Revisiting Ciliary Muscle Tendons and Their Connections With the Trabecular Meshwork by Two Photon Excitation Microscopic Imaging

Choul Yong Park,1,2 Jimmy K. Lee,2 Malik Y. Kahook,3 Jeffrey S. Schultz,2 Cheng Zhang,2 and Roy S. Chuck2

1Department of Ophthalmology, Dongguk University, Ilsan Hospital, Goyang, Gyunggido, South Korea
2Department of Ophthalmology and Visual Sciences, Montefiore Medical Center, Albert Einstein College of Medicine, Bronx, New York, United States
3Department of Ophthalmology, University of Colorado School of Medicine, Denver, Colorado, United States

PURPOSE. To elucidate the anatomy of the trabecular meshwork (TM) and its connection to ciliary muscle (CM) tendons with two photon excitation microscopic (TPEM) imaging.

METHODS. The human aqueous outflow pathway was imaged in an unfixed and nonembedded state by using an inverted TPEM. Laser (Ti:Sapphire) was tuned at 850 nm for emission. Backscatter signals of second harmonic generation (SHG) and autofluorescence (AF) were collected through 425/30-nm and 525/45 emission filters, respectively. Multiple, consecutive, and overlapping image stacks (z-stacks) were acquired to generate three-dimensional data sets.

RESULTS. Collagen and elastin structures of the TM were successfully visualized with TPEM. The TM and CM tendons were found to contain both collagen and elastin fibers. What appears to be juxtacanalicular tissue (JCT) was identified by its honeycomb-like appearance in AF images. Tracing CM tendons from their origins and to their insertions revealed that elastin fibers of CM tendons were connected to the elastin network within the trabecular lamellae. The CM tendons converged or diverged along their course, forming intricate networks with the TM. The CM tendon fiber density varied depending on its location within the aqueous outflow pathway with tendons near the JCT found to be the most dense, and in a fine-tooth comb arrangement.

CONCLUSIONS. By using TPEM imaging, new details of the human aqueous outflow pathway were elucidated. This high-resolution imaging technique revealed the intricate interconnections between the TM and CM tendons.

Keywords: trabecular meshwork, ciliary muscle, tendon, second harmonic, autofluorescence

Intraocular pressure (IOP) is a function of aqueous humor production and drainage. Most of the aqueous humor drains from the anterior chamber through the trabecular meshwork (TM). This is situated in the scleral sulcus, which extends from Schwalbe’s line to the scleral spur.1 The TM is divided into three distinct layers, from the innermost to the outermost layer: uveal meshwork, corneoscleral meshwork (CSM), and juxtacanalicular tissue (JCT).2–5

The TM is composed of trabecular lamellae and trabecular cells.6 The core structure of the TM lamellae consists of collagen and elastin fibers.7,8 The latter form an extensive network within the TM lamellae, connecting ciliary muscle (CM) tendons, the basement membrane of trabecular cells, as well as the inner wall of Schlemm’s canal.7,9–11 The intricate relationship between the CM tendon and TM was initially described by Rohen et al.10 The longitudinal CM tendons penetrate the TM to reach the inner wall of Schlemm’s canal. These tendons contain collagen and elastin fibers and are connected to TM’s elastic fiber network modulating the expansion of TM and permeability to aqueous humor.7 Contraction of CM moves the sclera spur posteriorly and pulls the TM lamellae inward, leading to enlargement of the potential space within TM and expansion of Schlemm’s canal.7,8,10,11 The CM–scleral spur–TM network is considered the main determinant of aqueous outflow control in primates.7,10–12 A recent study linked shortened scleral spur length and limited posterior movement capability to primary open angle glaucoma eyes, corroborating the complexity of the tissue that is the focus of this study.13

Three different types of CM tendons have been previously reported using a light and electron microscope.8 Type A tendons attach CMs to the sclera and scleral spur. Type B tendons are wide and flat strands, spanning the entire TM, and anchor within the corneal stroma. Type C tendons are incorporated into the outermost CSM or JCT.8 All three tendon types play important roles in the efficient transfer of the force generated from CM contraction to stretch the TM and expansion of Schlemm’s canal. However, owing to artifacts that arise from tissue processing such as dehydration and freezing, in both light and electron microscopic studies, live, three-dimensional configuration of these tendons have not been reported to our knowledge.

