Glaucoma

Active Lymphatic Drainage From the Eye Measured by Noninvasive Photoacoustic Imaging of Near-Infrared Nanoparticles

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Glaucoma, a leading cause of irreversible blindness in the world,1 is often associated with high IOP and impaired aqueous humor drainage.2 Treatments for glaucoma are aimed mainly at increasing aqueous humor drainage from the eye.3,4 Two aqueous outflow pathways have been identified as contributing to aqueous drainage and include the conventional pathway through trabecular meshwork into the venous system, and the unconventional pathway passing through the iris, ciliary body, choroid, and the sclera, also called uveoscleral pathway.5,6 An alternate route, called the uveolymphatic pathway has been proposed based on the finding of ciliary body lymphatics and the detection of intracamerally injected tracer in ciliary body lymphatic channels and cervical lymph nodes.7,8 As lymphatics in ciliary body were not detected by others,9,10 their existence remains controversial.11 The lymphatic system is essential for fluid homeostasis and immune competence and is primarily comprised of lymphatic vessels and lymph nodes.12 The lymphatic vessels reside in tissues of nearly every organ, where they drain excess fluid and large molecules into regional nodes.12,13 For a long time, it was assumed that there were no lymphatics in the eye. A renewed search using lymphatic endothelium markers combined with intracamerally injected tracer studies led to the discovery of lymphatic vessels in the ciliary body of the sheep and human eye.7 In vivo studies have been critical to further investigate lymphatic drainage. We previously evaluated lymphatic drain-
age from the eye in live sheep. Intracamerally injected radioactive albumin was measured in the lymph collected from cannulated lymphatic cervical vessels. These experiments in sheep, however, were challenged by the use of radioactive tracer and invasive techniques requiring deep anesthesia, mechanical ventilation, and prolonged immunosuppression, which may have influenced lymphatic drainage.

The mouse has been pursued as a model to study ocular lymphatic drainage due to similar human aqueous dynamics and pharmacology. Less invasive mouse studies using quantum dots and hyperspectral imaging of live mice, tracked lymphatic drainage from the anterior chamber to the ipsilateral submandibular node. While these experiments were more amenable than studies performed in sheep, limitations included the use of a tracer with potential toxicity and the inability to quantify drainage in vivo. Thus, the development of noninvasive quantitative techniques, with nonradioactive and nontoxic tracers would greatly aid our understanding of lymphatic drainage from the eye in health and disease.

Photoacoustic imaging is a recently developed imaging modality that combines the high contrast of optical imaging with the high spatial resolution of ultrasound. This technique allows imaging several centimeters in depth by way of acoustic waves generated in response to the absorption of pulsed laser light. Photoacoustic imaging has recently been used to map lymphatic drainage and spread of tumors to sentinel nodes using near-infrared contrast agents. Even so, quantitative data have rarely been reported. To our knowledge, there is no method available to measure lymph flow from the eye noninvasively. Here, for the first time, we detect and quantify lymphatic drainage from the mouse eye in the live state by photoacoustic tomography.

**Materials and Methods**

**Near-Infrared Tracer (Supplementary Material A)**

QC-1, a near-infrared quencher dye, (molecular weight [MW] 1.24 kDa; IRDye QC-1; LI-COR Biosciences, NE, USA) was used for photoacoustic tomography. The characterization of the QC-1 dye using in vitro phantom photoacoustic images showed a linear relationship between signal intensity and the QC-1 dye concentration from 0.078 to 120 μM (R² = 0.9273; Supplementary Material A). The highest concentration of 120 μM was comparable to the final concentration of the dye after injection into the mouse anterior chamber (aqueous humor volume in mice: 7 μL). The techniques used to characterize QC-1 are outlined in Supplementary Material B.

