Lymphatic Drainage from the Mouse Eye and the Effect of Latanoprost

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Laboratory Medicine and Pathobiology
University of Toronto

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Abstract

Glaucoma is a leading cause of world blindness, often associated with elevated eye pressure. Current glaucoma treatments aim to lower eye pressure by improving aqueous humor outflow from the eye. Ocular lymphatics have been demonstrated to contribute to aqueous humor outflow in human and sheep. It is not known whether any glaucoma drugs target this lymphatic drainage. The mouse is a valuable model with similar aqueous humor dynamics and pharmacology as human. Using in vivo hyperspectral fluorescence imaging combined with intracameral quantum dot injection, we identified an ocular lymphatic drainage in mouse. Immunofluorescence and confocal microscopy revealed lymphatic channels in the ciliary body, sclera, and orbit that may be responsible for this lymphatic drainage. We showed that latanoprost, a prostaglandin F$_{2\alpha}$ analog widely used to treat glaucoma, increases this ocular lymphatic drainage. Our findings provide the framework for future development of novel glaucoma drugs that stimulate the ocular lymphatic drainage.
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A final word of remembrance is for my grandfather, M.Y. Woo, and grandmother, P.C. Woo, who inspired me to pursue this degree and who are both much missed.
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List of Abbreviations

AqH  Aqueous humor
BSA  Bovine serum albumin
CAI  Carbonic anhydrase inhibitors
DAPI  4',6-diamidino-2-phenylindole
H&E  Hematoxylin and eosin
IOP  Intraocular pressure
LEC  Lymphatic endothelial cell
LYVE  Lymphatic vessel hyaluronan receptor
NIR  Near infrared
NPE  Non-pigmented ciliary epithelium
PBS  Phosphate buffered saline
PE  Pigmented ciliary epithelium
PG  Prostaglandin
PGA  Prostaglandin analog
PGF$_{2\alpha}$  Prostaglandin F$_{2\alpha}$
Prox  Prospero-related homeodomain transcription factor
PVA-DABCO  Polyvinyl alcohol-1,4-diazabicyclo[2.2.2]octane
QD  Quantum dot
RGC  Retinal ganglion cell
SC  Schlemm's canal
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<td>TM</td>
<td>Trabecular meshwork</td>
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<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine-5-(and-6)-isothiocyanate</td>
</tr>
<tr>
<td>TSA</td>
<td>Tyramide signal amplification</td>
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<tr>
<td>UVS</td>
<td>Uveoscleral</td>
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<td>VEGFR</td>
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Contributions

Zhexue (Josh) Zhang assisted in intracameral injection and initial in vivo imaging experiments.

Zazeba Chowdhury BSc. assisted in sectioning frozen tissues.
Introduction

1.1 Overview

Glaucoma is a leading cause of world blindness. The major risk factor is elevated intraocular pressure (IOP) [1]. Current medical and surgical therapies attempt to lower IOP by improving drainage through aqueous outflow pathways [2-4]. Lymphatics are channels known to drain extracellular fluid, solutes and proteins in most organs. Lymphatics in the human [5] and sheep eye [6] have been shown to contribute to aqueous humor (AqH) drainage. However, the existence of ocular lymphatic drainage remains elusive in mouse. Since mouse is a valuable model with similar AqH dynamics [7] and pharmacology [8, 9] as human, developing methods to visualize ocular lymphatic flow in vivo in additional to investigating the effect of the most commonly prescribed glaucoma drug, latanoprost, on the ocular lymphatic drainage would be highly relevant to glaucoma studies.
1.2 Glaucoma and Its Relation to Aqueous Humor Dynamics

Glaucoma is estimated to affect 60 million people in the year of 2010 to 80 million people in 2020 [10]. A major risk factor for the development and progression of glaucoma is elevated IOP [11]. High IOP leads to death of retinal ganglion cells (RGCs), damage of optic nerve axons, and eventually blindness [11]. Lowering IOP with medicated eye drops helps to preserve vision or slow progression of vision loss in glaucoma patients [12-14]. This is achieved mainly through increasing AqH outflow from the eye [14].

In a healthy state, AqH outflow against resistance generates an average IOP around 16 mmHg in human eyes [15]. IOP is required to maintain the curvature of the cornea and the refractive properties of the eye. IOP is determined by three factors: the rate of AqH production by the ciliary body, the resistance to AqH outflow across the trabecular meshwork-Schlemm’s canal system, and the level of episcleral vessel pressure [14]. Healthy IOP reflects a balance between inflow and outflow of AqH.

1.3 Aqueous Humor - Composition, Formation and Dynamics

AqH is a transparent fluid contained in the anterior and posterior chambers of the eye. It is responsible for the supply of nutrients to and removal of metabolic wastes from the avascular tissue of the eye such as lactate, pyruvate, and carbon dioxide [16]. It is crucial for the maintenance of the shape, IOP, and optical properties of the eye [17]. Moreover, it transports ascorbic acid into the anterior segment of the eye to scavenge free radicals, and plays a role in immune responses during inflammation and infection [18, 19].

The major components of AqH are organic and inorganic ions, carbohydrates, glutathione, urea, amino acids and proteins, oxygen, carbon dioxide, and water. In a number of mammalian
species [20-22], except from the eyes of rhesus monkeys [23], AqH was demonstrated to be slightly hypertonic to plasma. The greatest differences in AqH relative to plasma, moreover, are the concentrations of protein (20 times less) and ascorbate (20 to 50 times higher) [24]. The protein content of AqH has both quantitative and qualitative differences compared to plasma. Most AqH proteins are intrinsic glycoproteins of the vitreous, which are secreted by non-pigmented ciliary epithelium (NPE) [25]. In addition, molecules that play a role in maintaining the extracellular matrix, such as collagenase, have been identified in AqH and may influence trabecular meshwork (TM) outflow resistance and, consequently, the IOP [26].

AqH is formed and secreted by the ciliary processes which are composed of a double layer of epithelium over a core of stroma and a rich supply of fenestrated capillaries [27]. The outer layer of pigmented ciliary epithelium (PE) faces the stroma while the inner NPE faces the posterior chamber of the eye. The NPE cells contain numerous mitochondria, microvilli, and Na\(^+\),K\(^+\)-ATPase and are the main production site of AqH [28].

Three mechanisms are involved in AqH formation: diffusion, ultrafiltration and active secretion [29]. Diffusion and ultrafiltration are responsible for the accumulation of plasma ultrafiltrate in the stroma, behind the tight junctions of the NPE layer, from which the posterior chamber AqH is derived [30, 31]. These two processes are passive without involvement of active cellular participation. During the diffusion step, lipid soluble substances are transported through the lipid portions of the membrane of the tissues between the capillaries and the posterior chamber [32]. Next, with ultrafiltration, water and water soluble substances flow across fenestrated ciliary capillary endothelium into the ciliary stroma [32].
Active secretion, which accounts for 80% to 90% of total AqH formation [33-37], is comprised of three sequential steps. First, NaCl ions are transported into the PE cells from the stroma via secondary active transport (paired Na⁺/H⁺ and Cl⁻/HCO₃⁻ antiports) [38, 39]. Next, gap junctions between NPE and PE cells create a functional syncytium allowing for passage of NaCl ions from PE to NPE [38, 39]. Finally, Na⁺ and Cl⁻ ions are released into the posterior chamber through Na⁺, K⁺-activated ATPase and Cl⁻ channels [38, 39], which are influenced by bicarbonate formation with the enzyme carbonic anhydrase through the conversion of CO₂ and H₂O to HCO₃⁻ and H⁺ [40]. AqH then flows into the anterior chamber through the pupil [41], around the anterior chamber, and into the three drainage pathways via the anterior chamber angle: conventional TM outflow (pressure-dependent) [42, 43], unconventional uveoscleral (UVS) outflow (pressure-independent) [32], and the ocular lymphatic drainage [5, 6].

1.3.1 Trabecular Meshwork Outflow (Fig. 1-1)

Most of the AqH in humans exits the eye through the TM outflow pathway. This pathway is comprised of the TM, juxtacanalicular connective tissue, the endothelial lining of Schlemm’s canal (SC), the collecting channels and the aqueous veins [44-46]. The TM is made up by the uveal and corneoscleral meshworks, which form connective tissue lamellae or beams that are covered by flat TM cells which rest on a basal lamina [44-46]. These beams attach to one another in several layers, forming a porous filter-like structure to allow the passage of AqH. The TM structures also provide the main resistance for AqH outflow to build up IOP [47]. When the IOP is high enough, AqH will then flow across the TM into SC. Once in the lumen of SC, it was demonstrated in the cynomolgus (*Macaca fascicularis*) monkey that AqH then flows into the endothelial tubules that penetrate mostly through the sclera and join the episcleral venous plexus, where AqH is finally drained into the venous system [48].
1.3.2 Uveoscleral Outflow (Fig. 1-1)

While a fraction of AqH leaves via the TM outflow pathway, part of the AqH leaves the eye through the UVS pathway. The UVS outflow pathway is a drainage route for AqH from the anterior chamber that successively includes extracellular spaces within the iris root, the ciliary muscle, the anterior choroid and suprachoroidal space, and the adjacent sclera [49-52]. Fluid then leaks into the surrounding periorcular tissues directly through the sclera or through the loose connective tissue surrounding penetrating blood vessels and nerves [53]. The amount of AqH that leaves the eye via the UVS outflow routes varies in different species - mouse was reported to be 82% [7], primates such as cynomolgus monkey 55% [54], sheep 22% [6], and cat 3% [55]. In human eyes, various results were reported [56, 57].

The UVS pathway is relatively pressure-independent as it does not depend on the IOP to the same extent as the TM outflow [58]. TM outflow increases linearly as IOP increases while the UVS flow remains relatively constant. Nevertheless, at extreme conditions, such as removing almost all of the resistance offered by the ciliary muscle through cyclodialysis, clear signs of a pressure-dependence can be observed as the UVS flow was increased from 3 to 54% in rabbits [59] and a four-fold increase was seen in monkeys [60].