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Two photon excitation microscopic (TPEM) imaging typically uses near infrared laser (Ti-Sapphire) with ultra-short pulse duration (femtosecond) allowing for high-resolution images equivalent to confocal microscopy with wider depth of focus and minimal tissue damage. The tissue of interest can be imaged on a flat mount without additional tissue processing, hence preserving tissue relationships found in vivo. Autofluorescence (AF) of TPEM in human TM tissue is an effective way of imaging elastin fibers; however, second harmonic generation (SHG) best portrays collagen structures with minimal confounding from fluorescence attributable to other extracellular materials.

In this report, we evaluated the aqueous outflow pathway by using TPEM in human ex vivo cadaveric eyes. The obtained images elucidate new details of the TM collagen architecture and connection between the TM and subgroups of CM tendons.

MATERIALS AND METHODS

This study was approved by the institutional review board of Albert Einstein College of Medicine, Yeshiva University, and adhered to the tenets of Declaration of Helsinki.

Sample Preparation

Seven eye bank corneal rims were obtained from the Lions Eye Bank, Tampa, Florida, United States. All tissue samples were kept in a storage chamber with Optisol GS transfer media (Bausch & Lomb, Rochester, NY, USA) until imaging. None of the sample tissues had a clinical history of glaucoma (Table).

Two Photon Excitation Fluorescence Microscope Imaging

Second harmonic generation and AF imaging were performed using an inverted TPEM (FluoView FV-1000; Olympus, Central Valley, PA, USA). Tissue samples were placed on glass-bottom plates (35 mm; MatTek, Ashland, MA, USA) with the TM facing down. Laser (Ti-Sapphire) was tuned at 850 nm and emission was passed through a red dichroic mirror (690-nm) filter. A ×25 (numerical aperture = 1.05) water immersion objective was used to focus the excitation beam and to collect backward signals. The backward SHG signal was directed to a dichroic mirror (dm458) and second harmonic light was collected using a 410- to 440-nm bandpass filter. The backward AF signal was directed to a dichroic mirror (dm560) and AF was collected using a 503- to 547-nm bandpass filter. Multiple, consecutive, and overlapping image stacks (z-stack) were acquired using the same objective lens. When z-stacked images were acquired, samples were scanned in 1-μm step sizes in the z-axis to generate three-dimensional data sets. ImageJ software (http://imagej.nih.gov/ij/) provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) was used to analyze the acquired images. Because the inner wall of Schlemm’s canal is not flat, serial tangential sectional images contain oblique sections. Some tissues were incubated with 10 μg/mL Hoechst 33342 (catalog number 62249; Life Technologies, Grand Island, NY, USA) for 5 minutes at room temperature to counterstain nuclei.

Ciliary Muscle Tendon Density Analysis

To compare the tendon density in the different layers of TM, five random regions (513 × 513 μm) were selected from each quadrant and each layer of TM from seven tissue samples. The number of tendons was counted from each selected region and the average number of tendons was compared for JCT, uveal meshwork, and CSM.

RESULTS

The second harmonic signals demonstrated in great detail, the collagen architecture in tangential images (Fig. 1).
FIGURE 2. Second harmonic generation and AF images of the uveal and CSM. Tangential images from the TM area. The uveal meshwork (A–C) exhibits larger intertrabecular lacunae compared with the CSM (D–F). (A–C) Presumed collagen structures are located at the center of lamellae in the uveal meshwork (asterisk). Arrows indicate nodular autofluorescent protuberances in the uveal trabecular lamellae. (D–F) Presumed elastin fibers in the core of the CSM lamellae (arrowhead), with surrounding collagen structures. (A, D) SHG images, (B, E) AF images, (C, F) merged images. Images were taken from sample no. 4.

FIGURE 3. Oblique tangential sections of the TM. Both TM and Schlemm’s canal were imaged in this oblique section. (A–C) Presumed JCT (arrowheads) is situated between the CSM and corneal tissue, lining Schlemm’s canal. (D, E) Magnification of dotted box in (A) and (B). Delicate collagen structures (arrowheads in [D]) connecting the cornea to the trabecular lamellae. Some of these structures are terminal portions of CM tendons. Arrows (E) depict honeycomb-like structures. (A, D) SHG, (B, E) AF, (C) merged, (D, E) is the magnified images of rectangular area in (A, B). Images were taken from sample no. 4.
FIGURE 4. The collagen structure near anterior tip of TM attaching to cornea. The area containing presumed JCT and CSM was imaged by SHG (A) and AF (B). (A) Thick collagen strands (asterisk) bridge the cornea (top line demarcates border with cornea) with the CSM. Part of the CSM is visible in the lower right and shows high SHG and AF signaling. (B) Lacunae (arrows) in presumed JCT. The AF signal is weaker in this region compared with the CSM and no definite lamellar structure is visible. Thick collagen structures in (A) also show diffuse AF signal. (C) Merged image of (A) and (B) shows collagen-dominant (green) and elastin-dominant (red) structures. Images were taken from sample no. 6.