For in vivo experiments, to obtain a biocompatible tracer with optimal size to measure lymphatic drainage, QC-1 was conjugated to BSA (MW 66.4 kDa; Sigma-Aldrich Corp., St. Louis, MO, USA) following the manufacturer’s protocol (IRDye 800CW Protein Labeling Kit-High MW; LI-COR Biosciences). The QC-1 optical absorption spectrum measured at a concentration of 25 μM was used for photoacoustic spectral unmixing (Supplementary Material A). The dye/protein (D/P) ratio of QC-1/BSA was 1.88, and the final QC-1 concentration was 280 μM. The full details of measurement of the dye concentration and the dye/protein ratio are provided in Supplementary Material A. Tracer size importance was determined by QC-1 (MW = 1.24 kDa; 3 μL of 280 μM intracameral injection that showed significantly weaker signal intensity in the lymph node compared to that of QC-1 conjugated to BSA (MW 66.4 kDa; 3 μL of 280 μM; P = 0.0001; Supplementary Material B).

**In Vivo Experiments (Supplementary Material B)**

All animal experiments were approved by the Animal Care Committee at St. Michael’s Hospital and were conducted in accordance with the ARVO statement for the use of animals in ophthalmic and visual research. Fourteen CD-1 mice (7 male/7 female; Charles River Laboratories, QC, Canada) at 10 to 13 weeks of age were studied. Mice were kept on a 12-hour light/dark cycle with free access to food and water.

Under general anesthesia and using a surgical microscope, 3 μL of QC-1/BSA (280 μM; n = 1–4) or of QC-1 (280 μM; n = 4) was injected intracameral in the right eye through temporal cornea with a 33-G needle on a 10-μL NanoFill syringe (World Precision Instruments, Sarasota, FL, USA) following training by an ophthalmologist. Control experiments to detect the effect of possible leakage were performed by topical application of QC-1/BSA (3 μL; 280 μM). These control experiments showed negligible tracer leakage effect as seen by significantly larger slope and area under the curve (AUC) of tracer signal in the right cervical lymph node following intracameral injection (P = 0.0005; Supplementary Material B: Fig. S6). IOP was measured before and after injection of dye into the eye and showed no significant change. These were also performed under general anesthesia with 2% isoflurane (Pharmaceutical Partners of Canada, ON, Canada) and 100% oxygen (Supplementary Material B).

**In Vivo Photoacoustic Tomography (PAT)**

Full details of the animal preparation and in vivo PAT imaging (MSOT 128; iThera Medical GmbH, Munich, Germany) are provided in Supplementary Material B. The mice were awake between imaging sessions performed under general anesthesia as described above. Photoacoustic scanning of the head and neck regions was performed with 11 wavelengths: 680, 695, 735, 755, 775, 795, 825, 885, 925, 955, and 980 nm. Mice were imaged before, and 20 minutes, 2, 4, and 6 hours after injection. After each imaging session, mice were removed from the anesthesia unit and recovered in a clean cage placed over a heating pad (E-Z Anesthesia, Palmer, PA, USA) before being returned to the home cage with ad libitum food and water.

**Signal Visualization and Quantification**

Four of 14 mice were excluded from analysis due to failed injections, anesthetic complications, or mechanical problems during imaging. Data analysis was performed in the remaining 10 mice (4 male/6 female).

Photoacoustic images were reconstructed using the back-projection algorithm of the native software (ViewMSOT; iThera Medical GmbH). To visualize QC-1 signal compared with background, the reconstructed images were spectrally unmixed using the adaptive-filtered match algorithm and the images captured before tracer injection were used as the background.

To quantify the signal, the mean pixel intensities (MPIs) of the signal were extracted from each region (eyes and neck lymph nodes of both sides) using the linear regression-processed images. Photoacoustic images were spectrally unmixed with the linear regression algorithm of the native software, with the same 25 μM QC-1 spectrum as well as oxyhemoglobin and deoxyhemoglobin selected as input spectra. Details of the photoacoustic image analysis are provided in Supplementary Material B.