The UVS flow can also be affected by the degree of contraction of the ciliary muscle [61]. It was observed that increasing the ciliary muscle tone with pilocarpine almost completely blocked the UVS flow [62]. In fact, when combining the treatment with pilocarpine and PGF$_{2\alpha}$, pilocarpine abolishes all the effects of PGF$_{2\alpha}$ suggesting PGF$_{2\alpha}$ increases flow through the ciliary muscle [63]. Later experiments in cynomolgus monkey demonstrated that PGF$_{2\alpha}$ and PGF$_{2\alpha}$ analog, latanoprost, increases UVS outflow [64, 65].
1.3.3 Ocular Lymphatic Drainage (Fig. 1-1)

1.3.3.1 Non-Ocular Lymphatics

While the majority of AqH leaves via the TM and UVS outflow pathway, a portion of the AqH leaves the eye through the lymphatic pathway in human and sheep [5, 6]. The lymphatic system is responsible for returning the interstitial protein-rich fluid, extravasated plasma proteins and cells back to bloodstream. It also plays a role in immune surveillance of the body [66] and absorbs lipids from the intestinal tract [67]. The lymphatic system is composed of a network of collecting vessels known as initial lymphatics. They are blind-ended structures that lack fenestrations, a continuous basal lamina, and pericytes [68]. Initial lymphatics are composed of a single-cell layer of overlapping lymphatic endothelial cells (LECs) [69] which can be identified by detecting molecules that are specifically expressed by LECs such as prospero-related homeodomain transcription factor (Prox1) [70], vascular endothelial growth factor receptor-3 (VEGFR-3) [71], lymphatic vessel hyaluronan receptor (LYVE)-1 [72], and the membrane glycoprotein podoplanin [73]. LECs exhibit large interendothelial pores [74, 75], which make them highly permeable to large macromolecules, and migrating cells in the interstitium. Once the interstitial fluid and proteins get into the initial lymphatics, they are collectively known as lymph [76]. Lymph will then travel along the initial lymphatics, which coalesce into larger collecting ducts. The collecting ducts are described as pre-nodal ducts if they lead to the lymph nodes and as post-nodal ducts if they are drained away from lymph nodes. Collecting ducts contain a continuous smooth muscular layer, an adventitial layer, a basement membrane, and valves to aid in unidirectional flow [77]. The smooth muscle layer enables each functional unit of the collecting ducts, lymphangion, to pump lymph [78]. Lymph from the left side of the head, the left arm, left part of the chest region, and lower portion of the body enters the thoracic duct,
which in turn empties into the blood venous system at the juncture of the left internal jugular vein and left subclavian vein [68]. Lymph from the right side of the neck and head, the right arm, and parts of the right thorax enters the right lymphatic duct, which empties into the blood venous system at the juncture of the right subclavian vein and internal jugular vein [68].

1.3.3.2 Ocular Lymphatics in Relation to Ocular Lymphatic Drainage

With the specific lymphatic endothelial cell markers, such as such as LYVE-1 [72], and podoplanin [73] detected with D2-40 antibody [79], immunofluorescence staining and cryo-immunogold electron microscopy were performed to reveal numerous fine lymphatic channels through the ciliary body stroma in human and sheep eyes [5].

Intracameral injected fluorescent tracers were localized in the lumen of lymphatic channels, confirmed by LYVE-1 staining, in the ciliary body of the sheep [5]. When radioactive tracers were injected into the anterior chamber of the sheep, they were drained to the head and neck lymph nodes [5]. Subsequently, a sheep model was developed to quantitatively assess lymphatic drainage along with TM and UVS outflows. Following intracameral injection of $^{125}$I-bovine serum albumin (BSA), lymph and blood samples were continuously collected. Lymphatic and TM drainage were quantitatively assessed by measuring $^{125}$I-BSA recovery. This quantitative sheep model enabled assessment of relative contributions of lymphatic drainage (1.64% ± 0.89%), TM (68.86% ± 9.27%) and UVS outflows (19.87% ± 5.59%) [6]. Altogether, these studies demonstrated the presence of lymphatic channels in the eye and the role of these lymphatic channels played in fluid drainage from the eye.
1.4 Glaucoma Treatment

The goal in glaucoma treatment is to prevent further loss of vision [80, 81]. The preservation of vision is mainly achieved by lowering IOP to a level that is safe for the RGCs. Topically applied ocular medications are usually the first step in controlling the IOP [14]. If eye drops do not lower IOP sufficiently, then surgical procedures such as argon laser trabeculoplasty, trabeculectomy, and shunts implantations will have to be performed with possible sight-threatening post-surgical complications [82].

There are five classes of glaucoma drugs currently used to manage glaucoma. They are either decreasing AqH production or increasing the aqueous outflow. They are categorized into cholinergic agonists, $\alpha_2$-adrenergic agonists, inhibitors of carbonic anhydrase, $\beta$-adrenergic antagonists, and prostaglandin analogs (PGAs).

Cholinergic agonist, also known as miotics, is the oldest effective pharmacological treatment of glaucoma. The most commonly prescribed cholinergic compound is pilocarpine, which lower IOP by stimulating the M$_3$-type muscarinic receptors [83] of the ciliary muscle that widens the anterior chamber angle, resulting in increased conventional TM outflow [84]. Pilocarpine typically reduces IOP by about 20 - 30% in human [85]. While pilocarpine is an effective IOP-lowering agent, it is not commonly prescribed due to its side effects such as decreased visual acuity due to pupillary constriction [86] and a 3 or 4 times daily dosage requirement [84, 87, 88].

Adrenergic agonists in general decrease AqH production and increase UVS outflow [89, 90]. One of the $\alpha_2$-adrenergic agonists, apraclonidine hydrochloride (IOPIDINE® Ophthalmic Solution), decreases AqH production as well as episcleral venous pressure and improves UVS
outflow [84, 89, 90]. Apraclonidine affects vascular tone and might cause vasoconstriction [84]. Apraclonidine, however, is rarely used for long-term therapy because of its high rate of allergic blepharoconjunctivitis [91].

Carbonic anhydrase inhibitors (CAIs) decrease AqH production via enzymatic inhibition [92]. In the CE, carbonic anhydrase isoenzyme II catalyses the conversion of CO₂ and H₂O to HCO₃⁻ and H⁺, a process important for the production of AqH described previously. By inhibiting this conversion, AqH formation is decreased. Dorzolamide was approved as the first topical CAI for glaucoma therapy. At a dosage of three times daily, 2.0% ophthalmic solution lowers IOP by 18-22% [93]. Nevertheless, local side effects from topical CAIs include stinging, burning and itching still exist [93].

Beta-receptor antagonists reduce IOP by decreasing fluid formation through inhibition of cyclic adenosine monophosphate production in CE in the ciliary body [94]. Most topical ophthalmic β-adrenoceptor antagonists used today include timolol, levobunolol, metipranolol and carteolol. While β-adrenoceptor antagonists are very effective IOP-reducing agents, their popularity has declined due to their systemic side effects, such as exercise induced tachycardia, a reduction of cardiac output, congestive heart failure, hypotension, and heart block [95, 96]. In addition, depression, amnesia, and fatigue are the most common central nervous system adverse effects [97].

Prostaglandin analogs are a relatively new class of ocular hypotensive agents and were introduced into market in 1996. PGAs are currently the first-line treatment of glaucoma [98]. PGAs are all multicarbon-chain, lipophylic molecules, derived from arachidonic acid and sharing similar structural features to PGF₂α (C₂₄H₄₅NO₈). The four different PGAs approved for clinical
use - latanoprost, unoprostone isopropyl, travoprost, and bimatoprost, are typically the most potent class of IOP-reducing drugs available (Figure 1-2). They offer excellent control of diurnal IOP fluctuation [99, 100]. Except for minor ocular effects such as iris pigmentation changes, eyelash hypertrichosis, and ocular inflammation, they have few systemic side effects [101-103].

Amongst the PGAs, latanoprost is the only PGA to have received a formal first-line usage approval from the FDA. Latanoprost (17-phenyl-13,14-dihydrotrinor PGF$_{2\alpha}$) is an analog of the pro-drug PGF$_{2\alpha}$ isopropyl ester. The pro-drug penetrates the cornea and becomes biologically active after being hydrolyzed by corneal esterase [104]. Just like other PGAs, latanoprost increases fluid drainage from the eye in glaucoma patients [9] and this is ascribed to its action on the UVS pathway [105, 106] by loosening the intercellular spaces and induces remodeling of extracellular matrix adjacent within the ciliary body [104]. Latanoprost has been compared with timolol [101-103] and was found to be significantly more effective at lowering IOP with a lower dosage. Mean IOP reduction was 6.7± 3.4 mm Hg for latanoprost compared with 4.9 ± 2.9mm Hg for timolol after 6 months of treatment [101]. Moreover, latanoprost has been shown to be significantly more effective at reducing IOP than other classes of glaucoma medication such as dorzolamide [107] and brimonidine [108]. Most importantly, latanoprost has no deleterious effect on blood pressure, pulse, and pulmonary function in patients [109].

1.5 Prostaglandins and Non-Ocular Lymphatics

Prostaglandins (PGs) (PGA$_2$, PGB$_2$, PGF$_{2\alpha}$) [110], along with various compounds, such as serotonin [111], and thromboxane A$_2$ [112], are known to stimulate the lymphatic smooth muscle cells to increase the contractility; thereby, increasing the circulation of lymph. Of the PGs that were shown to excite lymphatic smooth muscle cells, PGF$_{2\alpha}$ has the greatest effects on
bovine mesenteric lymphatic smooth muscle cells [110], suggesting a significant effect of PGF$_{2\alpha}$ in facilitating lymph flow. As described previously, PGF$_{2\alpha}$ analogs such as latanoprost are the most potent, and most commonly prescribed worldwide to treat glaucoma. It is not clear whether PGs act on the lymphatic outflow pathway of the eye. Developing a model to understand the effect of latanoprost on the lymphatic drainage is critical in gaining insights into, and developing novel treatment strategies for glaucoma.

1.6 Background on Using a Mouse Model

The mouse model is the focus of the experiments laid out in this thesis. Although nonhuman primates most closely approximate human anatomy and physiology, experimentation and handling of nonhuman primates can be challenging and is expensive [113]. Common alternative models such as cats, dogs and rabbits are easier to handle and data for AqH dynamics are relatively easy to gather [114-116]. However, in those models, only a small proportion of total outflow exists via the UVS routes, with less than 10% of total outflow in cats [55] and rabbits [59] and around 15% in dogs [117]. Since ocular lymphatic channels and drainage have been demonstrated in the ciliary body in human and sheep [5, 6], it is possible that the ocular lymphatic drainage is closely related to the UVS outflow. With only a small proportion of total outflow via the UVS, cats, rabbits, and dogs will be expected to have even less proportion of total outflow via the ocular lymphatic drainage. Hence, these species may not be as helpful in understanding the ocular lymphatic drainage as mice, whose total outflow exits via the UVS was reported to be 82% [7].

