250 kHz, 1.5 µm resolution SD-OCT for in-vivo cellular imaging of the human cornea

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Abstract: We present the first spectral domain optical coherence tomography (SD-OCT) system that combines an isotropic imaging resolution of ~1.5 µm in biological tissue with a 250 kHz image acquisition rate, for in vivo non-contact, volumetric imaging of the cellular structure of the human cornea. OCT images of the healthy human cornea acquired with this system reveal the cellular structure of the corneal epithelium, cellular debris and mucin clusters in the tear film, the shape, size and spatial distribution of the sub-basal corneal nerves and keratocytes in the corneal stroma, as well as reflections from endothelial nuclei. The corneal images presented here demonstrate the potential clinical value of the new high speed, high resolution OCT system for non-invasive diagnostics and monitoring the treatment of corneal diseases.

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1. Introduction

Corneal blindness is the fourth leading cause of world blindness after cataract, glaucoma and age-related macular degeneration, with the greatest burden falling on developing countries, where it is estimated that > 90% of corneal blindness exists [1–3]. The etiology of corneal blindness is very broad: from infections and inflammations (bacterial, fungal and viral keratitis, trachoma, Mooren's ulcer and Steven's Johnson Syndrome) to inherited (stromal and endothelial (Fuch's) dystrophies) and degenerative (Keratoconus) conditions, to trauma [4] (mechanically or chemically induced). These conditions can affect adversely the morphology and physiology of either the entire cornea or can be limited primarily to selected regions of the anterior, middle or posterior cornea. At the early stages of development, corneal pathologies alter the morphology of corneal tissue at a cellular level, such as changes in the size, shape and spatial distribution of epithelial and endothelial cells, or keratocytes in the corneal stroma. Other changes affect the innervation of corneal tissue in terms of the length, tortuosity and density of corneal nerves [5,6]. Early diagnostics of corneal diseases is crucial to assigning the most effective therapy that can result either in cure or in slowing down the progression of incurable pathologies.

Currently, slit lamp biomicroscopy is the most routinely used technology for clinical evaluation of corneal pathologies, primarily due to its affordable price, ease of operation and the ability to view in real time the entire surface of the cornea. However, slit lamp biomicroscopes can examine only the most anterior layers of the cornea and offer very limited spatial resolution that is not sufficient to observe the cellular structure of corneal tissue. In-vivo confocal microscopy (IVCM) can be used for volumetric imaging of the human cornea and offers very high lateral resolution, sufficient to observe individual cells and even cellular nuclei in corneal tissue [5,7]. However, IVCM has a number of limitations: a) IVCM imaging requires physical contact with the corneal epithelium that can cause patients' discomfort or
pain, and increase the risk of infections and abrasions; b) limited field of view (typically \( \sim 400 \mu m \times 400 \mu m \)), therefore, imaging larger areas of the cornea requires acquisition and subsequent “stitching” of multiple images, thus increasing significantly the image acquisition and processing time; and c) depth location ambiguity due to poor axial resolution and slow scanning in axial direction relative to the fast axial eye motion.