Mean pixel intensities were calculated for each of the 4 postinjection times, 20 minutes, 2, 4, and 6 hours after QC-1/BSA injection and were transformed with the natural logarithm (logged MPI = ln[MPI + 100]). The logged MPIs were plotted over time for each region of interest for each mouse. The
natural log of the MPI was plotted from 20 minutes to 6 hours postinjection for each mouse, and a linear regression was performed. The slopes of the natural log transform of the QC-1 MPIs between 20 minutes and 6 hours postinjection were also calculated from these measurements. Additionally, the AUC was calculated using the raw QC-1 MPIs from 20 minutes to 6 hours postinjection. The average slopes and the average AUCs were compared between the right and left corresponding regions of interest using paired two-tailed t-tests. Statistical analyses were carried out with SAS (version 9.4, 2002-2012; SAS Institute, Inc., Cary, NC, USA), and GraphPad Prism (version 7.0c; GraphPad Software, Inc., La Jolla, CA, USA).

Ex Vivo Validation (Supplementary Material C)

As QC-1 is a nonfluorescent dye, it is not detectable by fluorescence imaging and so near-infrared fluorescent dye, CF770 (MW 3.14 kDa; Biotium, Fremont, CA, USA) was used. It was conjugated to BSA, a protein fixable with paraformaldehyde.41 The dye/protein ratio of CF770/BSA was 2.45, and CF770 concentration was 1.4 mM as determined using the method described for QC-1 (Supplementary Material A) using the maximum absorption wavelength of CF770, at 770 nm. CF770/BSA was injected into the right eye of four mice as described for QC-1 dye injections. Electrophoresis studies to determine evidence of tracer degradation revealed tracer stability (Supplementary Material C).

Ex Vivo In Situ Fluorescence Optical Imaging

CF770/BSA (3 μL; 1.4 mM) was injected into the right eye intracamerally in 3 CD1 mice, and euthanization was performed at 4 hours. Dissected head and neck tissue was imaged with near-infrared fluorescence optical imaging (Spectralis; Heidelberg Engineering GmbH, Heidelberg, Germany) as described in Supplementary Material C. A detailed study of the lymph nodes was performed based on previous anatomic descriptions of lymph nodes in mice.42,43 To assess the potential role of conjunctival lymphatics in draining intracameral tracer, fluorescence optical imaging was also performed in two mice 4 hours after temporal subconjunctival injection of CF770/BSA (Supplementary Material C).

Histopathologic Validation

Three microliters of CF770/BSA were injected intracamerally in the right eye as described above and mice were killed 2 (n = 2) and 4 hours (n = 2) later. BSA without dye was injected intracamerally into the right eye of two mice as negative controls and they were killed 6 hours later. The details of the tissue processing and sectioning are provided in Supplementary Material C.

Immunofluorescence Staining

The right and left neck nodes and the left inguinal node42,43 were harvested and processed as described in Supplementary

FIGURE 1. Photoacoustic images of coronal sections of the mouse head at the level of the eyes (identification number [ID] #7). Prior to right eye intracameral injection of QC-1/BSA, no signal is observed (A). Twenty minutes and 2 hours after injection, the bright signal in green is seen (B, C), with reduced signal at 4 and 6 hours (D, E). Asterisk indicates the location of the right eye without signal. Arrows point to tracer signal in the right eye. The vertical green scale indicates QC-1 signal intensity (arbitrary unit). Scale bar: 3 mm. R, right; L, left; S, superior; I, inferior.

FIGURE 2. Photoacoustic signal over 6 hours following QC-1/BSA injected into right eyes (green lines) of mice (n = 10) compared with un.injected left eyes (red lines). (A) Signal intensities converted to natural log of MPI reveal a clear separation of green from red lines with decay over time observed in green. The legends on the right side correspond to mouse ID#'s. (B) Slopes of the natural log signal intensity of right and left eyes from (A) show steep signal decay of the right eye. Box-plots show the median (solid line), mean (plus symbol), 25th and 75th percentiles (solid line box), and the minimum and maximum slopes (whiskers). *P < 0.001.
BSA, a strong QC-1 signal was noted in the right eye (Fig. 1B), with no detectable signal in the left eye. Additionally, the signal in the right eye decreased over time (Figs. 1B-E). Quantitative analysis showed greater mean pixel intensity of the right eye compared with the left eye at all time points for each mouse (Fig. 2A). The mean of intensity slopes as a function of time for the right eye was significantly steeper than that of the left eye (−3.74E−3 ± 1.62E−3 vs. −6.25E−4 ± 1.60E−3, respectively; \( P < 0.001 \); Fig. 2B). As seen in Figure 3, the AUC for the right eye was significantly greater than that of the left eye (7.75E6 ± 2.16E6 vs. 1.11E6 ± 2.70E5, respectively; \( P < 0.0001 \)).