The mouse model also offers several advantages for investigating ocular diseases because of the availability of transgenic mice, their similar genetic background and relatively quick
breeding [118]. The low cost and ease of handling of this species allows for large-scale experiments to be performed. With the development of techniques that permit reproducible measurement of aqueous flow, episcleral venous pressure, and total outflow facility in the mouse [7, 119] as well as reliable measurement of IOP [120-125], this species becomes an ideal species for the study of AqH dynamics. The mouse has a normal IOP range averaging approximately 10 to 20 mmHg, which is similar to that of healthy human eyes at 16 ± 3 mmHg [126]. Compared to the common models such as cows and rabbits, mice have better developed TM and SC in the eye [127]. In fact, mouse and human eyes have similar anatomic structure, outflow pathways, and response to IOP-lowering drugs [8, 128]. It has been demonstrated that topical application of PGF$_{2\alpha}$, which lowers IOP and increases UVS outflow in monkey and human eyes [9, 64], also lowers IOP in mouse eyes [8]. Finally, advances in in vivo imaging modules and nanotechnology have enabled visualization of the lymphatic drainage in small animals, including mice.

1.7 Justification of In Vivo Imaging for Study of Lymphatics

1.7.1 Conventional Lymphatic Drainage Imaging

Conventional lymphatic imaging involves the use of radionuclide scintigraphy and dyes. These methods can effectively image lymphatic vessels and delineate lymphatic drainage. Lymphoscintigraphy is a special type of radionuclide imaging which requires injection of gamma-emitting radionuclides labeled macromolecules into a local region of tissue to evaluate lymph drainage function [129-131]. For imaging with dyes, such as isosulfan blue, patent blue V, Evans blue or fluorescent dyes, dye molecules are typically injected intradermally or subcutaneously into the interstitial tissue of animals or human beings. A visual signal of the draining lymphatic vessels and lymph nodes can then be obtained from indirect micro-
lymphangiographies of superficial lymphatic vessels [132]. Cutaneous lymphatic vessels and lymphatic drainage from the skin can also be macroscopically visualized with this method [132-134]. However, there are various drawbacks for these methods. Lymphoscintigraphy has radiation exposure while both techniques have relatively low resolution. Moreover, the intrinsic fluorescence from dyes has very little penetration through tissue. Hence, invasive procedures such as incisions to expose organs or tissues of interests must be performed to visualize lymphatic drainage.

1.7.1.1 Cannulation of Lymphatics to Detect Injected Radioactive Tracers

In human, radioactive lymphography through direct cannulation of lymphatics to visualize lymphatic drainage is now abandoned due to the difficult and potentially life-threatening procedures [135]. In animals such as sheep [6], rabbits and cats [136], however, lymphatic drainage was studied by cannulation of lymphatic ducts. Following radioactive tracers injection into site of interest, continual collection of lymph through the cannula can provide quantitative data for understanding the role of lymphatic system in aqueous or cerebrospinal drainage. However, cannulation of lymphatics is difficult to perform in small animals such as mouse due to the small size of lymphatic vessels and the invasive nature of the procedure. Therefore, further non-invasive techniques are required to study lymphatic drainage in small animals.

1.7.2 Non-invasive In Vivo Imaging of Lymphatic System with Organic Fluorophores

With the advances of optical imaging, lymphatic system can now be studied non-invasively using organic fluorophores. In vivo optical imaging allows for imaging at molecular level, mapping of lymphatic drainage, and visualization of multiple nodes at a relatively high
resolution without radiation exposure [135]. Fluorophore such as indocyanine green has been approved in humans for assessing cardiac and hepatic function, and ophthalmic angiography [137-141]. Studies have also shown the potential of this dye to image lymphatics in animal models and in humans [142]. The principal downsides of optical imaging agents remain low depth penetration [143] and having emission spectra overlapping that of autofluorescence generated by tissue and melanin [144]. Probes that emit in the near infrared (NIR), approximately 600–800 nm, help to maximize the target to background ratio and have improved depth penetration [145] as cells, tissues, and biological fluids autofluoresce minimally when stimulated in the NIR [146]. NIR organic fluorophores such as Alexa Fluor 705, IRDye780, Cy7, and Cy5.5 thus can be used to detect lymphatic drainage. These organic fluorophores can also be conjugated with antibodies, proteins, and peptides to effectively enter and remain in the lymphatic system [143, 147]. However, optical resolution with organic fluorophores is hampered by narrow excitation spectra, broad emission spectra, and susceptibility to photobleaching [148]. Hence, the design of high-emission fluorophores is necessary for both animal research and clinical imaging applications [147]. The recent advance of nanotechnology has led to the development of quantum dots (QDs) that circumvent these drawbacks.

1.7.3 Non-invasive In Vivo Imaging of Small Animals with QDs

The unique physical characteristics of QDs such as broad excitation spectra, narrow size-tunable emission spectra and a high photobleaching threshold make them suitable for non-invasive in vivo imaging. Optical properties of QDs such as CdSe-ZnS core-shell nanoparticles are advantageous for long-term single or simultaneous multiple color imaging of live cells [149] and animals [150, 151]. QDs can be detected by hyperspectral imaging due to an intrinsic brightness greater than regular organic fluorescent dye [150, 152]. QD655 with a hydrodynamic
size of 19 nm provides optimal uptake and retention inside lymphatic vessels [151] and lymph nodes [153]. The emission spectrum of 655 nm penetrates a depth of up to 2 centimeters [151] and can be distinguished from autofluorescence mainly produced by melanin, which has an emission range of 360–560 nm [154], in pigmented animals [144].

1.8 Rationale and Hypothesis

1.8.1 Rationale

Lymphatic channels have previously been reported in sheep and human eyes [5]. The contribution of lymphatic pathway to AqH drainage from the sheep eyes has been quantified [6]. However, the existence of ocular lymphatic drainage and lymphatic channels are relatively unexplored in the mouse models. Since the ocular anatomy and aqueous dynamics between human and mouse are similar [7], in addition to the advantages of mouse models in glaucoma research [8, 155], experiments were undertaken in the first part of this thesis to examine the existence of a lymphatic drainage and lymphatic channels in the mouse eye.

Among the anti-glaucoma drugs, PGF$_{2\alpha}$ analogs are the most potent, and the most commonly prescribed worldwide. Latanoprost, an PGF$_{2\alpha}$ analog, increases fluid drainage from the eye in glaucoma patients [9]. Increased fluid drainage from the eye is ascribed to the action of latanoprost on the UVS pathway [106]. Although PGAs are well established to stimulate the lymphatic drainage in non-ocular tissues [156-158], it is not clear whether PGs act on the lymphatic outflow pathway [5]. Based on the demonstration of ocular lymphatic drainage from the mouse model [159], we aimed to determine the effect of latanoprost on the lymphatic drainage.
1.8.2 Hypotheses

**Hypothesis 1**

The first hypothesis is that lymphatic drainage from the mouse eye exists. The hypothesis will be tested through intracameral injection of QDs followed by in vivo hyperspectral imaging over a period of imaging time points to visualize the trajectory of the tracers.

**Hypothesis 2**

The second hypothesis is that lymphatic channels exist in the mouse eye and surrounding orbital tissue. This will be determined by performing immunofluorescence staining frozen eye and orbit sections for lymphatic channels and blood vessels with anti-podoplanin and anti-collagen IV antibodies, respectively. Stained sections will then be examined through confocal and bright field microscopy.

**Hypothesis 3**

The third hypothesis is that a PGF$_{2\alpha}$ analog, latanoprost, stimulates lymphatic drainage from the mouse eye. The in vivo lymphatic drainage rate will be compared through topical application of latanoprost or artificial tear as control, followed by intracameral injection of QDs and in vivo hyperspectral imaging. QD signal intensity will also be quantified in tissue sections using post-mortem hyperspectral imaging.
Figure 1-1. Aqueous Humor Outflow Pathways. AqH is produced by the ciliary body and it flows from the posterior chamber through the pupil into the anterior chamber. From there it flows out via three pathways at the anterior chamber angle: conventional outflow (red arrow) via the TM and SC; the unconventional UVS route (blue arrow) via the ciliary muscle and sclera; and the ocular lymphatic drainage pathway (green arrow) via the lymphatic channels in the ciliary body. (Adapted from reference [160])
Figure 1-2. Chemical structures of PGF$_{2\alpha}$ and commercially available PGAs.
Material and Methods

2.1 Subjects
The experiments reported in this thesis were designed and performed in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research with the approval by the institutional Animal Care Committee. The experiments utilized randomly bred male pigmented mice (129SVE from Taconic, Hudson, NY) with weight ranging from 19g to 28g. Housed under constant 12 light/dark cycles, they were provided with standard food and water ad libitum including the day before experiments. In the study of identification of ocular lymphatic drainage and channels in mouse, 27 mice, inclusive of the mice utilized in control experiments, were used. In the study of the effects of latanoprost on the ocular lymphatic drainage, 10 mice were used to determine the effects of latanoprost on IOP, 14 mice were used in the latanoprost-treated group and 14 were used as control group for in vivo imaging of ocular lymphatic drainage.
2.2 Quantum Dots
The nanotracers used in the experiments reported in this thesis are QDs (QD655, Invitrogen, Eugene, OR, USA) with core/shell CdSe/ZnS, are ellipsoid (6 nm x 12 nm), with maximal emission at 655nm. QDs were coated with carboxylic acids and negatively charged (QD-COOH, pH 9.0). Three μL of 8 μM solution in a 50 mM borate buffer was used for intracameral injection.

2.3 Topical Application of Substances Prior to QD Tracer Injection
In the study of mouse ocular lymphatic drainage, three drops of artificial tears (Refresh tears, Allergan Inc., ON, Canada) were topically administrated 17 hours before and 1 hour to 24 mice before the experiment took place to ensure the eyes stay moisturized. In the study of the effect of latanoprost, 28 mice were split into two groups. In one group, 3 drops of latanoprost 0.005% (Xalatan, Pfizer, QC, Canada) (n=14) were instilled to the eyes 17 hours and 1 hour prior to the tracer injection. The 14 mice instilled with artificial tears described in the mouse ocular lymphatic drainage experiments were used as the control group.