Optical coherence tomography (OCT) is a non-invasive optical imaging method capable of generating cross-sectional and volumetric images of biological tissue with cellular level resolution [9,10]. Over the past 25 years, OCT has found a wide range of biomedical applications [10], including imaging of the human cornea in health and disease [11,12]. Recent developments of broad-bandwidth light sources and high speed, large pixel number cameras resulted in development of spectral domain (SD) and full field (FF) ultrahigh resolution OCT (UHR-OCT) technology with axial resolution close to or below 1 µm, suitable for imaging the cellular and sub-cellular structure of biological tissue [13–17]. When used for corneal imaging, UHR-OCT is able to visualize \textit{in-vivo}, identify and in some occasions even measure the thickness of some of the major corneal layers such as the epithelium (EPI), the Bowman’s membrane (BM), the stroma (STR) and the Descemet’s membrane (DM) the DM–endothelial complex (DEC) [17–21]. It is also able to image \textit{ex-vivo} [22] and \textit{in-vivo} [19,21] and the cellular structure of different corneal layers, visualize \textit{in-vivo} [21] and count [23] keratocyte cells in the corneal stroma, measure \textit{in-vivo} physiological changes in the cornea [24], assess keratoconus induced damage to the human cornea at cellular level [19,25], as well as quantify the tear film thickness and track tear film dynamics [26]. Mazlin et.al [21] utilized a novel FF-OCT image \textit{in-vivo} healthy human cornea and were able to image keratocytes and nerves in the corneal stroma, as well as endothelial cells. However, due to the limited axial resolution and the susceptibility of the FF-OCT technology to strong reflections arising from scattering of the imaging beam from the collagen structure of the corneal BM, they were not able to image the tear film and the cellular structure of the corneal epithelium. Recently, our research group reported the use of a sub-micrometer axial resolution SD-OCT system to visualize \textit{in-vivo} for the first time the corneal pre-Descemet’s layer (PDL, also referred to as the “Dua” layer in some publications), which in the past has been identified and measured only in \textit{ex-vivo} human corneal preparations [27]. The Descemet’s membrane and the corneal endothelium were also clearly identified in cross-sectional UHR-OCT images in that study, and the thickness of all 3 posterior corneal layers was measured \textit{in-vivo} in healthy subjects. Furthermore, our group utilized the same UHR-OCT technology to image the cellular structure of the corneal epithelium in healthy and KC corneas, to assess the damage to the corneal stroma in subjects with mild to advanced stages of KC [19], as well as generate \textit{in-vivo} and without tissue contact, volumetric images of the limbal crypts and Palisades of Vogt in the healthy human limbus [28]. However, due to the fairly slow camera readout rate (34 kHz) of the UHR-OCT technology used for these studies [19,27,28], it was difficult to generate volumetric images of the cellular structure of the human cornea and limbus without imaging artefacts generated by the fast eye motion.

To image the cellular structure of the human cornea with UHR-OCT in clinical environment \textit{in-vivo}, volumetrically, over a wide filed-of-view and with minimal effect of eye motion related image artefacts, it is important for the OCT technology to provide both micrometer scale spatial resolution and high image acquisition rates. Swept source OCT technology (SS-OCT) offers impressive A-scan rates in the order of MHz [29]. However, the axial resolution of SS-OCT systems is typically \( > 5 \mu m \) in biological tissue, due to the limited spectral range of the tunable light sources available for the 800 nm and 1060 nm spectral regions. Spectral domain OCT (SD-OCT) systems with close to 1 µm axial resolution in biological tissue and A-scan rates of up to 70 kHz have been reported for various biomedical applications including corneal imaging [15–17, 20]. Furthermore, high speed CMOS cameras have also been utilized in SD-OCT systems to generate A-scan rates of up to \( \sim 500 \) kHz [30–33]. However, the axial OCT resolution in those cases was in the range of 3 µm to 8 µm,
insufficient to image in-vivo and volumetrically the cellular structure of biological tissue. Moreover, the limited number of illuminated camera pixels and small pixel size (typically 10 µm × 10 µm), resulted in significantly shorter scanning range and large signal to noise ratio (SNR) roll-off.

Here we describe in detail the optical design of a fiberoptic SD-OCT system that offers isotropic imaging resolution of ~1.5 µm in biological tissue, sufficient to visualize the tissue cellular and sub-cellular structure. The system utilizes a new 2048-pixel linear array CMOS camera that combines a 250 kHz readout rate with a tall pixel design, in order to generate rapid volumetric in-vivo images of biological tissue with reduced motion related artefacts. Both test data of the system’s performance and images acquired in-vivo with it from the corneas of healthy human subjects, are reported.

2. Methods

2.1 Design of the high-speed UHR-OCT system

The UHR-OCT system is based on a compact fiber optic Spectral Domain OCT design (Fig. 1). A 50/50 broad bandwidth, spliced fiber coupler (Gould Fiberoptics, USA) serves as the core of the Michelson interferometer. The system is powered by a femtolaser (Femtolasers GmbH, Austria) that provides a broad bandwidth spectrum centered at ~800 nm. A spool of ~100 m long fiber is connected between the femtolaser and the core of the UHR-OCT system to stretch the femtosecond pulses enough to approximate CW emission. The reference arm of the UHR-OCT system is comprised of a fiber collimator (achromat doublet, f = 10 mm, Thorlabs, USA), a neutral density filter (NDF), a beam expander (achromat doublets, f = 80 mm and f = 40 mm, Thorlabs, USA), a focusing lens (achromat doublet, f = 10 mm, Thorlabs, USA) and a mirror mounted on a miniature manual translation stage (Edmund Optics, USA). A custom dispersion compensation unit (Fig. 1, DCP) comprised of 2 BK7 prisms (Edmund Optics, USA) mounted on miniature translation stages is placed at the entrance of the beam expander to allow for passive, manual dispersion compensation. The group optical components in the reference arm marked with the dashed-line rectangle in Fig. 1 are mounted on a 12” manual translation stage (Edmund Optics, USA). The sample arm of the UHR-OCT system consists of a fiber collimator

