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Research article

Quantitative measurement of retinal ganglion cell populations via histology-based random forest classification

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ABSTRACT

The inner surface of the retina contains a complex mixture of neurons, glia, and vasculature, including retinal ganglion cells (RGCs), the final output neurons of the retina and primary neurons that are damaged in several blinding diseases. The goal of the current work was two-fold: to assess the feasibility of using computer-assisted detection of nuclei and random forest classification to automate the quantification of RGCs in hematoxylin/eosin (H&E)-stained retinal whole-mounts; and if possible, to use the approach to examine how nuclear size influences disease susceptibility among RGC populations. To achieve this, data from RetFM-J, a semi-automated ImageJ-based module that detects, counts, and collects quantitative data on nuclei of H&E-stained whole-mounted retinas, were used in conjunction with a manually curated set of images to train a random forest classifier. To test performance, computer-derived outputs were compared to previously published features of several well-characterized mouse models of ophthalmic disease and their controls: normal C57BL/6J mice; *Jun*-sufficient and *Jun*-deficient mice subjected to controlled optic nerve crush (CONC); and DBA/2J mice with naturally occurring glaucoma. The result of these efforts was development of RetFM-Class, a command-line-based tool that uses data output from RetFM-J to perform random forest classification of cell type. Comparative testing revealed that manual and automated classifications by RetFM-Class correlated well, with 83.2% classification accuracy for RGCs. Automated characterization of C57BL/6J retinas predicted 54,642 RGCs per normal retina, and identified a 48.3% *Jun*-dependent loss of cells at 35 days post CONC and a 71.2% loss of RGCs among 16-month-old DBA/2J mice with glaucoma. Output from automated analyses was used to compare nuclear area among large numbers of RGCs from DBA/2J mice ($n = 127{,}361$). In aged DBA/2J mice with glaucoma, RetFM-Class detected a decrease in median and mean nucleus size of cells classified into the *RGC* category, as did an independent confirmation study using manual measurements of nuclear area demarcated by BRN3A-immunoreactivity. In conclusion, we have demonstrated that histology-based random forest classification is feasible and can be utilized to study RGCs in a high-throughput fashion. Despite having some limitations, this approach demonstrated a significant association between the size of the RGC nucleus and the DBA/2J form of glaucoma.

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1. Introduction

Retinal ganglion cells (RGCs) are the final output neuron of the retina; their axons transmit action potentials to the brain that encode all of the light and feature detection signals that our retinas

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can sense. Thus, RGCs are essential to normal vision and over a century of work has been done to better understand their basic biology. Because they are post-mitotic, loss of RGCs in disease leads to irreversible vision loss. RGCs can be damaged by a wide range of insults (Levin and Gordon, 2002), several of which are very common, including glaucoma (Weinreb et al., 2014), Alzheimer's disease (Dehabadi et al., 2014), diabetes (van Dijk et al., 2012), and forms of traumatic brain injury (Mohan et al., 2013). The importance of RGCs in health and disease, coupled with their relative ease of accessibility, will continue to drive their study for many years to come.

Studies of RGCs, even in mouse models where extraneous variables can be minimized, face several challenges. It is currently estimated that there are likely up to 30 different sub-types of RGCs (Sanes and Masland, 2015). There are several advantages to being able to distinguish and categorize these sub-types, and great progress has recently been made in utilizing computer-assisted approaches for using dendritic field morphologies to accomplish this on individual neurons (Sumbul et al., 2014). Other challenges remain in the study of large populations of RGCs, two of which merit particular mention. First, in both health and disease, RGC density varies substantially. RGC density naturally varies ~4-fold by anatomic position (Drager and Olsen, 1981), and ~2-fold by genetic background (Williams et al., 1996). Thus, most measurements of cell density reflect a substantial amount of pre-existing variation. Moreover, even within genetically identical strains of mice, there are relatively high degrees of individual-to-individual variation in RGC density (Williams et al., 1996), further exacerbating this issue. A second major challenge is that, in mice, RGCs are distributed across a heterogeneous environment in which displaced amacrine cells are approximately equal in number to RGCs, and a variety of other cell types are also present (Drager and Olsen, 1981; Jeon et al., 1998; Schlamp et al., 2013).