QC-1 signal was not detected in neck nodes prior to or 20 minutes after injection (Figs. 4A, 4B), and was first detected in the right neck node 2 hours after injection (Fig. 4C). The signal in the right node was increased at 4 compared with 2 hours postinjection (Fig. 4D) and remained at 6 hours (Fig. 4E). Signal from the right neck node was greater than the left after the 2-hour time point for each mouse (Fig. 5A). The intensity slopes as a function of time were significantly larger in right neck nodes compared with the left (3.71E−3 ± 1.86E−3 vs. 1.38E−3 ± 1.18E−3, respectively; \( P = 0.0051 \)) as shown in Figure 5B. Mean AUC was significantly greater for the right compared with left neck node measurements (4.06E6 ± 1.26E6 vs. 1.25E6 ± 5.8E5, respectively; \( P < 0.0001 \)) as seen in Figure 6.

### Ex Vivo In Situ Validation of Tracer Presence

#### Ex Vivo Optical Fluorescence Imaging

Near-infrared fluorescence imaging of the dissected head and neck specimens \( (n = 3) \) 4 hours after intracameral injection of CF770/BSA into the right eye showed tracer signal in the right submandibular, right accessory submandibular, and right deep cervical lymph nodes (Supplementary Material C).

#### Histopathologic Validation of Tracer Presence

Two hours after right eye injection, tracer CF770/BSA was detected within the right submandibular lymph node surrounded by a collagen IV-positive capsule (Fig. 7A). The presence of the tracer in the right submandibular node was observed in all four mice. No CF770/BSA tracer signal \( (n = 1, at 2\) hours postinjection; Figs. 7B, 7C) or trace signal \( (n = 1, n = 2\) at 2 and 4 hours postinjection, respectively) was observed in left submandibular and inguinal nodes. No near-infrared fluorescence signal was detected in nodes of control animals killed 6 hours after injection of unlabelled BSA into the right eye \( (n = 2) \). CF770/BSA extracted from the right submandibular node at 4 hours remained intact with molecular weight of 69 kDa, similar to freshly prepared tracer (Supplementary Material C).
This work is the first to quantitatively assess lymphatic drainage from the eye, in a noninvasive manner by photoacoustic tomography combined with a near-infrared tracer. In vivo imaging of multiple regions and at multiple time points before and after tracer injection in the same animal subjects was used to measure the increase in tracer signal in the right submandibular node compared with the left. Following intracameral injection, tracer in the right eye was decreased, with a simultaneous increase in the right submandibular node.

Methods such as magnetic resonance imaging and optical lymphangiography using contrast agents have been used to visualize the lymphatic network in other organ systems. Although fluorescence imaging techniques have been performed to study dermal lymph drainage dynamics, their application to assessing ocular fluid drainage into lymph nodes has been challenging.

Photoacoustic imaging has been used to detect sentinel nodes with or without the presence of cancer. Rare studies were able to quantify lymphatic drainage of intradermally injected nanoparticle contrast agents, such as gold nanocages and oligomer nanoparticles, to a peripheral node by photoacoustic imaging. Our approach to measuring ocular fluid drainage into the lymph nodes has multiple advantages. We used a small volume (3 μL) of tracer injected into anterior chamber under visual guidance without blood contamination. Earlier work in skin lymphatics employed photoacoustic technology with a single element ultrasonic transducer that required longer scanning time to acquire a three-dimensional image. In our study, the advanced photoacoustic tomography used a 128-element ultrasonic array, which allowed multispectral whole-body tomographic imaging with the capacity to separate the tracer signal from endogenous chromophores in the eye and lymph nodes. Furthermore, we used albumin, the most abundant natural plasma transport protein rather than oligomer nanoparticles and gold nanocages with unknown biocompatibility. Albumin as a tracer has been previously used to study lymphatic function and to measure aqueous humor flow and its drainage via conventional, and uveoscleral pathways, including the uveolymphatic pathway. Albumin was conjugated to a small near-infrared QC-1 dye (MW 1.24 kDa) that showed a linear relationship between the dye concentration and the signal intensity within the range of concentrations used in this study.

