Label-Free In Vivo Imaging of Corneal Lymphatic Vessels Using Microscopic Optical Coherence Tomography

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Purpose. Corneal neovascularization, in particular lymphangiogenesis, is a limiting factor in corneal transplant survival. Novel treatment approaches focus on (selective) inhibition and regression of lymphatic vessels. Imaging clinically invisible corneal lymphatic vessels is a prerequisite for these strategies. Using a murine model, this study investigates whether corneal lymphatic vessels can be imaged using microscopic optical coherence tomography (mOCT).

Methods. Corneal neovascularization was induced by intrastromal placement of 11.0 nylon sutures in one eye of BALB/c mice. After 2 weeks, cross-sectional images and volumes of the corneas with a 0.5 mm lateral and axial field of view were acquired using a custom-built mOCT system enabling a resolution of 1 μm at a B-scan rate of 165/s. Three of the six animals received an additional intrastromal injection of India ink 24 hours before the measurement to stain the corneal lymphatic system in vivo. Immunohistochemistry using CD31 and LYVE-1 was used to validate the mOCT findings.

Results. Using mOCT, lymphatic vessels were visible as dark vessel-like structures with the lumen lacking a hyperreflective wall and mostly lacking cells. However, individual, slowly moving particles, which most likely are immune cells, occasionally could be observed inside the lumen. In lymphatic vessels of ink-stained corneas, hyperreflection and shadowing underneath was observed. Ink-filled lymphatic vessels were colocalized in consecutive corneal flat mounts of the same specimen.

Conclusions. Corneal lymphatic vessels can be imaged using mOCT. This novel approach opens new options for noninvasive clinical imaging of corneal lymphatic vessels for diagnostic and therapeutic indications.

Keywords: optical coherence microscopy, optical coherence angiography, cornea imaging, corneal lymphatic vessels, lymphangiogenesis

The avascularity of a healthy cornea allows light transmission toward the retina. However, pathologic hem- and lymphangiogenesis may occur due to disease, infection, or injury.1 This may cause reduced visual acuity2 and ultimately blindness or lead to graft rejections after corneal transplantation.3 Anti-inflammatory drugs currently are under investigation with the aim of preventing these complications.4–7 Visualization and quantification of angiogenesis is mandatory to assess the therapeutic effects of these drugs, during drug development toward the retina. However, pathologic hem- and lymphangiogenesis may occur due to disease, infection, or injury. This may cause reduced visual acuity2 and ultimately blindness or lead to graft rejections after corneal transplantation.4 Anti-inflammatory drugs currently are under investigation with the aim of preventing these complications.4–7 Visualization and quantification of angiogenesis is mandatory to assess the therapeutic effects of these drugs, during drug development or at the clinic.5,6

While pathologic corneal blood vessels are visible clinically using slit-lamp biomicroscopy, lymphatic vessels are invisible due to their thin walls and transparency of the lymphatic fluid.4 For this reason, imaging with conventional light microscopic methods is extremely challenging. In contrast to blood vessels, relatively little is known about lymphatic vessels. In this respect, the transparent corneal tissue can be used as a model to investigate the anatomy and physiology of the lymphatic vascular system.

Currently, ex vivo human corneal grafts or corneal tissue samples from animals are investigated by histology and immunostaining.8 In vivo, contrast-enhancing tracers must be used to enable optical, radiologic, or nuclear medical imaging.9–13 Label-free in vivo imaging is made feasible by microscopic optical methods, including confocal microscopy, which offers high lateral resolution and even enables single cell analysis in lymphatic vessels.14 However, due to limitations on speed, the acquisition of three-dimensional (3D) volumes using confocal microscopy is time-consuming and vulnerable for axial motion artifacts, which are hard to correct. Alternatively, laser speckle imaging can visualize lymphatic vessels,15 but has no depth resolution. Today, two-photon microscopy (TPM) is the method of choice for intravital imaging of lymphatic vessels, allowing key players of inflammation and the transmigration of immune cells into functional lymphatic vessels to be visualized over time in 3D image volumes.16 However, complex and expensive components and concerns about safety presently restrict TPM to preclinical research in animals.
In contrast with TPM, optical coherence tomography (OCT) is a clinically established method that is used widely in ophthalmology. The general ability of OCT to image lymphatic vessels, for example, in mouse or human skin, has been demonstrated by different groups. Their segmentation method has been based on the analysis of statistical properties of the noise due to the absence of an OCT signal in the lymphatic vessel in adjacent B-Scans. However, because this method detects signal-free regions, it will not work in sparse structures, such as the stroma of the cornea. The resolution in these studies was marginal and the vessel networks could not be reconstructed thoroughly. Direct flow measurements in lymphatic vessels by Doppler OCT are possible, but the low flow-velocities or even the absence of flow in lymphatic vessels render OCT angiography-based detection of lymphatic vessels difficult and unreliable.