FIGURE 5. Connections between the TM and CM tendons. (A–C) Fibers (arrows) originating from CM ends are shown. These fibers form tendons and contain both collagen (A) and elastin fibers (B). (D–F) In magnified images, tendons (arrows) originating from CM ends, cross the CSM lamellae. Collagen connections, which were difficult to confirm previously (D), reveal a fine elastin network that appears to connect tendons to the TM lamellae, as shown in (E) (dotted squares). Tendon projections from CM tips were noted to bend acutely to attach to the trabecular lamellae (E) (arrowheads). (A, D) SHG images, (B, E) AF images, (C, F) merged images. Images were taken from sample no. 5.
Figure 6. Serial tangential sections (5-μm interval) of the region where CM tendon attaches the cornea (near Schwalbe’s line). Serial sectional images taken from inner (A) to outer (J) TM trace the tendon insertions. Tendon fibers (arrows) from CMs attach to the cornea (asterisks) near Schwalbe’s line. With the image plane moving out, tendon bundles are visible (A, D, G, J). Diffuse AF area marks presumed JCT and its border with the cornea (B, E, H, K). Diffuse and fine elastin structures are visible in this area (dotted oval area) (H, K). Nuclei (Hoechst 33342 stained) occupy the empty spaces in JCT (inset in [K]). (A, D, G, J) SHG images, (B, E, H, K) AF images, (C, F, I, L) merged images. Images were taken from sample no. 6.

Figure 7. The spatial relation between CM tendons and CSM. (A–D) Serial tangential sectional images (1-μm step) from inner to outer CSM reveal tendons (arrows) intertwined with the trabecular lamellae (asterisks). Hoechst 33342-stained nuclei (arrowheads) of trabecular cells occupy the intertrabecular space. Branching of the tendon is noted. AF (red) and SHG (green) were merged. Images were taken from sample no. 6.
Elsewhere, tendons converged or sclera near Schwalbe’s line (Fig. 6), presumably as type B tendons within the aqueous outflow pathway. The average number of density of CM tendon fibers varied depending on their location walls of Schlemm’s canal (Fig. 9; Supplementary Video S2). The vertical collagen lamellae connecting the anterior and posterior trabecular lamellae were also found to be intertwined with CM. The previously reported fine elastin fiber network in JCT was visible in AF images (Figs. 3, 4; Supplementary Video S1). The previously reported fine elastin fiber network in JCT may be the origin of the faint and diffuse AF signal in this area. In this study, we obtained high-resolution images of the human TM and CM tendons by using TPEM. By tracing their entire path, from origin to insertion, we were able to characterize the intricate interconnections between CM tendons and the TM. This was done with AF and SHG imaging in fresh tissue, without artifacts of exogenous labeling.

Our current findings are supported by previous studies.17–20 Elastin fibers in the TM have been previously studied by comparing AF with SHG images.14,18,23 Huang et al.18 found that areas with high AF signals colocalized with both SHG signal-voids and eosin- (elastin staining) labeled fluorescent areas. Others have shown that elastin fibers form a network distributed throughout the JCT.19,24 The presumed elastin and collagen fibers were classified based on their imaging characteristics; however, the actual fiber types were not verified via immunohistochemistry. The “presumed” collagen fibers most likely represent fibrous collagenous structures with multiple collagen subtypes. It is also known that some elastin fibers are surrounded by a sheath of type VI collagen.25 The clinical relevance of this is that their morphology changes in pathologic conditions. The shear is known to thicken with age and in primary open angle glaucoma.7 One suggested role of the elastin fiber network is to connect CM tendons to the TM, establishing a CM-TM functional unit.7,24,26

In our study, collagen fiber connections between CM tendons and trabecular tissue were not verified. Considering the dynamic nature of the CM-TM functional unit, our finding suggests that the elastin network is the key connection between these two structures. However, trabecular tissue contains other extracellular components; therefore, further study is necessary. Particularly, the dynamic change of the tendon-lamellae complex in response to experimentally
induced CM contraction will be helpful in clarifying the tendons’ role. The minimally invasive nature of TPEM would permit real-time tracking changes of tendons and trabecular tissue in response to a specific stimulus.

