Surgucheva et al., 2008). The POU domain class 4 transcription factor Brn3a labels the nuclei of the vast majority of RGCs, up to 85–97% of Fluoro-gold marked RGCs in rodents (Galindo-Romero et al., 2011; Nadal-Nicolas et al., 2009, 2012b; Schlamp et al., 2013). Despite controversy on the disappearance of the marker before actual RGC death in the pathological retina, Brn3a has become a popular marker for studying RGC degeneration (Della Santina et al., 2013; Mead et al., 2014a; Nadal-Nicolas et al., 2009; Salinas-Navarro et al., 2009a; Zhang et al., 2015).

Although RGC counting can be done on retinal sections, retinal flatmounts offer distinct advantages for quantifying RGCs such as alleviating the need for sectioning and providing a view of the whole RGC layer at a glance. When manually counting RGCs in flatmounts, small image sections or “frames” are randomly selected from the central, mid-peripheral and peripheral retina. RGCs are then counted within these frames and the RGC density is averaged and often extrapolated for the entire retina as a percentage *versus* control (Feng et al., 2013; Pirhan et al., 2015; Ryu et al., 2012; Shen et al., 2015; Wilson et al., 2014). Although this is an easy method that works for all RGC visualization methods, it is very laborious, subject to inter- and intra-observer variation, and insensitive to sectorial differences in RGC density (Salinas-Navarro et al., 2009b). To overcome these issues, automated counting routines have been developed (Bizrah et al., 2014; Danias et al., 2002, 2003; Salinas-Navarro et al., 2009b). However, most of the available scripts use commercial – often expensive – software and those using free software have not been validated for Brn3a-immunopositive (Brn3a+) cell counting. The script presented in this manuscript is fast, works in a user-independent manner, can quantify the entire retinal flatmount, and is fully validated for Brn3a+ RGCs.

Within this study, it is first demonstrated that the developed script could quantify Brn3a+ RGCs on both widefield and confocal images of murine retinal flatmounts by validation via a correlation analysis of manual *versus* semi-automated RGC counts and an assessment of inter-observer variability. The validated script was then applied to evaluate RGC degeneration in three commonly used GON models in order to test its general applicability to score RGC survival in experimentally induced retinopathies. Overall, this new, freely available semi-automated script for RGC quantification is shown to be reliable and able to detect a wide range of RGC densities in several GONs.

2. Materials and supplies

2.1. Animals and surgery

This study was conducted in compliance with the European Communities Council Directive of 22 September 2010 (2010/63/EU) and the Belgian legislation (KB of 29 May 2013), and was approved by the KU Leuven institutional ethical committee. Adult (2–4 months) C57/Bl6J mice (JAX™ Mice Strain, stocknr. 000664, acquired via Charles River Laboratories) and CD-1 mice (Hsd:ICR, acquired from Envigo) were obtained from the university breeding colony, which is regularly supplemented with newly purchased mice. All animals were kept under a 12/12 light-dark cycle, with *ad libitum* access to food and water.

All surgical procedures were performed under general anesthesia, induced via intraperitoneal injection of 75 mg/kg body weight ketamine (Anesketin, Eurovet) and 1 mg/kg medetomidine (Domitor, Pfizer). Anesthesia was reversed with 1 mg/kg atipamezol (Antisedan, Pfizer). Antibiotic ointment (Tobramycin, Trobrex, Alcon) was applied to both eyes to prevent infection of the eye and corneal desiccation. Eyes from a separate, naive group of animals, as well as the untreated eye of experimental animals were used as controls.

N-methyl-D-aspartate (NMDA) injection was performed in C57/Bl6J mice as described (Nakazawa et al., 2007). Briefly, NMDA (3.5 mM, 5.0 mM and 6.5 mM in phosphate-buffered saline (PBS); Sigma-Aldrich) was injected intravitreally into the superior quadrant of the eye after application of topical anesthesia (oxybuprocaine 0.4%, Unicaïne, Thea). The injection was performed with a glass capillary with a 50–70 μm outer diameter, connected to a Hamilton syringe. To avoid damage to the lens, the capillary tip was inserted 1 mm behind the limbus under a 45° angle. A total volume of 2 μl was injected at a rate of 0.5 μl per second. Animals were sacrificed at 1 day post injury (dpi).

Optic nerve crush was performed in C57/Bl6J mice as described previously (De Groef et al., 2016; Dekeyster et al., 2015b). First, a small temporal incision was made in the conjunctiva and, by carefully pulling the conjunctiva, the eye was rotated nasally. Next, a curved forceps was used to expose the optic nerve. A mechanical crush was performed 1 mm behind the globe, for 5 s, using a self-closing forceps (Fine Science Tools) in order to provide constant and consistent pressure of 120 g/mm^2. Retinal perfusion was checked before and after the procedure via fundoscopy and animals with ischemia were excluded from the study. Animals were sacrificed at 4 and 7 dpi. Untreated eyes in the same animal were used as control.

Ocular hypertension by laser photocoagulation of the perilimbal and episcleral vessels was induced in albino CD-1 mice, the most commonly used strain for this model (Dekeyster et al., 2015a; Salinas-Navarro et al., 2009a). Briefly, the pupil was dilated by a droplet of 1% tropicamide (Colircusí tropicamida, Alcon Cusí), followed by photocoagulation of the vessels with a 532 nm diode laser (Quantel Medical). Approximately 70 laser spots were delivered, with an exposure time of 0.5 s and a power intensity of 0.3 W. Fundoscopy was performed before and after the procedure to ensure intact retinal perfusion. The intraocular pressure was measured before and up to 7 days after the procedure, using a rebound tonometer (Tono-Lab, Icare). Animals were sacrificed at 7 dpi.