Increasing axial and lateral resolution by broad band light sources and the use of high numerical aperture (NA) imaging objectives are able to push OCT resolution into the cellular regime. However, increasing the lateral resolution decreases the axial field-of-view. It has been demonstrated that different strategies can be used to decouple lateral resolution and depth of field. Interferometric synthetic aperture microscopy (ISAM) and holoscopy use phase-based reconstruction algorithms to achieve full lateral resolution over an arbitrary depth, but the former is not fast enough and the latter suffers from multiply-scattered light. It has been shown that using Bessel or Bessel-Gauss beams can elongate the focus considerably, but there is a reduction in sensitivity and disturbing sidebands arise in the point spread function. The annular shaped illumination of micro-OCT works similarly to Bessel beam illumination and has achieved 2 μm resolution over an extended field. In our study, we use a custom-built microscopic OCT (mOCT) with an optimized NA of 0.5 and an axial resolution of 1 μm. An excellent image quality was achieved, although lateral resolution degraded outside the focal plane. By increasing resolution and speed, mOCT enables the visualization of morphologic structures at a cellular level including lymphatic vessels.

We demonstrated the feasibility of our custom-built mOCT system to visualize lymphatic vessels in vivo, label-free, and in 3D in vascularized murine corneas. Successful characterization of lymphatic vessel morphology and its quantification will support research in testing antilymphangiogenic drugs, the investigation of graft rejection, and will help to monitor patients suffering from corneal neovascular diseases prospectively.

**METHODS**

**mOCT System**

The custom-built Fourier domain system for microscopic OCT (Fig. 1) used in this study achieves a resolution of approximately 1 μm in all spatial dimensions. For a high axial resolution, a supercontinuum light source (EXW-4 OCT; NKT Photonics, Birkerød, Denmark) is used. Approximately 100 mW output in the spectral range from 500 to 1000 nm was coupled via a spectral split box (SuperK Split; NKT Photonics) into a single mode fiber (FD-7; NKT Photonics). The output of the fiber was collimated onto a pair of galvanometer scanners (6210H; Cambridge Technology, Bedford, MA, USA) and then imaged to the back focal plane of a 0.5 NA microscope immersion objective (HCX APO L20x/0.5 W UV-1; Leica, Wetzlar, Germany). Backscattered light from the cornea was detected by a custom-built spectrometer (Thorlabs GmbH, Dachau, Germany), which supports a spectral range of 550 to 950 nm. The spectrometer has 2048 spectral channels and an A-Scan rate of 127 kHz can be achieved. The axial imaging range was 0.71 mm in air, and the sensitivity reached 85 dB.

In this study, 3D volumes with 512 × 512 A-scans and time series of 2D cross-sectional images with up to 165 fps B-scan-rate (512 A-scans per B-scan) were acquired. The OCT data were evaluated by ThorImage (Thorlabs GmbH). In some datasets, a subsequent averaging from three to five images was performed using the public domain software ImageJ. The region growing method was applied to conduct volumetric segmentation of lymphatic vessel segments using the public domain medical image processing and visualization toolbox MeVisLab (MeVis Medical Solutions AG, Bremen, Germany). For region growing, it was necessary to set seed points manually within the vessel. 3D volumes were visualized by IMARIS 7.6 (Bitplane, Zurich, Switzerland).

For comparison purposes, OCT images of the whole anterior segment of the eyes were acquired with a prototype of a small animal OCT (HSM01, OptoMedical Technologies GmbH, Lübeck, Germany), reaching a resolution of 6 μm axial and 12 μm lateral.

