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Methods in eye research

An open-source computational tool to automatically quantify immunolabeled retinal ganglion cells

Ana C. Dordea [a], Mark-Anthony Bray [b], Kaitlin Allen [a], David J. Logan [b], Fei Fei [c], Rajeev Malhotra [d], Meredith S. Gregory [c], Anne E. Carpenter [b], Emmanuel S. Buys [a, *]

[a] Anesthesia Center for Critical Care Research, Department of Anesthesia, Critical Care, and Pain Medicine, Massachusetts General Hospital Research Institute, Harvard Medical School, Boston, MA, USA
[b] Broad Institute of MIT and Harvard, Cambridge, MA, USA
[c] Department of Ophthalmology, Schepens Eye Research Institute, Harvard Medical School, Boston, MA, USA
[d] Division of Cardiology, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

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ABSTRACT

A fully automated and robust method was developed to quantify β-III-tubulin-stained retinal ganglion cells, combining computational recognition of individual cells by CellProfiler and a machine-learning tool to teach phenotypic classification of the retinal ganglion cells by CellProfiler Analyst. In animal models of glaucoma, quantification of immunolabeled retinal ganglion cells is currently performed manually and remains time-consuming. Using this automated method, quantifications of retinal ganglion cell images were accelerated tenfold: 1800 images were counted in 3 h using our automated method, while manual counting of the same images took 72 h. This new method was validated in an established murine model of microbead-induced optic neuropathy. The use of the publicly available software and the method's user-friendly design allows this technique to be easily implemented in any laboratory.

1. Introduction

Glaucoma is the leading cause of irreversible blindness worldwide, affecting an estimated 60 million people (Tham et al., 2014). While there are many forms of glaucoma, all are associated with an optic neuropathy characterized by the loss of retinal ganglion cells (RGCs) and their axons, resulting in optic nerve degeneration and irreversible vision loss. Animal models of glaucoma that simulate the optic neuropathy observed in human disease facilitate the elucidation of possible mechanisms of RGC loss and enable researchers to develop and evaluate neuroprotective therapies. The ability to specifically identify and accurately count RGCs is essential to assess the death or survival of RGCs in models of the disease.

Various techniques for visualizing and quantifying RGCs have been reported, including retrograde labeling and immunolabeling (Buckingham et al., 2008; Huihui et al., 2011). In a healthy, non-diseased retina, fluorogold retrograde labeling is a specific and accurate method to label and automatically quantify RGCs (Buckingham et al., 2008; Danias et al., 2003). However, this method is technically challenging, requiring intracranial surgery and, in a glaucomatous retina, retrograde labeling can result in the labeling of retinal microglia in addition to RGCs, due to the phagocytosis of degenerating RGCs (Peinado-Ramon et al., 1996; Thanos, 1991a,b). An alternative quantification method of RGC viability may be carried out with immunolabeling of RGCs in retinal flatmounts using an RGC-specific antibody directed against β-III-tubulin, which labels the RGC somata and nerve fiber extensions. The specificity of the β-III-tubulin antibody has been confirmed by colocalization of β-III-tubulin staining with fluorogold-labeled RGCs (Huihui et al., 2011).

While the immunolabeling method is technically simpler than fluorogold labeling, manual quantification of immunolabeled murine RGCs is onerously time-consuming. In a small-scale pre-clinical project with only 2 experimental groups, an investigator would be required to manually quantify RGCs in approximately 1000 images, necessitating at least 48 h of effort. In addition, manual counting can be inconsistent, with significant inter- and even intra-observer variability, due to the use of differing quantification techniques and/or inhomogeneous staining.

* Corresponding author.
E-mail address: ebuys@mgh.harvard.edu (E.S. Buys).

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2. Materials and supplies

2.1. Animals

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. Wild-type mice bred on a 129S6 background (129S6/SvEvTac, Taconic Farms Inc.) were used in this study. All animals were treated in accordance with the Institutional Animal Care and Use Committees (IACUC) of Massachusetts General Hospital (Subcommittee on Research Animal Care), and the Schepens Eye Research Institute.

2.2. Processing and imaging of retinas

Animals were sacrificed under CO_2. Eyes were enucleated and retinas dissected from the anterior segments at the conclusion of the study: day 32 post-injection. Resultant retinal cups were incised to create four quadrants of similar size and were fixed in 4% paraformaldehyde at 4 °C overnight. Retinas were then treated with 1% Triton-X-100 and 5% fetal BSA in PBS for 1 h, followed by a 2 h incubation with DAPI (1:500) and the primary antibody against an RGC marker, β-III-tubulin (anti-TUJ1+, Millipore, Billerica, MA, 1:500), and 1 h incubation with the Alexa Fluor® 594 Goat Anti-Mouse IgG secondary antibody (Life Technologies, Carlsbad, CA, 1:500) at room temperature. Retinal whole-mounts were then flattened on SuperFrost Plus slides (VWR, Batavia, IL), coverslipped with mounting medium for fluorescence (VectaShield®, Vector Laboratories, Burlingame, CA) and imaged under the Leica TSC SP5 confocal microscope at ×63 magnification. Imaging was performed on the mid-peripheral area of the retina (around 0.5 mm distal from the optic nerve head) divided into 4–5 distinct areas across all four quadrants. Each retina was imaged in 20–25 frames of 0.0696 mm^2.