2.4 Intracameral Injection of QD Tracer
Intracameral tracer injections were performed under general anaesthesia using either inhalation of 3% or 1.5% isoflurane (Abbott Labs Inc., Saint-Laurent, QC, Canada), in 70% N₂O and balance of O₂. The hair on the head, neck, abdomen regions and forelimbs were gently shaved with a hair trimmer (Chromini Type 1591, Wahl, Sterling, IL, USA) [144]. Under an operating microscope (Leica M655, Wetzlar, Germany), 3 μL of QD655 was injected into the anterior chamber of the left eye using a 33 gauge needle (Hamilton Company, Reno, NV, USA) and either a 100 μL or 25 μL Hamilton syringe (Hamilton Company, Reno, NV, USA). QD toxicity suggested by in vitro studies [161, 162] was not specifically studied, though acute signs of ocular toxicity were not observed.
2.5 IOP Measurement

Under general anaesthesia as described above, IOP was measured non-invasively using a handheld rebound tonometer (TonoLab, Type TV02, Icare Finland Oy, Helsinki, Finland) [120-125] before the tracer injection and at the end of experiments to monitor any IOP changes for all in vivo imaging experiments in this thesis. Generally, the tonometer probe was applied perpendicularly to the centre of the cornea under operating microscope. Multiple readings were taken until six steady consecutive measurements could be obtained, and the mean value of these six measurements was calculated. No statistically significant difference in IOP was observed between measurements performed before tracer injection and 6 hours after tracer injection for latanoprost-treated and control mice (latanoprost-treated group: before tracer injection: 17.9 ± 6.6 mmHg and 6 hours after tracer injection: 15.1 ± 5.2 mmHg, t-test, p>0.2; control group: before: 17.6 ± 4.2 mmHg, and 6 hours after tracer injection 15.5 ± 4.5 mmHg, t-test, p>0.2).

For experiments performed to confirm the IOP reduction following topical application of latanoprost in 10 randomly bred male pigmented mice (Figure 2-1). One drop of 0.005% latanoprost (Cayman Chemical Co., Ann Arbor, MI, USA) or 0.02% benzalknonium chloride in 1x phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA) was topically applied to the left eye at 5 pm and 16 hours later. Right and left eye IOPs were measured prior to each instillation, and 1, 1.5, 2, 3 and 5 hours following last instillation. All IOPs were measured one minute after induction of general anesthesia to minimize the effects of anesthesia on IOP [8]. The effect of isoflurane on IOP during in vivo imaging was not studied in the present experiments. The IOP decrease in the latanoprost-treated group (n=5) was compared to that in the control group (n=5). Mean IOP in latanoprost-treated mice was compared to controls at each time point using two-sample t-tests assuming equal variances.
**Figure 2-1. Timeline for IOP measurements.** Timeline for inhalation anesthesia (IN), IOP measurement (IOP), and topical application (TA).

2.6 In Vivo Hyperspectral Fluorescence Imaging [163, 164] (Figure 2-2)
Prior to in vivo imaging, a hair trimmer (Chromini Type 1591, Wahl, Sterling, IL, USA) was used to shave the head, neck, abdominal and forelimb areas [144]. Under general anaesthesia in vivo hyperspectral fluorescence imaging was performed before injection, and various time points after injection of QD into the anterior chamber of the left eye. Mice were anesthesitized through induction of isoflurane for 60 sec in a chamber and maintenance through an air tube for 3 - 4 minutes during hyperspectral system imaging (Maestro™, Cambridge Research & Instrumentation Inc., Woburn, MA, USA). Anesthesia was discontinued after the imaging and mice were placed back in their cage between imaging sessions. The anesthesia was repeated only during the imaging sessions at various time points. The in vivo imaging time points in the study of identification of lymphatic drainage were: before injection, and 6 and 24 hours after injection (n=3) or before injection, 5, 20, 40, and 70 minutes; 2 and 6 hours after injection (n=14) of QD into the anterior chamber of the left eye. In determining the effect of latanoprost, the time points were before tracer injection, and at 5, 20, 40, 70, 120, and 360 min after injection (n=28). Two subjects were excluded from analysis due to Maestro – computer interface problems (Animal...
ID#16, 17), with 4 excluded due to QD leakage from the eye following injection (#24, 25, 28, 29). Time to QD detection was defined as the earliest in vivo detection of QD signal to the neck region after eye injection. QD signal detection rate (60/time to in vivo detection) (hours\(^{-1}\)) was calculated to assess lymphatic drainage.

All mice were monitored throughout the experiment. No abnormal behavior was observed due to general anesthesia. The excitation filter and the emission filter were 503–555 nm, 580 nm long pass, respectively. The tunable filter was automatically stepped in 10-nm increments from 500 to 800 nm. The exposure time was 900 ms. Animals under anaesthesia were gently placed in a light-tight chamber. Captured images were analyzed using the Maestro 2.4 Imaging Software (Cambridge Research & Instrumentation Inc., Woburn, MA, USA), with unmixing algorithms to separate autofluorescence from QD signal [165, 166]. Green signal was set to represent spectra of autofluorescence mainly from melanin [144] while red signal was set to represent the QDs spectrum.
Figure 2-2. Timeline for in vivo hyperspectral imaging. Timeline for inhalation anesthesia (IN), topical application (TA), IOP measurement (IOP), intracameral injection (IC), in vivo hyperspectral imaging (HI), and euthanasia (E).

2.7 Control Experiments for QD drainage

To ensure QDs drain similarly in the right eye, QDs were injected into the anterior chamber of the right eye in 5 mice. In vivo imaging was performed before injection, 2 and 6 hours after injection (n=5). Two mice were excluded from analysis due to injection problems (#2, 5).

Because some leakage from anterior chamber into the conjunctiva can occur during intracameral injections, control experiments were conducted to verify that QDs draining to the lymph node was not due to conjunctival lymphatics. To test this, QDs were topically administrated onto the conjunctiva (n=5). In vivo imaging was performed before injection, 2 and 6 hours after injection.

2.8 Tissue Collection and Processing

2.8.1 Head and Neck Specimen

Mice were sacrificed with CO₂ inhalation at either 29 (n=3 from study in identification of lymphatic drainage) or 6 hours (n=14 for latanoprost-treated, n=14 for tear-treated, n=10 in control experiments) after injection of QDs. The upper body including head and neck, thorax and front limbs was dissected. Specimens were immersion-fixed in 4% paraformaldehyde (Electron...
Microscopy Sciences, Hatfield, PA, USA) for 48 h at 4°C. The specimens were then cryoprotected by immersion in 10% glycerol (Bioshop, Burlington, ON, Canada) and 2% dimethyl sulfoxide (Fisher Scientific Company, Ottawa, ON, Canada) in 0.1 M phosphate buffer for 4 days and 20% glycerol and 2% dimethyl sulfoxide in 0.1 M phosphate buffer for 6 days. The upper body of mouse was scanned using Maestro™ for the location of QDs after skin removal to get rid of the autofluorescence signal generated by the melanin pigment in hair and skin [144]. Neck tissues containing QD signal were harvested. The tissue block and the remainder of the upper body were scanned again to confirm the presence of QD signal and its absence, respectively. Mouse heads were harvested followed scanning. To orient the neck tissue block, its apex and right side were marked by red and blue surgical pathology ink, respectively (Triangle Biomedical Sciences, Inc., Durham, NC, USA).

2.8.2 Orbit Specimen

Orbit specimens were collected and cryoprotected in an identical manner as the head and neck tissue. To orient the orbit tissue block, its superior and nasal side were marked by yellow and blue surgical pathology ink, respectively.

2.9 Frozen Sections

2.9.1 Head and Neck Specimen

Tissue blocks from the neck region [167] containing QD signal were frozen in isopentane cooled by dry ice [5] and embedded into cryomatrix resin (Shandon Cryomatrix, Thermo Scientific, MI, USA). All blocks were serially sectioned (140 μm thick) using a sliding microtome (Leica DM2400, Leica, Germany). Sections were mounted onto double-frosted glass slides (Fisher Scientific Company, Ottawa, ON, Canada) with a PVA-DABCO (Polyvinyl alcohol-1,4-
diazabicyclo[2.2.2]octane) anti-fade mounting medium (Sigma-Aldrich, St. Louis, MO, USA). All sections were imaged under the Maestro™ and sections with QD signal were selected for histological analysis.

2.9.2 Orbit Specimen

Mouse orbit specimens were frozen in isopentane cooled by dry ice and embedded into cryomatrix resin. Blocks were serially sectioned (140μm thick) through the coronal plane using a sliding microtome. Sections were mounted onto double-frosted glass slides with a PVA-DABCO anti-fade mounting medium.

2.10 Quantification of Tracer Drained into Lymph Node

Serial neck region sections scanned by hyperspectral imaging (450 ms exposure time) were analyzed in a masked manner for QD signal intensity on 696 x 520 pixel size images (Image J 1.43 u National Institutes of Health, Bethesda, USA). The area and mean gray value of the region with QD signal was measured using thresholds of 60 and 255, respectively. QD signal intensity for each mouse was calculated by adding the product of the area and mean gray values in serial sections with QD signal.

2.11 Immunofluorescence and Nuclear Staining

2.11.1 Submandibular Lymph Node

To verify the presence of QDs in lymph node, selected sections containing the submandibular lymph node were washed three times in 0.1 mol 1x PBS (pH 7.4) for 6 min each, and incubated for 10 min in 0.2% TritonX-100 (Fisher Biotech, NJ, USA) in PBS. After three more washes in PBS for 6 min each, sections were incubated in 3% hydrogen peroxide (EMD, Darmstadt,
Germany) in PBS for 15 min. Sections were then washed 3 times for 6-min in PBS, and incubated for 1 h with 1% blocking reagent (Tyramide signal amplification (TSA) kit, Invitrogen, Oakville, ON, Canada) in PBS. Sections were incubated in collagen IV antibody (1:100, rabbit polyclonal, Abcam, Inc., Cambridge, MA, USA) with 1% blocking reagent overnight at 4°C. After three 5-min washes in PBS, sections were treated in the dark with a 1:100 dilution of the Tyramide Alexa Fluor 555 (TSA kit) for 10 min in amplification buffer. Sections were washed three times in PBS for 5 min and incubated for 20 min with a 1:1000 dilution of Sytox Green (a nuclear staining, Invitrogen, Eugene, OR, USA). Sections were washed three times in PBS for 6 min each before cover-slipping with a PVA-DABCO anti-fade mounting medium as described above. All sections were treated at room temperature and with mild agitation at all steps unless otherwise noted. Negative controls were obtained by omitting primary antibody.