![Fig. 1. Schematic of the 250 kHz SD-OCT system. CL – collimator; DCP – dispersion compensation prisms; FC – broadband fiber coupler; FFT – fast Fourier transform; L1 to L4 – broadband achromat doublets; M – mirror; MEFO - Multi-element Focusing Objective; MO – microscope objective; NDF – neutral density filter; PC – polarization controllers, X,Y – a pair of galvanometric scanners; VPHG – volume phase holographic grating.](image-url)

(achromat doublet, f = 10 mm, Thorlabs, USA), a pair of galvanometric scanners (Cambridge Technologies, USA), a beam expander (achromat doublets, f = 40 mm and f = 80 mm, Thorlabs, USA) and a 10×/0.26 NIR corrected microscope objective. Fiber optic polarization controllers are used to optimize the shape and amplitude of the system’s point-spread-function (PSF). The detection end of the UHR-OCT system is comprised of a customized, commercially available spectrometer (Cobra-S 800, Wasatch Photonics, Durham, USA) integrated with a 2048-pixel, monochrome, line-scan CMOS camera (OCTOPLUS CL, e2v, Teledyne Dalsa, Canada). The camera offers a tall pixel design (10 µm x 200 µm) and a maximum readout rate of 250 kHz. A frame grabber (X64 Xcelera-CL + PX8 Full, Teledyne Dalsa, Canada) is used to acquire images at the maximum camera rate. A custom LabVIEW code was developed for operation of the UHR-OCT system. For in-vivo imaging of the human cornea, the optical power of the imaging beam was set to ~800 µW, significantly lower than the maximum permissible exposure power as specified by the ANSI (American National Standards Institute) standard [34].

2.2 Imaging procedure

This study was approved by the Research Ethics Committee at the University of Waterloo and was carried out in compliance with the tenets of the Declaration of Helsinki. Healthy subjects (n = 10), aged 20 to 45 years, were recruited for this study. All subjects passed a slit-lamp biomicroscopy screening and provided written consent for participation in the study. During the OCT imaging sessions, a head band and a fixation target were used to minimize the subject’s head and eye motion. Cross-sectional (1000 A-scans × 1024 pixels) and volumetric (750 A-scans × 750 B-scans x 1024 pixels) images were acquired from ~0.75 mm × 0.75 mm regions of the cornea, located slightly inferiorly with respect to the corneal apex in order to avoid direct reflection artefacts at the corneal apex. The acquisition time for a volumetric OCT image of 750 A-scans × 750 B-scans was ~2.8 s, taking into account the 80/20 raster scan ratio. Because of the limited depth-of-focus of the microscope objective, the OCT imaging probe was adjusted manually in axial direction to a specific depth location of the cornea and the reference path-length was adjusted accordingly. Multiple volumetric OCT images were acquired from different depth locations in the cornea from each of the subjects.

2.3 Image processing

Images were generated from the raw OCT data and numerically dispersion compensated up to the 5th order with a custom MATLAB algorithm. A cross-correlation algorithm was applied to the 3D OCT images to compensate for eye motion. No additional image post-processing was used for the UHR-OCT images presented in this paper. The volumetric and enface images were generated from the 3D data sets with Amira (Amira Inc.)