**Animal Model**

For animal experiments, female BALB/c mice aged 6 to 8 weeks were used. The local animal care committee approved all animal protocols, which were in accordance with the Association for Research in Vision and Ophthalmology’s (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

The following procedure was performed to induce inflammatory corneal neovascularization as described previously. Each animal was deeply anesthetized with an intraperitoneal injection of Ketanest S (8 mg/kg) and Rompun (0.1 mL/kg). Three 11-0 nylon sutures (Serag Wiessner, Naila, Germany) were placed intrastromally with two stromal incisions extending over 120° of corneal circumference each (Fig. 2). The outer suture point was placed near the limbus, and the inner suture point near the corneal center, equidistant from the limbus, to obtain standardized angiogenic responses. Sutures were left in place for 14 days.

Three of the six mice received an additional intrastromal injection of India ink (Drawing Ink N° 17, Pelikan, Hannover, Germany) into the center of the cornea (Fig. 2) 24 hours before intravitral mOCT imaging. By these measures, the ink was shown to be taken up by the developing lymphatic vessels. Imaging was performed under long-term anesthesia with 7% fentanyl (50 mg/mL; Bayer Health Care, Leverkusen, Germany), 9% midazolam (5 mg/mL; Curamed, Karlsruhe, Germany), and...
4.5% medetomidin (Domitor, 1 mg/mL; Pfizer, Berlin, Germany) in 400 μL NaCl per hour intraperitoneally. The animals underwent tracheotomy and were ventilated (MiniVent; FMI, Seeheim-Ober-Beerbach, Germany). Tracheotomy and ventilation were required because the applied long-term anesthesia included relaxation of muscular activity preventing sufficient spontaneous breathing of the animals. The chosen ventilation rate corresponded to the normal breathing rate of approximately 150 strokes per minute and was not changed or discontinued during image acquisition. The animals were placed in a heated (37°C) animal holder (MouseFix; Steven GmbH, Ochtrup, Germany) below the immersion objective used for mOCT imaging. To ensure optical coupling and avoid ocular surface desiccation, artificial tear gel (Vidisic; Bausch + Lomb, Berlin, Germany) was used. After the experiments, the mice were euthanized and the corresponding eyes were enucleated for histology as described below.

Immunohistochemistry

Corneas were harvested at the end of the experiment. Corneal flat mounts were rinsed, fixed in acetone, rinsed in PBS and blocked with 2% bovine serum albumin. Staining was performed with FITC conjugated CD31 (Santa Cruz Biotechnology, Heidelberg, Germany), FITC conjugated CD11c (Biolegend, San Diego, CA, USA), unconjugated rabbit anti-mouse LYVE-1 (AngioBio, San Diego, CA, USA), or CD3 (Abcam plc, Cambridge, UK).
Cambridge, UK) overnight. The next day, corneas were washed in PBS, stained with anti-rabbit Cy3 (Jackson Immuno Research, West Grove, PA, USA), washed and mounted onto slides using fluorescence mounting media (DAKO, Santa Clara, CA, USA). Images were taken with a fluorescence microscope (BX53; Olympus, Hamburg, Germany) and a confocal laser scanning microscope (Meta 710, Carl Zeiss Meditec, Jena, Germany).

RESULTS

mOCT Performance

Due to use of the high NA microscope objective, a broad-band light source, index matching, and the high transparency of the cornea, a resolution of approximately 1 μm in all dimensions was achieved, which has been verified experimentally in the focal plane using nonscattering phantoms. Scattering or the presence of defocus outside the focal plane decreases the effective resolution. To minimize motion artifacts due to respiration, acquisition times were kept as short as possible. At the given A-scan rate of 127,000/s, a B-scan rate of 165/s was possible when acquiring 500 A-scans per B-scan. Consequently, depending on the desired resolution, a volumetric acquisition took, for example, three seconds. For an adequate lateral sampling and an adequate acquisition time, the maximum size of the field of view was 0.5 mm, although larger fields were possible at the expense of resolution or speed. The axial imaging range was 0.5 mm in tissue.

Due to the high lateral resolution, the depth of field for optimal resolution was reduced to approximately 8.5 μm. For optimal imaging of the full cornea, the focal plane must be shifted to detect different layers (see different focal positions in Figs. 3B–D). The layer with the highest lateral resolution can be selected from the 3D data set in post-processing. In contrast to the lateral resolution, the axial resolution remains constant over the full image depth. Individual cells and collagen structures were visible (Figs. 3B, 3C), although speckle noise may mask very small cell-like structures.