2.11.2 Right Eye and Orbit Specimen

Frozen right eye and orbit sections (n=5) were selected for immunofluorescence and nuclear staining based on the following criteria: 1) conjunctiva, ciliary body, sclera, and orbital tissue must be present under examination by bright field microscopy; and 2) ocular structures must be intact and not fragmented. Immunofluorescence and nuclear staining were performed as described above except primary antibody used to label lymphatic channels and blood vessels were podoplanin (1:100, hamster polyclonal, eBioscience, San Diego, CA, USA) and collagen IV antibody (1:100, rabbit polyclonal, Abcam, Inc., Cambridge, MA, USA), respectively. Secondary antibody targeting podoplanin and collagen IV were goat anti-hamster Alexa Fluor 488 and anti-rabbit Alexa Fluor 647 (1:100 dilution, Invitrogen, Oakville, ON, Canada), respectively. Sections were incubated for 20 min with a 1:500 dilution of propidium iodide.
2.12 Bright Field Microscopy
Hematoxylin and eosin (H&E) stained sections were viewed with upright brightfield microscope (BX51, Olympus, Tokyo, Japan). Images were taken with MicroFire® True Color Firewire Microscope Digital CCD Camera 1600 x 1200P (Optronics, California, USA). Exposure times were set to automatic.

2.13 Combination of Fluorescence and Bright Field Microscopy
To visualize and confirm the QDs outflow pathway in the left injected eye and orbit, frozen coronal sections (140µm thick) of left orbit specimens from control group (n=14) were examined using an upright fluorescence microscope (BX51, Olympus, Tokyo, Japan). Two subjects were excluded due to QD leakage from the eye following injection (#24, 28). A DAPI (4',6-diamidino-2-phenylindole) filter was used to capture blue emission signal from autofluorescence of tissues while a TRITC (Tetramethylrhodamine-5-(and-6)-Isothiocyanate) filter was used to capture red emission from QD signal in left orbit specimens only. Images were captured with a DP72 digital camera using cellSens software (Olympus, PA, USA) and a 4x, 10x, 20x, or 40x objective lens. Bright field images were superimposed on fluorescence images to visualize tissue structure. Exposure times for QDs and tissue in left orbit specimens were manually set to avoid over saturation of signals (Table 2-1).

In right orbit sections without QD signal, exposure time for tissue was manually set to 15ms with 40x objective lens.
Table 2-1. Exposure times (ms) at various magnifications for frozen sections of orbit specimen.

<table>
<thead>
<tr>
<th>Objective lens</th>
<th>Tissue</th>
<th>QDs</th>
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<tbody>
<tr>
<td>4x</td>
<td>50</td>
<td>50 - 75</td>
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<td>40x</td>
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2.14 Confocal Microscopy
Confocal microscopy was utilized to verify the presence of QDs in lymph node, and to identify lymphatic channels in the eye and orbit specimen from immunofluorescence-labeled sections. Stained sections were viewed with a confocal laser scanning microscope (TCS SL, Leica, Wetzlar, Germany) and images of 1024 x 1024 pixel resolution were captured. Excitation wavelengths used were 488 nm for Alexa Fluor 488 and Sytox green nuclear stain, 543 nm for Alexa Fluor 555, and 633nm for Alex Fluor 647. QD655 was excited with the shortest available wavelength, 488 nm, and the emission window was specifically set at 620 – 680 nm to capture its maximum emission spectrum.

2.15 Statistical Analysis
Mean QD signal detection rate in latanoprost-treated mice was compared to controls using two-sample t-tests assuming unequal variances. Mean total QD intensity (log scale) in the latanoprost
group was compared to the control group using two-sample $t$-tests assuming equal variances. 
P<0.05 was considered statistically significant. Statistical analysis was carried out with Microsoft Excel (Version: 14.0.6112.5000; Microsoft Office Professional Plus 2010, Microsoft Corporation, Seattle, WA, USA). Results were graphed with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA).
Results

Whole body in vivo imaging confirmed the presence of strong QD signal in the left eye at 5, 20, 40, and 70 minutes, 2 hours after tracer injection. Signals were consistent with the QD spectral profile (Figure 3-1D,H). At these time intervals, no QD signals were detected in other parts of the body. At six hours after injection, while a strong QD signal was still detected in the injected left eye (Figure 3-1B), a focus of QD signal was noted only in the left neck region in 14 out of 17 mice (Figure 3-1F). The spectral analysis of the signal in the left eye and left neck confirmed the QD spectral profile (Figure 3-1D,H). Twenty-four hours following injection, the strong QD signal was still observed in the left neck region (Figure 3-1G), while QD signal intensity observed in the left eye was reduced (Figure 3-1C).

A consistent background pattern of green signal from facial and limb hair, and faint orange signal from mouth, forelimb and ear was observed (Figure 3-1A-C, E-G).
Control Experiments

QD signal was noted only in the right neck region 6 hours following injection in all mice (n=3) successfully injected with QDs in the right anterior chamber. Post-mortem imaging with skin removal confirmed, in all mice (n=3), the presence of QDs in the neck tissue where the right submandibular lymph node was located.

No QD signal was observed during the in vivo imaging sessions in the head and neck region of the control mice (n=5) with QDs were topically applied onto the conjunctiva. Moreover, QD signal was not observed in anywhere else in the body except remaining at the site of topical application. Post-mortem imaging with skin removal revealed no QD signal in the head and neck tissue or in any organ or tissue for all mice (n=5).
Figure 3-1. In vivo hyperspectral fluorescence imaging of head and neck in dorsal (A-C) and ventral (E-G) views. Imaging performed prior to QD injection (A) shows no signal in the left eye (arrow). At 6h (B) and 24h (C) following injection, QD signal in red is noted in the left eye (arrow). Strong QD signal is noted in the left neck region (arrow) at 6h (F) and 24h (G), and is not seen prior to injection (E). Scale = 10 mm. Emission spectral profiles of the signals in the eye (arrow in B) and in the left neck region (arrow in F) at 6 hours are shown in dotted lines in (D) and (H), respectively. Spectral profiles of both signals confirm the QD spectral profile (red), compared to the background signal profile (green) (D, H).
Having observed QD signals draining from the anterior chamber to the neck tissue, we next aimed to determine the anatomical structures where the intracameraly injected QDs drained. Based on post-mortem imaging using hyperspectral imaging, intense QD signals were observed within a block of neck tissue on the left side (Figure 3-2A) after skin removal. This soft tissue area adjacent to the submandibular salivary gland was isolated (Figure 3-2B,C). Hematoxylin and eosin (H&E) stained sections revealed classic lymph node architecture (Figure 3-2D).

**Figure 3-2. Post-mortem analysis of neck tissue.** Neck tissue containing QD signals (red) (A) was harvested (B), and isolated (C). Hematoxylin and eosin stained section shows a lymph node (D). Scale = 10 mm (A-C) and 250 μm (D).
To further confirm our histological findings, immunofluorescence and confocal microscopy were performed on frozen sections of the neck tissue containing the submandibular lymph node. QDs in red were detected inside the submandibular lymph node (Figure 3-3). Immunofluorescence labeling with antibody against collagen IV outlined the morphology of a lymph node (Figure 3-3) while counterstaining with Sytox green demonstrated architecture of the node (Figure 3-3). QDs were consistently detected in the cortex region at the subcapsular sinus close to one pole of the submandibular lymph node. No QD signal was noted in the paracortex or medulla regions.

**Figure 3-3. Immunofluorescence of left submandibular lymph node.** QDs in red are contained within the lymph node surrounded by capsule in blue (anti-collagen IV) against a green background of cell nuclei of the node parenchyma (Sytox green). Scale = 250 μm.
Altogether, the data suggests a lymphatic drainage from the mouse eye draining QDs from the left anterior chamber to the left submandibular lymph node. Nevertheless, we cannot exclude the possibility that the Maestro was unable to detect minute amount of QDs that is below detection threshold in the contralateral lymph node or elsewhere in the body. To further understand this lymphatic drainage, we first set out to determine the locations within in the eye and orbit of where QDs were drained from the anterior chamber. Through examination by fluorescence and bright field microscopy, of frozen coronal sections of left orbit specimen from the control group (n=12), we observed QD drainage from the anterior chamber to various ocular compartments 6 hours following QD injection. QDs were observed in ciliary body (Figure 3-4A,B) in all 12 mice, in choroid in 11 of 12 mice (Fig. 3-4C,D), and in the sclera of all mice (Figure 3-4C,D). QDs were observed in the posterior orbit in 10 of 12 mice (Figure 3-4E,F). The signal appeared to be a continuation from choroid, to sclera, and to orbital tissue and onward. Although QD signal was observed in the sclera, we were not able to distinguish whether the QDs were located in the anterior or posterior portion of sclera based on coronal sections. Transverse sections will be needed to pinpoint the portion of sclera into which QDs were drained. QDs were not observed in the lens, retina, or vitreous. QD signal was not observed in control non-injected right eye and orbit specimens. In the frozen sections, lymphatic or blood vessels could not be observed under the magnification of the bright field microscope. As such, further studies with higher magnification are needed to determine whether or not QDs are located in ocular lymphatic channels and that intraocular lymphatics play a role in draining the QDs from the anterior chamber to the submandibular lymph node. However, the existence of lymphatic channels in the mouse eye has not been explored. This opened up the opportunity for us to first identify whether lymphatic channels exist in the mouse eye.
Figure 3-4. QD distribution in left eye 6h following intracameral injection in control mice treated with artificial tears. Merged images from fluorescence and light microscopy showed QDs (red), uveal tissue (black), all other ocular tissues (blue) and optically empty space between tissues (bright field). QDs observed in boxes in panels A, C, and E are shown at higher magnification in B, D, and F, respectively. Scale bar = 500 μm (A,C,E), 100 μm (B,F), and 50 μm (D). Ciliary body (CB), lens (L), choroid (C), sclera (S), retina (R), and orbit (O). (Published in supplementary material in reference [168])
With the observation of QD signals in the ciliary body, sclera, and orbit (Figure 3-4), we focused on identifying lymphatic channels in these structures. The morphologic criteria used to establish a structure as a lymphatic vessel were as follows: 1) endothelial cell-lined immunostained vessel; 2) a very thin-walled vessel with central lumen, usually with a wavy, irregular contour and often collapsed; and 3) a poorly developed or absent basal lamina indicated by absence of collagen IV staining.