Here, we set out to test the feasibility of a novel approach, based on computer-assisted detection of nuclei and classification by the random forest method, for studying RGC populations. The term “random forest” refers to an ensemble learning method that operates by building a multitude of decision trees, each built from random subsets of data describing the objects being classified, and ultimately assigning classes based upon the consensus of “votes” from all of the trees (Pedregosa et al., 2011; Touw et al., 2013); we have often found the random forest to be superior to other classification schemes in retinal image analysis (Niemeijer et al., 2011). In the inner retina, the nuclei of RGCs are morphologically distinct from those of other cells (Drager and Olsen, 1981); as such, a random forest-based approach was expected to be able to make use of multiple histologically discernable features for inclusion in tree building to distinguish the RGCs from other cell types. To explore this possibility, we made use of RetFM-J (Hedberg-Buenz et al., 2015), a semi-automated software module for the publicly available image processing program ImageJ (Abràmoff et al., 2004), that detects, counts, and collects quantitative data on 41 features of nuclei in the inner retina of H&E-stained whole-mounted retinas. Using these features, we developed a command-based software tool, RetFM-Class, which uses data from RetFM-J to perform random forest classification of cell type. Among initial tests of performance, we applied random forest classification to retinas of normal C57BL/6J mice, the most widely studied inbred strain of mice, as well as mice with controlled optic nerve crush (CONC) injury. We also studied retinas from DBA/2J mice with a naturally occurring form of glaucoma, and discovered that the mean nuclear size of surviving RGCs decreased in glaucoma. Modeling suggests that these glaucomatous changes occurred through a mechanism involving a subtle enlargement of all nuclei and preferential death of cells with the largest nuclei.

2. Materials and methods

2.1. Animal husbandry

Inbred C57BL/6J and DBA/2J mouse strains (The Jackson Laboratory, Bar Harbor, ME) were housed at the University of Iowa Research Animal Facility, where they were maintained on a 4% fat NIH 31 diet provided *ad libitum*, housed in cages containing dry bedding (Cellu-dri; Shepherd Specialty Papers, Kalamazoo, MI), and kept in a 21 °C environment with a 12-h light: 12-h dark cycle. Mice with conditional deletion of *Jun* in the retina, B6129;*Jun(Six3-Cre)* (abbreviated throughout as $Jun^{-/-}$ and *Jun*-deficient), were on a mixed C57BL/6J - 129 background and were generated by crossing mice with a floxed allele of *Jun* (Behrens et al., 2002) with mice expressing *Cre recombinase* under the control of the *Six3* early retinal promoter (Furuta et al., 2000). Control littermate mice for experiments involving *Jun* contained the *Six3-cre* transgene and at least 1 copy of the wild-type *Jun* allele (abbreviated throughout as $Jun^{+/?}$ and *Jun*-sufficient). This colony was housed at the University of Rochester, where they were maintained on a 4% fat NIH 31 diet provided *ad libitum*, housed in cages containing dry bedding (Cellu-dri; Shepherd Specialty Papers, Kalamazoo, MI), and kept in a 21 °C environment with a 12-h light: 12-h dark cycle. All experiments included mice of both genders. All mice were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. All experimental protocols were approved by the Animal Care and Use Committee of the University of Iowa or the University of Rochester.

2.2. Microscopy and imaging of retinal whole-mounts for measurement of total retinal area

Retinas were collected for whole-mount preparations as previously described (Hedberg-Buenz et al., 2015), with attention to keeping times for staining, mounting, and documentation as uniform as possible, and keeping experimental and control samples evenly dispersed between histologic batches. Whole-mounted retinas stained with H&E were scanned at 40× magnification with a digital whole-slide scanner (ScanScope CS, Aperio, Vista, CA). The total area of each retina was measured by outlining the sample, determining the stained area using the “Color Deconvolution v.9” algorithm, and then subtracting the area of the optic nerve. The areas of the central, mid-peripheral, and peripheral zones of eccentricity were calculated using circular regions of interest that divided each petal into approximately thirds. The length of each of the 4 petals (outer edge of optic nerve to edge of retina) was measured with the *ruler* tool and used to calculate average petal length. The central zone was defined as the area from the outer edge of the optic nerve to 1/3 of the average petal length, the mid-peripheral zone as the area from the edge of the central zone to 2/3 of the average petal length, and the peripheral zone as the area from the edge of the mid-peripheral zone to the edge of the tissue. The peripheral zone was calculated by subtracting the areas of central and mid-peripheral zones from the total retinal area.

2.3. Classification of nuclei with RetFM-Class

Stained whole-mount retinal preparations were imaged by light microscopy and images were subjected to analysis using RetFM-J as previously described (Hedberg-Buenz et al., 2015). Quantitative measurements of 41 features relating to morphology and stain appearance (Supplemental Table 1) were computed for each extracted nucleus, so that further processing and classification could be performed. Included features were not specific to H&E, but