Juxtacanalicular tissue is a thin layer, 2 to 15 μm in thickness, between the inner wall of Schlemm’s canal and the outermost layer of the CSM. This layer, sometimes referred to as the cribriform layer, is highly porous.27 Because the height of Schlemm’s canal is shorter than the height of the TM, the anterior part of the JCT is thinner and nonfunctional, whereas the posterior part of the JCT is thicker and functional. There was no landmark to represent the JCT in TPEM analysis. Previously, Tan et al.23 used 6-0 nylon suture cannulation to discriminate Schlemm’s canal from the JCT. However, this technique can deform normal TM architecture.

In our study, the JCT was defined by its anatomical position and was designated as the area between the last layer of the CSM and cornea or sclera. In this area, we did not see the prominent array of fibers that was evident in the CSM. Instead, diffuse AF signaling was observed, which may represent the elastic fiber network that was previously described as an “elastic fiber-like plexus” or “cribriform plexus” by Rohen et al.8 Additionally, AF signal-void pores in the presumed JCT were found to be occupied by cells. We also found that collagen fibers in the distal portion of CM type B tendons crossed the JCT to attach to the cornea or sclera. We propose

![Figure 9](image_url)

**Figure 9.** Oblique tangential sectional images (2-μm step) of TM and CM tendon. (A) Three-dimensional reconstruction of z-stack images (17-μm thickness) is shown with orthogonal views (horizontal and vertical crossing the center). (B–G) SHG images were taken from near inner wall (B) to outer wall (G) of Schlemm’s canal. The outermost CSM is visible (small arrows [B, C]). Ciliary muscle tendons (arrowheads) connecting the roof and the floor of Schlemm’s canal (signal-void area, asterisks) are densely bundled. Schlemm’s canal is visible between tendons (asterisk [D–F]). One thick collagen structure (open arrow) can be traced to the CSM (B) eventually merging with collagen structures constituting the outer wall of Schlemm’s canal (F). These collagen structures are scleral fibers and appear distinctly different from their tendon counterparts. (G) Outer wall of Schlemm’s canal is lined with thick scleral collagen fibers. Images were taken from sample no. 1.
that the elastic fiber network is the web that holds together the inner wall of Schlemm’s canal, the JCT, and CM tendons.\(^7\)\(^{24}\)

Another interesting finding of our study is that CM tendons are densely distributed in a fine-tooth comb pattern. Our results suggest that the relation between tendons and trabecular lamellae is more complicated than previously described.\(^7\) They are connected three-dimensionally by an extensive elastic network and the CSM lamellae are interwoven.
with tendons. Additionally, we found that the density of tendon structures increased from the CSM to the JCT, with the most compact arrangement observed near the inner wall of Schlemm’s canal. The lack of a definite mechanical structure of inner wall in Schlemm’s canal has raised the question of how the canal can remain patent. The increased density of CM tendons suggest that they serve as the scaffold for the superficial TM lamellae. They can bolster the JCT and prevent collapse of the inner wall of Schlemm’s canal in the densely packed deep TM area.

The major advantage of TPEM is eliminating distortion from fixation, dehydration, and mechanical sectioning. In conventional light or electron microscopy, tissue alteration with fixative and freezing can distort the normal architecture of TM. However, near infrared wavelength in SHG imaging enables optical sectioning of each layer of TM. Clear images are acquired without disrupting or distorting three-dimensional relationships, which will allow for volumetric analysis of specific targets based on their three-dimensional presentation in situ. However, lack of perfusion and IOP during imaging and isolating the tissue from surrounding sclera compromises the exact representation of TM morphology under normal physiologic conditions.

Our study has several limitations. Donor corneas were harvested without anterior segment perfusion, potentially distorting the architecture of TM and CM tendons with postmortem changes. In addition, future investigation of individual molecular requirements of different types of CM tendons by using immunohistochemistry may enhance our understanding of TM physiology. The lack of dynamic studies of CM tendons and Schlemm’s canal changes after pharmacologic intervention is another drawback. This may provide a new insight about dynamic regulation of aqueous humor outflow. However, our current study can serve as a platform for future dynamic investigation.

In summary, we elucidate the aqueous outflow pathway with TPEM. The entire path of CM tendons was traced from the muscle ends to their insertion sites. In addition, the intricate interconnections between the TM and CM tendons were demonstrated. The implications, both morphologic and functional, are discussed. Further work applying this imaging modality to record CM tendons’ dynamics in disease, or with pharmaceutical interventions can enhance our understanding of the regulation of aqueous humor outflow.

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