With the criteria above, we first made sure the primary antibodies, podoplanin and collagen IV, were specifically targeting lymphatic channels and blood vessels, respectively. We selected sections with conjunctiva as a positive control. The morphology of conjunctiva was confirmed by collagen IV-positive basement membrane and multiple layers of nucleus counterstained by propidium iodide (Figure 3-5A). Conjunctiva sections showed podoplanin-immunoreactive lymphatic channels that were distinct from collagen IV-positive conjunctival blood vessels in 5 out of 5 subjects (Figure 3-5B). Negative controls omitting primary antibody showed no non-specific staining (Figure 3-5C).
Figure 3-5. Podoplanin-positive lymphatic channels in conjunctiva as positive control (A) Con juncta va immunostained with collagen IV antibody (blue), counterstained with PI (red) (arrow indicates the substantia propria where lymphatics are seen). (B) A podoplanin-positive lymphatic channel in green (arrow) is distinct from a collagen IV-positive blood vessel in blue (asterisk). Both signals are absent in the negative control without primary antibodies (C). Scale bar = 30 μm (A), and 10 μm (B,C).
Having determined the specificity of the primary antibodies, we examined the existence of lymphatic channels in the ocular structures in the following order: ciliary body, sclera, and orbital tissue. Within the ciliary body stroma, podoplanin-immunoreactive lymphatic channels were noted (Figure 3-6B) and they were distinct from collagen IV-positive blood vessels (Figure 3-6C). These lymphatic channels were thin-walled vessels without collagen IV staining. Lymphatic channels were observed in the ciliary body of all mice (n=5). Non-specific staining was absent in negative controls omitting primary antibody (Figure 3-6D).

Podoplanin-positive lymphatic channels were observed in the episclera region (Figure 3-7A,B), and were distinct from collagen IV-positive blood vessels (Figure 3-7C). These lymphatic channels were endothelial cell-lined vessels that had a central lumen and wavy and thin-walled contour. They lacked basal lamina indicated by absence of collagen IV staining. Lymphatic channels were noted in 3 out of 5 mice. Negative controls omitting primary antibody showed no non-specific staining (Figure 3-7D).

Finally, podoplanin-positive lymphatic channels were found in the orbital tissue. The Harderian gland, which was surrounded by a fine connective tissue capsule stained by collagen IV, was used as a landmark to locate the posterior orbit (Figure 3-8A). Podoplanin-immunoreactive lymphatic channels were observed in the posterior orbit, adjacent to the Harderian gland, in 5 out of 5 subjects (Figure 3-8B). These orbital lymphatic channels were distinct from collagen IV-positive blood vessels (Figure 3-8B). Lymphatic channels in the posterior orbit were thin-walled, were collapsed in contrast to the blood vessels with rounder contour, and were not collapsed. Non-specific staining was absent in negative controls (Figure 3-8C). Other regions of the posterior orbit were not examined in this study; thereby, we cannot exclude the possibility of the existence of lymphatic channels elsewhere in the orbit.
Figure 3-6. Podoplanin-positive lymphatic channels in ciliary body. (A) Ciliary body immunostained with collagen IV (blue) and counterstained with PI (red) (arrow indicates where lymphatics are seen in ciliary body bounded by ciliary processes (CP) and sclera (S)). (B) Lymphatic channel in green (arrow) is identified with anti-podoplanin immunofluorescence. (C) Lymphatic channel in green (arrow) is distinct from collagen IV-positive blood vessel in blue (asterisk). Both are absent in the negative control without primary antibodies (D). Scale bar = 100 μm (A), and 5 μm (B,C,D).
Figure 3-7. Podoplanin-positive lymphatic channels in sclera. (A) Sclera (S) adjacent to the ciliary processes (CP) was identified with merging fluorescent and bright field microscopic images. (B) The structure was confirmed in the identical section stained with collagen IV (blue) counterstained with PI (red) (arrow indicates where lymphatics are seen in the sclera). (C) Lymphatic channel in green (arrow) is identified with anti-podoplanin immunofluorescence, distinct from collagen IV-positive blood vessel in blue (asterisk). Both signals are absent in the negative control without primary antibodies (D). Scale bar = 50 μm (A, B), and 10 μm (C, D).
**Figure 3-8. Podoplanin-positive lymphatic channels in posterior orbit.** (A) Posterior orbit section containing the retina (R), choroid (Ch), and Harderian Gland (H) stained with collagen IV (blue) and counterstained with PI (red) (arrow indicates where lymphatics are seen in the posterior orbit). Sclera was not visible in the staining. (B) Podoplanin-positive lymphatic channel in green (arrow) is distinct from collagen IV-positive blood vessel in blue (asterisk). Both signals are absent in the negative control without primary antibodies (C). Scale bar = 200 μm (A), and 10 μm (B,C).
The existence of lymphatic channels in the eye and orbit provided a framework for future studies in determining the role of ocular and orbital lymphatics in the ocular lymphatic drainage observed from in vivo hyperspectral imaging [159]. Most importantly, our data so far provided evidence of a lymphatic drainage from the mouse eye. Next, we asked whether latanoprost, a PGF$_{2\alpha}$ analog, stimulates ocular lymphatic drainage. We first confirmed the IOP lowering effect of latanoprost to ensure the drug has its action in our mouse strain. Latanoprost-treated left eyes (n=5) showed a significant difference in mean IOP compared to control left eyes (n=5) at 2 hours following last instillation (9.1±1.1 mmHg vs. 14.9±2.4 mmHg; (Mean±SD), P < 0.001, two-sample $t$-test) (Figure 3-9). No significance difference was observed in other time points for left eyes and in all time points for right eyes.

**Figure 3-9. Effect of latanoprost on IOP.** Latanoprost-treated left eyes (n=5, black squares) showed a significant difference in mean IOP compared to control left eyes treated with 0.02% benzalkonium chloride in 1xPBS (n=5, open circles) at 2 hours following last instillation. *P<0.001.
Next, we examined the effect of latanoprost on the ocular lymphatic drainage in mouse. In the latanoprost-treated group, QD signal in the left neck region was detected in 2 mice within 20 minutes of QD eye injection (Figure 3-10A), and in 7 additional mice by 70 minutes (Figure 3-10C and Figure 3-11). Control mice treated with artificial tears showed no neck signal at 20 minutes (Figure 3-10B), 40 minutes, or 70 minutes (Figure 3-10D) after QD injection (Figure 3-11). By 6 hours, all latanoprost-treated (Figure 3-10E) and control mice (Figure 3-10F) except #36 showed neck signal (Figure 3-11). No QD signal was detected except at the injection site using in vivo hyperspectral imaging prior to observation of QD signal in the left submandibular lymph node. QD signal detection rate was increased in the latanoprost-treated group compared to controls (1.23±1.06 hours⁻¹ vs. 0.30±0.17 hours⁻¹, mean±SD, P<0.02) (Figure 3-12).
Figure 3-10. In vivo hyperspectral imaging of mouse neck region. Following QD injection into the left eye, latanoprost-treated mice showed QD signal (red) in the left neck region (arrow on ventral view) at 20 minutes (A, #20), 70 minutes (C, #43), and 6 hours (E, #39) by in vivo hyperspectral fluorescent images. In controls treated with artificial tears, QD signal was not detected at 20 min (B, #38) or 70 minutes (D, #42) post-injection imaging times, but was present at 6 hours (F, #34). Green signal corresponds to background and red to QD signal. Scale = 10 mm.
Figure 3-11. In vivo QD signal detection over post-injection imaging times. In most latanoprost-treated mice (black squares), QD signal was detected in the neck at earlier post-injection times compared to controls treated with artificial tears (open circles).
Figure 3-12. **In vivo QD signal detection rate.** Histogram shows mean and standard deviation of QD drainage rate (hours$^{-1}$) for latanoprost-treated group (black, n=11) and control group treated with artificial tears (white, n=11) groups. *P<0.05.
Frozen sections were examined to ensure QDs were drained from the left anterior chamber to the left submandibular lymph node in both groups. Indeed, QDs were detected inside, and beneath the capsule of the left submandibular lymph node in both latanoprost-treated (Figure 3-13A) and control (Figure 3-13B) mice. Collagen IV immunofluorescence staining outlined the capsule with characteristic bean-shaped morphology of a lymph node (Figure 3-13A,B) while counterstaining with Sytox green demonstrated lymph node cellular architecture (Figure 3-13A,B).

**Figure 3-13. Localization of QD in lymph node of latanoprost-treated and control mice.**
QDs in red are located in the left submandibular lymph node in both latanoprost-treated mouse (A, #37) and control mouse treated with artificial tears (B, #23). Both lymph nodes are surrounded by capsule in blue (anti-collagen IV) against a green background of cell nuclei (Sytox green). Scale = 250 μm.
In addition to demonstrating an increased lymphatic drainage, we aimed to provide evidence of an increased amount of QDs being drained to the submandibular lymph node. When examining the QD intensity in the left submandibular node, mean total QD intensity was significantly greater in latanoprost-treated mice compared with controls (10.55±1.12 vs. 9.48±1.24, mean±SD, \( P<0.05 \)) (Figure 3-14). QD signal was not detected in the right submandibular node in both groups. Overall, our findings from in vivo and post-mortem analysis show that latanoprost stimulates the ocular lymphatic drainage from the mouse eye [168].

Figure 3-14. QD intensity in the submandibular node in latanoprost-treated group and control group treated with artificial tears. Box-plot shows the median, 25th, and 75th percentiles (solid line box), and the minimum and maximum intensity (whiskers) for the natural log-transformed total QD intensity gray value measured. *\( P<0.05 \).
Discussion

The present thesis provides, for the first time, evidence of lymphatic drainage from the mouse eye [159]. A non-invasive approach combining intracameral injection of nanotracers and hyperspectral fluorescence imaging was developed to visualize in vivo lymphatic flow from the eye. We were first to identify lymphatic channels in the mouse ciliary body, sclera, and orbit that may be responsible for this ocular lymphatic drainage. The establishment of lymphatic outflow from the eye provided opportunities to investigate whether any drugs target this drainage. Through the application of the novel in vivo lymphatic imaging technique, we demonstrated for the first time, latanoprost, the most potent and widely prescribed glaucoma drug, stimulates ocular lymphatic drainage [168]. Collectively, our findings support the use of this mouse model in combination with the in vivo imaging technique for future screening studies of drugs that may target the ocular lymphatic circulation.