The choice of QC-1 dye with maximum absorption at 737 nm within the optical window from 600 and 1350 nm with minimal light attenuation may have improved the detection of the signal. Furthermore, the ex vivo in situ near-infrared fluorescence optical imaging and histopathologic studies confirmed the presence of intracameral injected C770/BSA in the right submandibular lymph nodes. Electrophoresis of the lymph nodes showed that most of the conjugated tracer harvested from the right submandibular lymph nodes 4 hours after injection remained intact with molecular weight of 69 kDa (Supplementary Material C). Furthermore, the free/
unconjugated QC-1 dye of small MW (1.24 kDa) injected intracameraly was not detected in the lymph node using PAT (Supplementary Material B). These findings are in keeping with a previous rat study in which 40 kDa but not the smaller 5 kDa tracers were detected in head and neck lymph nodes. Of note, after subcutaneous injection, small particles with MW less than 2 kDa are predominantly cleared by uptake into blood capillaries. Particles and molecules of increasing size have restricted uptake into capillaries and instead are absorbed into the lymph capillaries, especially particles greater than 16 kDa. Future studies are needed to explore the role of ocular blood capillaries in the clearance of intracameraly injected smaller tracers.

Our results confirm our previous work describing the presence of intracameraly injected quantum dots in the ipsilateral neck node in mice. The distribution of the trapped quantum dots in the subcapsular sinus close to one pole of the node matches closely the afferent flow into the node. It is reasonable that the smaller tracer CF770/BSA would make its way to the parenchyma. As both tracers have negative surface charge, the difference in the distribution of the tracers within nodes may be due to differences in size (approximately 14 vs. 19 nm) and in biocompatibility. Particles from the afferent lymphatics passively flow into the subcapsular sinus of the draining lymph node. At the sinus-cortex interface, soluble proteins smaller than 70 kDa in mass can enter a reticular conduit system that transports directly to the lymph node parenchyma. In addition to size considerations, biocompatibility differences may influence their mode of transport, extracellular versus intracellular, and their accumulation patterns. The surface chemistry of albumin and its negative charge may also be relevant to the biodistribution in eye tissues. In addition to surface chemistry, parameters such as dose, exposure, and the immune response may help to explore the kinetics and its distribution within the eye and orbit, lymphatic organs, other tissue components, and blood. A significant component of albumin injected into the eye exits the anterior chamber via the conventional pathway to blood, and the albumin filters from blood to various tissues including the neck nodes and other systemic nodes. It is well known that tracer in lymph enters the systemic circulation via the thoracic and right lymphatic ducts, and subsequently filters from the blood into the lymph nodes. The left cervical nodes were used as a control for filtration of the albumin from the blood. The inguinal nodes were also used as controls because the nodes draining the hindlimb have low plasma-protein ratio. The trace amount of signal in the inguinal and left submandibular lymph nodes suggests that tracer detected in the right submandibular lymph node was largely due to tracer entry from afferent lymphatics rather than the bloodstream.

By photoacoustic imaging, we were able to monitor multiple regions of interest, such as both eyes and the neck nodes simultaneously. The decreasing signal in the right eye showed an exponential decay as previously demonstrated by other techniques, such as fluorometry. In this study, we assumed that the rate of aqueous flow is constant during the 6-hour experiment, although it is known that some diurnal fluctuation exists. The calculation of flow is further simplified because the detected signal in the eye follows a simple first-order exponential decay as seen by other techniques, such as fluorometry.

Among limitations encountered, the spatial resolution of PAT used in this study was not sufficient to visualize different tissue components of the eye and orbit in the albino mouse strain. Imaging species with larger eyes, such as rats, may improve assessment of the various ocular tissue components. Photoacoustic imaging can also detect endogenous chromophores, such as melanin, and the imaging of pigmented rat strains may help to visualize the melanin-containing uvea from other tissue components and to measure the signal of the near-infrared tracer in the uveal tissue to monitor uveoscleral drainage.