2.3. Microbead injections

Mice were anesthetized by intraperitoneal injection of a mixture of ketamine (100 mg/kg; Ketaset; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (9 mg/kg; TranquiVed; Vedco, Inc., St. Joseph, MO) and eyes were dilated by topical application of proparacaine (0.5%; Bausch & Lomb, Tampa, FL). Elevation of IOP was induced unilaterally in adult 129S6 mice by injection of polystyrene microbeads (FluoSpheres; Invitrogen, Carlsbad, CA; 15 μm diameter) into the anterior chamber of the right eye of each animal under a surgical microscope. Microbeads were reformulated at a concentration of 5.0×10^6 beads/ml in phosphate-buffered saline (PBS). The right cornea was gently punctured near the center using a sharp 30-gauge needle (World Precision Instruments Inc., Sarasota, FL). An air bubble was injected via the micropipette connected with a Hamilton syringe, itself coupled to a syringe pump to avoid overflow of the AqH from the anterior chamber prior to injection of microbeads. A precise volume (2 μl) of microbeads was injected through the pre-formed hole into the anterior chamber using the micropipette. Mice were placed on a heating pad for recovery after the injection, and antibiotic Vetropolycin ointment (Dechra Veterinary Products, Overland Park, KS) was applied topically onto the injected eye to prevent infection.

2.4. IOP measurement

Mice were anesthetized with isoflurane inhalation (2%), which was delivered in 95% oxygen with a precision vaporizer. IOP measurement was initiated within 2 min after animal lost toe pinch and blink reflex. IOPs were acquired with a TonoLab rebound tonometer (iCare, Franconia, NH, USA). Five TonoLab readings were averaged to obtain a single IOP value per eye. IOP measurements were carried out at days 4, 10, 15, 18, 22 and 28 of the 32-day study.

2.5. Statistical analyses

All statistical analyses were performed using GraphPad Prism (version 6.0). Statistical tests included the Student's *t*-test, Pearson correlation, or multivariate linear regression. In order to compare correlation coefficients, the Fisher method of *r*-to-*z* transformation was employed, using the following equation:

$$z_n = \frac{1}{2}\ln\left(\frac{1+r}{1-r}\right)$$

Pairwise correlations with their respective samples sizes (n_1 and n_2) were compared with the following test statistic:

$$Z = \frac{(z_1 - z_2)}{\sqrt{\frac{1}{(n_1-3)+(n_2-3)}}}$$

Correlation dispersion was used to quantify differences between automated and manual counts for each retina. Correlation dispersion was determined using the following equation:

$$CD = \sqrt{[y(\text{manual}) - y(\text{automated})]^2}$$

where y represents the cell count in RGC/mm^2.

$P < 0.05$ was considered significant throughout the study. Data are presented as mean ± s.d.

3. Detailed methods

3.1. Generation of a rapid and robust automated quantification technique

To minimize variability and enable faster and more efficient quantification of immunolabeled RGCs, we sought to develop and validate a reproducible automated quantification method. The approach uses the freely-available softwares, CellProfiler and CellProfiler Analyst (CPA) (Carpenter et al., 2006; Kamentsky et al., 2011; Lamprecht et al., 2007), and can be used for high-throughput image analysis. The CellProfiler image analysis template was designed to recognize all DAPI-positive (nuclear marker) and β-III-tubulin-labeled cylindrical RGC somata (Box 1).

The workflow was as follows: the CellProfiler template was loaded and images from experimental set-ups were fetched into the "File list". Automated processing of each image was initiated ("Analyze Images"): the morphological features for each cell, including the nuclei and cellular shapes, as well as intensity-based and textural features from both the DAPI and β-III-tubulin channels were recognized and measured by CellProfiler; the complete set of these measurements for each cell is defined as the "cytoprofile"

Box 1
CellProfiler software.

The CellProfiler software is freely available to download at www.cellprofiler.org. The image analysis template used for the automated quantification presented in the manuscript, the latest software updates and the source code are available to download at http://www.cellprofiler.org/published_pipelines.shtml under the "B3-tubulin_RetinalGanglionCell_Assay.cpproj" nomenclature.