Imaging of Naive Pathologic Corneal Blood and Lymphatic Vessels

Under mOCT imaging, dark structures appeared in the same regions of the cornea where blood vessels were visible. The structures were extended with a vessel-like shape (Fig. 4A). The cross-sections of most observed voids were elliptical (data not shown). With 10 to 20 μm, the length of the shorter axis was approximately half that of the long axis, which was between 25 and 35 μm. In contrast with blood vessels, which had a relatively fast and dense flow of blood cells, the hyporeflective tubes were sparsely filled with cells (see Supplementary Video S1) or even empty (Fig. 4A). They furthermore lacked hyperreflective vessel walls, which typically are seen in blood vessels (Fig. 4B).

In minimum intensity projections of the recorded image volumes, long continuous structures were visible (Fig. 5A), which we consider to be lymphatic vessels due to their specific morphology. After semiautomatic segmentation as described above, the structures can be visualized within the recorded image volume (Fig. 5B, Supplementary Video S2).

The spatial and temporal resolutions were high enough to visualize the flow of individual cells in blood and lymphatic vessels in time series of B-scans. Besides erythrocytes, cells that were slowly rolling along the blood vessel wall also were observed (see Supplementary Videos S1, S3).

![Figure 4](http://arvojournals.org/)

**Figure 4.** Longitudinal mOCT cross-section of a naïve corneal lymphatic vessel (not averaged; [A]) and a corneal blood vessel (5× averaged; [B]). Lymphatic vessels appear as dark, mainly cell-free structures, whereas blood vessels are densely populated with fast-flowing cells. In contrast with lymphatic vessels, blood vessels exhibit hyperreflectivity in the endothelium and vessel wall (see Supplementary Fig. S3 for real-time flow movie).

![Figure 5](http://arvojournals.org/)

**Figure 5.** (A) Minimum intensity projection for visualization of the morphology of a lymphatic vessel in full length within the imaged volume. (B) Overlay of original mOCT data (white) with a segmented lymphatic vessel segment (red) as a 3D projection (see Supplementary Fig. S2 for more views of the volume).
Imaging of Ink-Stained Lymphatic Vessels

In animals that received an intrastromal injection of ink 24 hours before imaging, mOCT exhibited increased reflections at the rims of the ink depots and a significant shadowing of the structures below (Fig. 6A). In addition, spurious signals were observed at the upper rims of the image and throughout the A-scans. They are caused by self-interference and signal saturation due to the high reflection at the ink-tissue interface. At the OCT wavelength used, India ink hardly scatters at all, yet it is strongly absorbent and reflective at the interface to the tissue (data not shown). Hence, ink-filled volumes appear void. Only the interface of the ink with the surroundings creates strong OCT signals due to the ink’s significantly higher index of reflection. The ink absorption masks all structures below it. In the ink-injected animals, lymphatic vessels also exhibited strongly hyperreflective walls, shadowing of the structures underneath them, and characteristic signals at the upper rim of the images (Fig. 6B).

Histological Correlation

To confirm the intravital finding that the injected ink is drained by corneal lymphatic vessels, we performed immunohistochemical stainings on corneal flat mounts. Eyes with vascularized corneas that were injected with India ink were harvested, and corneas were stained with LYVE-1 for lymphatic vessels and CD31 for blood vessels as described above. Afterwards an overlay of the fluorescence images depicting the specific staining for blood and lymphatic vessels and the bright field image showing the India ink as dark structures was recorded. Thereby, we could demonstrate the colocalization of LYVE-1+ vessel-like structures with India ink in the cornea (Fig. 7).

DISCUSSION

Imaging perfused blood vessels in human corneas is feasible using the strong optical absorption of blood and the Doppler signal caused by motion of blood cells. In contrast, imaging lymphatic vessels remains a challenge, due to the lack of absorption and motion contrast. We demonstrated the feasibility of high resolution mOCT to image lymphatic vessels in vivo, in 3D, and label-free at a cellular level using a murine model for corneal neovascularization. The high resolution of approximately 1 µm in all spatial dimensions allows identification of lymphatic vessels and demarcation from blood vessels using the following criteria.