The existence of lymphatic drainage from the mouse eye is in keeping with lymphatic drainage reported in sheep [5, 6]. The submandibular lymph node [167] appears to be the initial
draining lymph node from the eye as strong QD signal was observed following intracameral injection. The presence of QDs in other tissues at concentrations below the threshold of the imaging system used cannot be excluded. Our study shows that QDs used to detect lymphatic drainage from peritoneal and pleural spaces, and skin [169-171], are useful to study ocular lymphatic drainage, a newly described route for fluid out of the eye. However, our results differ from the literature regarding the location of the lymphatic drainage. We only observed the intracameral injected QDs draining toward the submandibular lymph node, but not cervical or facial lymph nodes [5, 6, 172]. It is possible that the retention of fluorescent dextrans or radioactive albumins in lymph nodes is different from QDs. QDs are known to accumulate in the first lymph node they were drained to and have excellent retention [173]. This may explain why QDs were only observed in one lymph node. Tracers with various sizes, charges, and optical properties [6, 52, 151, 174] can be used to examine whether or not locations of drainage via the ocular lymphatics in mouse are affected by selection of tracers.

The distribution of QDs in the subcapsular sinus close to one pole of the submandibular lymph node matches closely the afferent flow into the lymph node [175]. Our results are in keeping with the distribution of the QDs across the subcapsular sinus of the sentinel lymph node following subdermal injection [150]. It is possible that the size and charge of QDs may affect their distribution pattern and this requires further study. It is known that mouse skin and fur can reduce the QD detection sensitivity [176, 177]. This is supported by the stronger QD signal we observed following skin removal in post-mortem imaging. For this reason, we cannot rule out the possibility that some QDs were drained earlier than the initial detection time following tracer injection. This reduction in QD655 sensitivity may not be optimal for quantitative analysis of in vivo hyperspectral imaging [178].
The data from frozen left eye and orbit specimens revealed that following intracameral injections, QDs were observed in the orbit and in the tissue compartments that typically comprise the UVS outflow pathway - the ciliary body, choroid, and sclera. The tissue compartments from the UVS pathway in which the fluorescent tracer appeared in our findings are the same as observed in the NIH Swiss mouse eyes [128], supporting the use of QDs, like fluorescent dextran, as tracers for time course study of AqH drainage. Our results, however, differ in the intensity of fluorescence from QDs in tissue compartments. In the present thesis, an elimination of fluorescence within the elements of UVS outflow pathway [128] was not observed at 6 hours following intracameral injection. Instead, QDs fluorescence remained at moderate to high intensity amongst the tissue compartments. A possible reason for this may be the injection volume. Instead of 1.5 µL of tracer used, it is possible that 3 µL of QDs is sufficient for a continuous transport of tracers through the UVS pathway; thereby, maintaining the observed intensity in the frozen sections. Another possibility is that the size of the tracer may be important. The ocular distribution of dextrans following intracameral injection varies with various sizes of the tracers [52], 40-kDa dextran was shown to be optimally recovered in the anterior uvea, anterior sclera, and posterior sclera for normal eyes; while 150-kDa dextran was recovered most efficiently in inflamed eyes [52]. Therefore, additional experiments using tracers with various sizes may further clarify the properties of macromolecular transport of AqH outflow to the various ocular tissue compartments.

The identification of lymphatic channels in the mouse ciliary body using podoplanin, a specific lymphatic endothelial cell marker supports the previous findings of lymphatics identified in the human and sheep ciliary body using D2-40 to specifically detect podoplanin and LYVE-1 antibody for the lymphatic endothelial hyaluronan receptor [5]. The characteristics of the
lymphatic channels in mouse ciliary body are in keeping with lymphatics in human and sheep ciliary body as they are not covered by collagen IV-positive basement membrane, distinguishing them from blood vessels [179, 180]. Larger lymphatic collecting ducts covered by collagen IV-positive basement membrane were not observed in our study. Further experiments using electron microscopy may be helpful in locating larger collecting ducts in the orbit.

The presence of lymphatics in the sheep ciliary body provides a unique pathway for the drainage of AqH [5, 6]. In sheep, tracers injected in the anterior chamber are drained to the head and neck lymph nodes via the lymphatic in the ciliary body. Other studies have confirmed that intraocular antigens can traffic to local draining lymph nodes [181, 182]. It is possible that the injected tracer in the mouse eye draining to the submandibular lymph node [159] is partially due to the lymphatics in the ciliary body. When taking into account of both identification of lymphatics in ciliary body and the observation of QDs in the ocular compartments composed of the UVS pathway, for instance, ciliary body, choroid, sclera, it is possible that QDs were drained through the lymphatic channels in the mouse ciliary body. This network of lymphatics may branch off to join with lymphatic channels in the orbital tissues in the posterior orbit. Altogether, these lymphatic channels can drain QDs from the anterior chamber to the submandibular lymph node. Further studies are needed to determine whether the lymphatics in the ciliary body and orbit contribute to AqH drainage.

Previous studies using corrosion casting and serial section microscopy have indicated that fluid can be drained from the suprachoroidal space through preformed scleral channels at the inner aspect of the anterior sclera into the intrascleral venous plexus [183, 184]. Later studies using 5'-nucleotidase, an early marker for LECs [185], failed to identify these scleral channels as lymphatics [186]. In the present study, lymphatic channels were identified at the episclera with
podoplanin. These lymphatics are not covered by collagen IV-positive basement membrane, distinguishing them from blood vessels [179, 180]. The presence of lymphatic channels in the episclera may provide a unique pathway for the drainage of fluid out of the suprachoroidal space into the sclera and finally into the orbital tissues [187]. Further studies are needed to determine the exact location of the distributions of lymphatic channels in the sclera to better understand the drainage of fluid out of the eye.

Mouse conjunctival lymphatics were identified recently with LYVE-1 and podoplanin [188]. We confirmed podoplanin-positive conjunctival lymphatic channels in this study in which they were also used as positive controls. Though LYVE-1 can also detect macrophages [188], it was demonstrated that podoplanin staining was negative on the LYVE-1+ non-endothelial cells. Since our study used podoplanin as the lymphatic endothelial cell marker, the possibility of the luminal structures identified in this study being macrophages is unlikely.

In the orbit, lymphatics have previously been identified in the lacrimal gland and the dura mater of the optic nerve in human [185, 189], and in the Harderian gland of rats [190]. Lymphatics in the extraocular muscles and in the connective tissues of the extraocular muscle cones in mice [188] were not investigated in the present study due to problems with processing and sectioning of the extraocular muscles and their connective tissues. Nevertheless, using podoplanin as a lymphatic endothelial cell marker, we identified the existence of lymphatic channels near the Harderian gland in mice. The presence of the lymphatic channels in the orbit may provide a drainage pathway of the ocular/orbital tissue fluid. Since AqH drained via the UVS pathway exits the portal in the sclera [62], it is also possible for these orbital lymphatic vessels to drain fluid originating from the exit sites of the scleral portals. Further studies are needed to determine the origin and destination of lymph drained via the orbital lymphatics.
The fact that the eye is frequently affected by inflammations [191], and intracamerally injected antigens are drained to neck lymph nodes [159, 192, 193], suggest that ocular lymphatics [5] identified in this thesis may play a role in immune responses. Indeed, experimentally induced inflammation of ciliary body in cynomolgus monkeys increases the UVS outflow [194]. Hence, our findings may be important for studying ocular inflammation. In terms of intraocular tumors, metastasis can occur via spreading to regional lymph nodes [195, 196]. The risk of metastasis and mortality are higher when tumors invade the ciliary body [197, 198]. Podoplanin staining ciliary body of posterior uveal melanoma with and without extraocular extension showed lymphangiogenesis in the ciliary body [199]. Our findings of an ocular lymphatic drainage [159] and lymphatic channels in the normal ciliary body and sclera support the notion of intraocular peritumoral lymphatics originating from preexisting lymphatics in the ciliary body.

The existence of an ocular lymphatic drainage in mouse [159] led to opportunities to study pharmacology of this drainage pathway. Here we provided the first evidence that a topical PG, latanoprost, a widely prescribed glaucoma drug, stimulates lymphatic drainage from the eye [168]. Quantification of the total QD intensity from all serial lymph node sections demonstrated significant increase of QD signal in left submandibular lymph node in latanoprost-treated group compared to controls. In addition, the fact that QD signal was not detected elsewhere in both latanoprost-treated and control mice suggests that topical application of latanoprost increases the drainage rate without altering the location of QD drainage. The increased lymphatic drainage rate from the eye to the lymph node in the latanoprost-treated group provides evidence that latanoprost stimulates lymphatic drainage from the eye. Cellular changes induced by latanoprost may be implicated in this process. Ciliary muscle relaxation [200], by PG action on
Prostaglandin F (FP) receptors [200-203] may contribute indirectly to increased ocular lymphatic drainage. PGs act on lymphatic endothelial and contractile cells surrounding lymphatic channels responsible for the peristaltic movement of lymph [204]. Latanoprost remolds extracellular matrix, increasing matrix metalloproteases (MMP), proMMP-1 and proMMP-3 [205], while decreasing collagens, fibronectin, laminin, and hyaluronan [206, 207]. It is unknown whether these cellular changes observed following long-term latanoprost treatment, are relevant to increased lymphatic drainage observed in this study.

The increased detection rate of intracamerally injected QDs in the left submandibular lymph node in the latanoprost-treated group compared to controls, suggests that latanoprost may act directly on the lymphatic vessels within the eye [5]. In addition to this uveolymphatic “pathway”, our findings from fluorescent and bright field microscopy on the QDs outflow pathways in the left eye and orbit specimens confirm that a portion of tracers were drained by the UVS pathway to sclera [128] and then to the orbit. It is possible that some of these tracers in the orbit are drained via the orbital lymphatics [185, 188, 190] to the regional lymph node. Further studies are needed to determine the role of orbital lymphatics in AqH drainage.