2.4 In-vivo confocal microscopy (IVCM) and histology

IVCM images of the cornea were acquired from healthy subjects with the Heidelberg Retinal Tomograph III (HRT III), using the Rostock Cornea Module (Heidelberg, Germany). The size of the imaged area in the cornea was 400 µm x 400 µm. The instrument was set up according to standard techniques outlined in the manufacturer’s operation manual and described elsewhere [35,36]. A high viscosity gel, Tear-Gel, was applied to the front surface of the microscope lens, prior to mounting a new, sterile Tomocap. One drop of a topical anesthetic (0.5% proparacaine hydrochloride) was instilled into the participant’s eye prior to the imaging procedure. The HRT III system was aligned so that the TomoCap made slight contact with the cornea and the imaging probe was translated in axial direction to focus the imaging beam at different axial positions inside the cornea.

Histological images were also acquired using the following protocol. Healthy corneas were harvested postmortem and after initial fixation in 10% neutral buffered formalin, the tissue was embedded in paraffin, serially sectioned into 5 µm thick sections, and stained with
hematoxylin and eosin (H&E). Using standard immunohistochemical staining protocol, paraffin sections were also placed on charged slides and immunostained for GFAP (glial fibrillary acidic protein) to identify and assess the location and spatial distribution of corneal nerves. The histological slides were evaluated using a bright field microscope (Leica DM1000, ICC50 HD, Leica Microsystems Inc, Canada).

3. Results

3.1 System’s resolution and SNR

The performance of the UHR-OCT system was evaluated by measuring the system’s spatial resolution and SNR and the test results are presented in Fig. 2. Figure 2(A) shows the normalized reference arm (black line) and sample arm (red line) spectra measured at the detection end of the UHR-OCT system by using a silver mirror as the test sample. The overlap between the two spectra has a spectral bandwidth of ~140 nm and is centered at ~790 nm, thus corresponding to a free space theoretical axial resolution of 1.98 µm. The OCT axial PSF was measured experimentally by using a silver mirror as the test sample and the result is shown in Fig. 2(B). The black line PSF was measured only after coarse hardware dispersion compensation using the dispersion compensation unit (DC) in the reference arm. The red line PSF in Fig. 2(B) was generated after numerical dispersion compensation up to the 5th order using a custom MATLAB based algorithm. The FWHM of the axial PSF was 2.1 µm in free space, which corresponds to ~1.5 µm in biological tissue, assuming an average refractive index of n = 1.38 and not taking into consideration any wavelength dependent local variations of the refractive index.

Results from the system’s sensitivity test as a function of scanning depth are presented in Fig. 2(C). The SNR was measured using a silver mirror as the imaged object. The maximum SNR of 98 dB was measured ~100 µm away from the zero-delay line at the maximum camera data rate of 250 kHz and with incident optical power of 800 µW. Due to the broad spectrum and limited number of camera pixels the spectrum was spread over, the SNR roll-off in free space was measured to be ~10 dB over a scanning range of 1.4 mm.

![Image](image_url)

Fig. 2. Sample and reference arm spectra measured at the detection end of the SD-OCT system (A). Axial PSF measured in free space (B). Sensitivity roll-off (C). Image of an USAF target acquired with a 10x microscope objective (D). Magnified view of the USAF target marked with the blue square (E). Red and green lines show the intensity profiles of group 7, elements 6 and 7 of the USAF target image (F).
The lateral resolution of the UHR-OCT system was measured using a standard United States Air Force resolution target (USAF, Thorlabs, USA). An image of the USAF target acquired at 250 kHz is shown in Fig. 2(D). A magnified view of the central region marked with the blue square in Fig. 2(D) is shown in Fig. 2(E). The line pairs of groups 6 and 7 or Element 7 are clearly resolved. The red and green lines in Fig. 2(E) mark the locations from which the intensity profiles shown in Fig. 2(F) were extracted. These results suggest that the lateral resolution measured in free space is < 2 µm.