Due to the transparency of lymphatic fluid, lymphatic vessels appeared dark compared to the surrounding tissue. Furthermore, in contrast with blood vessels, which exhibit a thick and hyperreflective vessel wall, no visible wall could be determined in case of lymphatic vessels. Although a resolution of 1 µm should allow visualization of the simple squamous cell
The layer of the lymphatic endothelium within the focal plane, the thin single cell layer was not visible with mOCT. Probably, lack of sufficient contrast and masking by speckle noise impeded visualization.

An additional, but not sufficient criterion to differentiate lymphatic from blood vessels is the number of cells inside. We observed most lymphatic vessels empty; in some cases, however, individual cells, flowing at slow speed, were found, which is in line with former reports.14 Altogether, the above listed features typically are attributed to lymphatic vessels14,19,20 and mOCT has the resolution to image them clearly.

However, few or nonflowing cells and consequently low intensity regions also may result from nonperfused blood vessels or edema, which can be interpreted falsely as lymphatic vessels. While the absence of a clearly visible and relatively thick hyperreflective vessel wall is proposed as a main criterion for the demarcation of lymphatic and blood vessels, the number of cells and their flow speed could be used as secondary criteria using a score system.

Furthermore, the achieved resolution and image quality allowed the volumetric segmentation of lymphatic vessel segments as negative contrast structures at high accuracy. This is the basis for identification of lymphatic vessels by their characteristic continuous tubular structures and a prerequisite for the desired quantification of vessel networks.

All derived criteria for the demarcation of blood and lymphatic vessels using mOCT are summarized in the Table.

After the uptake of India ink, the observed voids displayed hyperreflective walls and shadowing underneath. Furthermore, spurious signals were observed at the upper rims of the image and throughout the A-scans. These features are characteristic of ink accumulation in tissue volumes and suggest that the empty tubular structures observed with mOCT are part of the lymphatic system.

The visualization and identification of lymphatic vessels in this proof-of-concept study was made feasible by the outstandingly high resolution, which was achieved by an immersion objective with a high numerical aperture. Consequently, the depth of field was comparatively small, and only within a part of the axial image range is the image as bright and clear as desired. In addition, the lateral field of view was limited (e.g., 500 × 500 μm²). Hence, mOCT enabled a detailed analysis of the size and shape of individual vessel segments, but not of vessel networks. Imaging the full vessel network would require lateral and axial stitching of sequentially acquired volumes. This would be possible in animal studies, but difficult in clinical applications.

To increase applicability, contact-free imaging without immersion would be desirable. We found that an acquisition speed of 127,000 A-Scans/s was sufficient to prevent motion artifacts and to enable volumetric imaging in the immobilized mice. Clinical application, however, presumably would require an even higher imaging speed and use of an eye tracker or sophisticated algorithms for motion correction. For this, live B-scans for locating and differentiating lymphatic and blood vessels using flow characteristics and the signature of vessel walls could be combined with volumetric imaging at a lower resolution for analyzing morphology and network topology. Locating, identifying, and finally quantifying the corresponding structures to gain all desired information then would be done iteratively.

In conclusion, corneal lymphatic vessels can be imaged by mOCT. Due to the transparency of the lymphatic fluid and their thin walls, lymphatic vessels appear as dark vessel-like regions without typical vessel wall features in cross-sectional scans. Lymphatic vessel structures can be made visible by minimum intensity projections of image volumes or by 3D semiautomatic segmentation of the voids.

Compared to existing approaches, the resolution of mOCT is comparable to confocal microscopy; however, mOCT enables fast volumetric imaging. Unlike two photon microscopy, mOCT is safe and based on clinically established OCT. With respect to other OCT approaches for imaging lymphatic vessels, the outstandingly high axial and lateral resolution allows direct identification of lymphatic vessels at a cellular level in the context of the surrounding tissue using specific morphologic features.

In summary, mOCT with a resolution of approximately 1 μm allows for label-free noninvasive intravital imaging of pathologic corneal lymphatic vessels, which is of high interest in research and in the clinic, for example, to test antilymphangiogenic drugs, investigate corneal graft rejection, and monitor patients suffering from corneal neovascular diseases. We currently are working on translation of the mOCT technique to a clinically useful device that can be applied to humans.

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