The hydrodynamic size and intrinsic brightness of QDs provide optimal retention in lymphatic vessels and lymph nodes [151] in addition to emit signal that can penetrate up to 2 cm of tissue [150] making this in vivo imaging method very sensitive to changes in lymphatic drainage. The optical resolution of this hyperspectral imaging system, however, restricted in vivo visualization of QDs in TM, UVS pathway, and iris. While localization of QDs in the UVS pathway was demonstrated in coronal orbital sections, QDs in anterior chamber angle structures such TM and SC that are involved in the conventional pathway could not be assessed, and the use of transverse frozen sections may help to locate QDs in these structures. Without visualizing
QDs in the TM, we cannot exclude the possibility that some QDs were drained through the conventional pathway into the venous circulation and be taken up by the liver and spleen [208]. However, no QD signal was observed in the liver or spleen from any mice in the latanoprost-treated (n=11) or control group (n=11) during in vivo and post-mortem imaging performed 6 hours following intracameral injection. It is possible that concentrations of QDs in the spleen and liver were below the detectable threshold by the imaging system used. Further experiments with more sensitive techniques [208, 209] may be needed to investigate the QDs localization in the liver and spleen following intracameral injection.

AqH dynamics have been studied in several species [210, 211] including the mouse [155]. Our observation that an increase in lymphatic drainage rate following latanoprost application matches temporally with a decrease in IOP suggests that enhanced ocular lymphatics may be implicated in the IOP-lowering effect of latanoprost in normal mice [8, 155]. As mouse aqueous dynamics are similar to humans [7], the presence of lymphatic drainage from the mouse eye provides the framework for developing glaucoma treatments through pharmaceutical manipulation of the lymphatics to decrease IOP. In addition, since lymphatic drainage from the eye may play an important role in controlling pressure in the eye, further investigations in glaucoma mouse models [212] will help to determine whether stimulating lymphatic drainage from the eye using latanoprost, is implicated in its IOP lowering effect in glaucoma.
4.1 Limitation of the Studies

In demonstrating, in vivo, a lymphatic drainage from the mouse eye, our experiments relied on both the hyperspectral imaging system and the nanotracer, QDs. We acknowledge that both tools inherently include some limitations. The detection of QDs by the hyperspectral imaging system are dictated by two factors. First, the intrinsic brightness of QDs can penetrate the tissue of a depth up to 2cm [150]. Hence, our detection of QDs are restrained by the proximity to the surface of the tissue structure in which QDs locate. Second, the intensity of QDs penetrated through the tissue and skin has to reach a certain detection threshold. Taken together, it is possible that QDs were drained into deep tissues from where QD signals cannot penetrate toward the surface. Similarly, traces of QDs that are below the detection threshold could be drained to other tissues aside from the submandibular lymph node. Therefore, we cannot exclude the possibilities that some QDs may have been drained into structures other than the submandibular lymph node within the 6 hour in vivo imaging time period. Further studies involved sacrificing animals in earlier time points, sectioning whole body, and increasing the exposure times can be helpful in determining whether QDs can be detected elsewhere other than the submandibular lymph node.

The optical resolution of the hyperspectral imaging system is another limitation we faced when we aimed to confirm the aqueous outflow of QDs within the eye during in vivo imaging. The system is sensitive to the changes in QD intensity in gross anatomical structure such as lymph nodes. However, the optical resolution of the system is unable to provide sufficient magnification to visualize finer ocular structures. Hence, we were unable to confirm in vivo AqH outflow through the TM and UVS pathway. Post-mortem studies with examination of frozen eye
and orbit sections will help understand and characterize the trajectory of QD within in the eye and orbit following intracameral injection.

In the identification study of lymphatic channels, it is important to note that the value of immunofluorescence relies heavily on the specificity of the primary antibody toward the lymphatic endothelium. In our study, anti-podoplanin stained specifically the lymphatic channel but not the blood vessel. We did not select tissues without lymphatics to eliminate the possibilities of false positive results from anti-podoplanin staining for non-specific non-endothelial cells. Nevertheless, we relied on the morphological criteria, such as having a full central lumen and partially-collapsed structure, to determine whether podoplanin-positive structures are indeed lymphatic channels. Further improvement of this study would be to use a different primary antibody targeting lymphatic endothelium, for instance, anti-LYVE-1, and compare results to further confirm the existence of lymphatic channels in the eyes and orbits.

Finally, in vivo quantification was restrained in studying the effect of latanoprost on the lymphatic drainage. The intrinsic brightness of QDs led to oversaturation of signals in the eye during in vivo imaging. Hence, QDs intensity changes in the eye could not be quantified and compared between the latanoprost-treated and control mice. Further studies using tracers with less intrinsic brightness and adjustment of imaging parameters will be helpful in determining the changes of QDs intensity in the eye.
4.2 Future Directions

Role of Ocular Lymphatics in AqH Drainage

Ocular lymphatics identified in this thesis may play an important role in draining AqH; thereby, controlling pressure in the eye. Hence, determining their role in AqH drainage is highly relevant for glaucoma studies. It has been shown in sheep that intracamerally injected tracer are located in the lumen of lymphatic channels in the ciliary body [5], which demonstrated lymphatic channels playing a role in AqH drainage. It is highly likely that intracamerally injected QDs in the mouse left eye will also be located in the lymphatic channels in the conjunctiva, ciliary body, and sclera. Immunofluorescence targeting lymphatic channels in the frozen eye sections with QDs followed by examination of these sections with confocal microscopy will be needed to localize QDs in the lumen of lymphatic channels.

The localization of QDs in the posterior orbit raises the possibility of orbital lymphatics involving in AqH drainage. The location to where tracers are drained by the orbital lymphatics is currently unknown. QDs can be directly injected into the orbital tissue to determine the AqH outflow pathways through there.

Mapping AqH Outflow Pathway to Submandibular Lymph Node

Understanding the general pathways in which QDs drained from the anterior chamber to the submandibular lymph node is crucial. This can be achieved through intracamerally injecting tracers conjugated with specific antibody against LECs such that the tracers can bind to the lymphatics as they are being drained. It is possible that the lymphatic drainage begins in the extracellular spaces of the ciliary muscle and enters into the suprachoroidal space and anterior choroid. Then the network exits the periocular tissues directly through the sclera or through the
loose connective tissue surrounding penetrating vessels and nerves. QDs will then be drained toward the tissues toward the submandibular lymph node.

**Dissection of Ocular Lymphatic Drainage from UVS Outflow**

Since PGF$_{2\alpha}$ analogs affect both lymphatic vessels [156-158] and UVS drainage [105, 106], it may be difficult to separate the component of increased UVS pathway from the direct effect on lymphatics. Future studies using other drugs such atropine [62], that have been shown to increase the UVS pathway via a different mechanism from PGF$_{2\alpha}$ analogs, or the use of selective drugs that do not affect UVS outflow but have direct effect on lymphatics may be helpful to pharmacologically dissect out ocular lymphatic drainage from UVS outflow. Better characterization of lymphatics in the eye and in the orbit in mouse and the localization of PGF$_{2\alpha}$ receptors and other receptors in the lymphatics in the eye and orbit may help to identify new therapeutic targets to modulate lymphatic drainage from the eye.

**Studying Ocular Lymphatic Drainage in Glaucoma Mouse Models**

Existing glaucoma mouse models provide opportunities to study whether ocular lymphatics can be targeted to control IOP; thereby, delaying onset or progression of glaucoma. In human, it is known that glucocorticosteroid administration may lead to the development of ocular hypertension and open-angle glaucoma [213-216]. It has been demonstrated that application of dexamethasone, a glucocorticosteroid, to the mouse eye will lead to ocular hypertension [217]. The mechanism behind the increased IOP was thought to be morphologic and biochemical changes in the TM, which led to reduction in AqH outflow [218]. Similarly, transgenic mice, such as a mouse strain expressing the Tyr423His myocilin point mutation can also be used [219, 220]. Myocilin is a secreted glycoprotein [221, 222] that is expressed in high
amounts in the TM [223-225]. In human, the secreted myocilin is present in the aqueous humor [226-228]. Mutated myocilin was demonstrated to accumulate in the TM cell cytoplasm and was prevented from secreting into the extracellular space in mouse [219], which leads to detachment of the endothelial cells of TM and contributes to development of glaucoma [219]. These glaucoma models are especially useful for studying the effect of ocular lymphatics in controlling IOP as only the TM was predominately affected while the UVS outflow remains unaffected. These studies would allow the screening various compounds that stimulate the ocular lymphatic drainage and lower IOP in the glaucomatous mice.

**Immune Response, Tumor Metastasis, and the Eye**

The findings of an ocular lymphatic drainage provided opportunities to study ocular immune responses and metastasis. It has been demonstrated that experimentally induced inflammation of ciliary body in cynomolgus monkeys increased UVS outflow [194]. Studies involving inducing ocular inflammation in mouse and assessing lymphatic drainage with in vivo hyperspectral imaging can be helpful in understanding the relationship between ocular inflammation and the lymphatic drainage. Intraocular tumors can metastasize via spreading to regional lymph nodes [195, 196]. Lymphatics in the ciliary body may play a role in metastasis of uveal melanoma [199, 229]. Studies involving stimulating lymphangiogenesis or ocular lymphatic drainage followed by observing the extraocular extension and metastasis would be helpful in understanding the role of ocular lymphatics play in spread of the tumour [230]. The results may enable development of anti-angiogenic drugs or drugs that slow ocular lymphatic drainage to prevent metastasis of intraocular tumors.
4.3 Conclusion

This work provides direct evidence of lymphatic drainage from the mouse eye. Lymphatic drainage from the eye has for the first time been visualized in vivo with QDs and hyperspectral fluorescence imaging techniques. Lymphatic channels identified in the ciliary body, sclera, and orbit in mice may play a role in AqH drainage. Using this model in conjunction with in vivo imaging, a stimulatory effect of latanoprost on ocular lymphatic drainage was shown. The findings in this thesis support the use of this mouse model as a potential platform for future screening studies of drugs that target the ocular lymphatic circulation. Due to limited sensitivity to detect QD signal in deep tissue by in vivo hyperspectral imaging system, it may be difficult to localize QD signal in deep anatomical structures. Future development of biocompatible tracers and more sensitive hyperspectral imaging methods may nevertheless circumvent these drawbacks and will enable translation of pre-clinical findings to clinical trials devoted to the treatment of blinding eye disease.
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