3.2 Corneal images

3.2.1 Epithelium

Figure 3(A) shows a representative cross-sectional H&E stained histological image of the anterior healthy human cornea. The image shows the cellular structure of the corneal epithelium (EPI), as well as the Bowman’s membrane (BM) and keratocytes in the corneal stroma (STR) marked with red arrows. Figure 3(B) shows a representative cross-sectional UHR-OCT image, that was acquired in-vivo from the anterior cornea of a healthy subject at the maximum camera readout rate of 250 kHz. The cellular structure of the corneal EPI is clearly visible on the UHR-OCT B-scan. Reflective white dots are also observed inside the cells, most likely corresponding to reflections from the cellular nuclei. The interface between the corneal epithelium and the Bowman’s membrane appears as a thin, highly reflective, almost solid white line, while the interface between the BM and the stroma is only marked by the first, most anterior layer of keratocyte cells, marked with red arrows. The high axial resolution (1.5 µm physical resolution), also allows for visualization of the thin tear film layer (Fig. 3(B), blue arrow) and measurement of its thickness, which in this particular case was ~4 µm. A volumetric image of the corneal epithelium is shown in Fig. 3(C). In addition to the clearly visible cellular morphology of the EPI layer, the image also shows hyper-reflective (white) structures within the tear film, marked with yellow arrows. An enface view of the tear film surface is shown in Fig. 3(D). Some of the reflective structures have more or less round shape of different dimensions and they, most likely, correspond to clumps of cellular debris. Other reflective structures appear as thin, occasionally branching lines of various length. We hypothesize that those structures may correspond to clusters of mucins that are present in the tear film. Figures 3(E)-3(G) show enface UHR-OCT images of the corneal epithelium that were acquired at different depths within the EPI layer. The image in Fig. 3(G) shows the cellular structure of the basal cell layer of the EPI. Reflective white dots are visible inside the cells and most likely they correspond to reflections from cellular nuclei. Figure 3(H) shows a larger field of view (~250 µm × 250 µm) enface UHR-OCT image of the basal cell layer of the corneal epithelium, which correlates well with an IVCM image acquired at a similar location and in the cornea and with similar magnification.
3.2.2 Corneal nerves

The high spatial resolution of the UHR-OCT system combined with the high image acquisition rate also allows for in-vivo imaging of corneal nerves. Figure 4(A) shows a representative volumetric UHR-OCT image of a healthy human anterior cornea. Figure 4(B) shows the same data set with the upper layers of the corneal EPI digitally removed up to the basal cell layer of the epithelium. The long reflective (white) lines in the image correspond to sub-basal corneal nerves that are located inside the EPI basal cell layer and run parallel to the interface between the corneal EPI and the BM. Figure 4(C) shows a representative GFAP stained histological cross-sectional image of the anterior cornea. The GFAP stain appears brown in the image and marks the corneal nerves. Figures 4(D) and 4(E) show larger field-of-view, enface images of sub-basal corneal nerves acquired with the UHR-OCT system and IVCM respectively. Although the contrast in the UHR-OCT image is not as high as the IVCM one, it may be sufficient for precise segmentation and morphometry of the corneal nerves.
3.2.3 Corneal stroma

Figure 5 shows UHR-OCT and IVCM images of keratocytes in the corneal stroma. Volumetric UHR-OCT images of the anterior and middle stroma are presented in Fig. 5(A) and 5(D) respectively. Corresponding maximum projection UHR-OCT images from the two locations in the corneal stroma are shown in Fig. 5(B) and 5(E) respectively. Keratocytes of different shape, size and spatial distribution are observed in these UHR-OCT images and correspond closely to IVCM images (Fig. 5(C) and 5(F)) acquired from similar locations in the subject’s cornea. Thin stromal nerves are also visible in both the UHR-OCT and IVCM images (red arrows) acquired from the anterior stroma.

3.2.4 Posterior cornea

Figure 6 shows UHR-OCT and IVCM images of the posterior cornea. A cross-sectional UHR-OCT image of the entire cornea is shown in Fig. 6(A). Since in this case the imaging beam was focused on the posterior cornea, the anterior part of the cornea appears of very low contrast in the image due to the limited depth of focus of the 10x microscope objective. A region of interest (ROI) is marked with the red dashed line rectangle and a magnified view of that ROI (5x stretching in axial direction) is presented in Fig. 6(B). The UHR-OCT image in Fig. 6(B) clearly shows the boundaries of the corneal endothelium (END). The speckle pattern observed inside the END layer is generated by light that was scattered or reflected from endothelial sub-cellular structures, such as cellular nuclei, mitochondria, etc. The Descemet’s membrane (DM) is also clearly visible, and the distinct speckle pattern observed in this corneal layer that most likely corresponds to the collagen fibrillae. The pre-Descemet’s layer is an acellular corneal layer located between the DM membrane and the posterior stroma and its boundary with the corneal stroma is identified by the most posterior layer of keratocytes. The PDL was identified and characterized morphometrically ex-vivo from histological corneal images by Dr. Dua, therefore some publications also refer to the PDL as the “Dua” layer. The PDL is clearly visible in the UHR-OCT image shown in Fig. 6(B), that was acquired in-vivo from a healthy subject. The red arrows in the image mark keratocytes.
that form the boundary between the PDL and the posterior stroma. The corneal layers identified in the UHR-OCT cross-sectional image correspond very well with a typical H&E histological image (Fig. 6(C)). Similar UHR-OCT images of the posterior human cornea were recently acquired in-vivo from healthy subjects in a pilot clinical study with a sub-micrometer axial resolution OCT system developed by our research group [27]. Due to the limited camera readout rate of that OCT system (34 kHz), relative to the speed of ocular saccades, at that time it was not possible to acquire in-vivo volumetric images of the posterior human cornea. The new UHR-OCT offers 8x improvement in the image acquisition rate and thus allowed for acquisition of volumetric images of the corneal END.