The in vivo detection of photoacoustic tracer signal in ipsilateral submandibular and accessory submandibular lymph nodes was validated by ex vivo near-infrared fluorescence imaging (Supplementary Material C). However, in vivo PAT imaging could not detect the tracer in the deep cervical node that was revealed using ex vivo near-infrared fluorescence. This finding suggests depth-related signal attenuation. Furthermore, this imaging approach was not sensitive enough to detect tracer in the blood that may have entered via the conventional pathway. The estimated maximum blood concentration of QC-1 (<0.45 μM; 3 μL of 280 μM QC-1/BSA divided by the total blood volume) may be below the detection limit in our experimental conditions. Furthermore, the background in the blood rich in oxyhemoglobin and deoxyhemoglobin may be too high to detect a minimal amount of the QC-1 signal. In future studies, the choice of a contrast agent with a narrow peak in the vicinity of the isosbestic point of the absorption spectra of deoxy- and oxyhemoglobin, and increased photoacoustic efficiency may improve the detection of the tracer in the blood, and extend the application of this technique to also assess the conventional outflow pathway.

Although our study shows that tracer injected into the anterior chamber drains to ipsilateral cervical lymph nodes, information regarding the specific route is lacking. Further studies are needed to understand the afferent lymphatic pathways that lead into the neck nodes. It is also not clear whether in the mouse eye, lymphatics similar to those found in human and sheep eyes are involved. Tracer entry via conjunctival lymphatics in periocular tissue has been observed ex vivo in rats. Albumin injected into the eye in this study,
would be expected to also enter the conjunctiva where lymphatic vessels are plentiful, after passing through the uveoscleral route. It was interesting to note that while both intracameral and subconjunctival tracer injections showed signal in the submandibular, accessory submandibular, and deep cervical lymph nodes, the subconjunctival route used as a control, resulted in a more diffuse filling pattern of tracer within the submandibular, accessory submandibular, and deep cervical lymph nodes, and in addition, made its way to the superficial parotid lymph node (Supplementary Material C).

Postmortem studies 24 hours after injecting fluorescent tracer into rat eyes revealed tracer in superficial cervical, facial, and deep cervical nodes. Differences in findings may be due to species, physicochemical tracer characteristics, and time interval following injection.

Transcellular transport of the tracer by antigen presenting cells in the eye and periocular tissue, or by lymphatic endothelial cells may each contribute to the signal seen in the node. Further studies are needed to determine the extent of transcellular albumin movement via the lymphatic system.

While these considerations limit the ability to precisely quantify the rate of lymphatic flow compared with other outflow pathways, relative changes in the rate of flow were readily detected. This imaging platform might be used to detect changes in aqueous flow and lymphatic flow induced by pharmacologic intervention. Further studies are needed to determine the optimal parameters to screen for drugs that may decrease or enhance lymphatic drainage from the eye. With these improvements, this assay would enable investigators to better define the physiologic regulators of fluid drainage from the eye. It offers an approach to understanding the relationship of the lymphatic system to numerous eye diseases modeled in mouse and may help uncover drugs with novel mechanisms of action to treat glaucoma.

Our findings show an in vivo photoacoustic quantitative imaging assay capable of dynamically measuring fluid movement from inside the eye into draining neck lymph nodes. This work provides evidence that fluid transported out of the eye drains at least in part into the lymphatic system. Information regarding the exact nature of the fluid pathway from the anterior chamber to the lymph node is an area of great interest—whether uveal lymphatics, periocular transport, periorbital transport, play a role, remains to be determined. Further studies are needed to elucidate the connection between the eye and the lymphatic system.

In conclusion, lymphatic drainage of aqueous outflow was visualized and quantified in the live state and noninvasively using photoacoustic imaging for the first time. Quantification of signal intensities in the right submandibular nodes showed an increase, along with an exponential decrease of the tracer signal in the right eye over time. This in vivo assay shows promise in the study of fluid dynamics in the normal and diseased eye. It also offers a novel approach to stimulate the search for new drugs that target lymphatics and lower IOP to prevent blindness from glaucoma.

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References