![Image of UHR-OCT and IVCM images](image.jpg)

Fig. 5. UHR-OCT volumetric images of the corneal stroma showing keratocyte cells in the anterior and middle stroma respectively (A and D). Corresponding enface UHR-OCT images with red arrows marking thin stromal nerves (B and E). IVCM images of the anterior and middle stroma respectively (C and F) showing keratocytes and thin stromal nerves (red arrows).

Figure 6(D) shows an IVCM image of the corneal endothelium that was acquired from a healthy subject. The image shows the honeycomb arrangement of the hexagonal endothelial cells. The thin red lines in the image connect cellular nuclei of the first neighbors of one endothelial cell. Figure 6(E) shows an enface UHR-OCT image of the corneal endothelium that was acquired in-vivo from a healthy subject and from a depth location corresponding to the middle of the END layer. Instead of clearly identifiable endothelial cells with hexagonal shape, a pattern of highly reflective dots is visible in that image. The thin red lines in the image connect the first neighbors of one such dot. An FFT map generated from the image in Fig. 6(E) is shown in Fig. 6(F). The radius of the ring structure visible in the FFT map, corresponds to the mean distance between the reflective white dots in the UHR-OCT image (Fig. 6(E)). The length of the radius was measured to be ~20 µm, which correlated very well with the average size of the healthy endothelial cells that was determined from the IVCM image shown in Fig. 6(D). Figure 6(G) shows a maximum intensity projection OCT image acquired ex-vivo from healthy post-mortem corneal tissue sample. This image was generated by averaging the OCT signal in axial direction over the entire thickness of the endothelial layer. The image clearly shows the hexagonal structure of the corneal endothelial cells.
4. Discussion and conclusions

The combination of isotropic, micrometer-scale imaging resolution and high image acquisition rate offered by the novel UHR-OCT system described here, allowed for in-vivo volumetric imaging of the cellular and sub-cellular structure of the human cornea without the need for physical contact with the tissue. To our knowledge this is the first report of UHR-OCT images that show the cellular morphology of all corneal layers, as well as, reveal structures in the tear film. Recently, Mazlin et.al [21], proposed a novel design of a full-field
OCT (FF-OCT) system, designed for in-vivo non-contact, volumetric imaging of the cellular structure of the human cornea [21]. One major advantage of the FF-OCT technology is its ability to acquire larger field-of-view, cellular resolution images of the cornea that are free of eye motion artefacts. This is due to the fact that FF-OCT generates fast enface 2D images and scans slower in axial direction. By using FF-OCT, Mazlin et al., were able to acquire impressive images of keratocytes and nerves in the corneal stroma, and of the honeycomb arrangement of the corneal endothelial cells. However, due to the limited axial resolution and the susceptibility of the FF-OCT technology to strong reflections arising from scattering of the imaging beam from the collagen structure of the corneal BM, they were unable to image the tear film and the cellular structure of the corneal epithelium.

The high spatial resolution offered by the SD-OCT system described here, allows for detection of reflections from endothelial cell nuclei, as shown in Fig. 6(E). The shape of the nuclei does not appear spherical in this image due to presence of optical aberrations that have not been compensated. Note that Fig. 6(E) shows a single-plane enface image, acquired at a depth location corresponding to about half way through the thickness of the corneal endothelial layer (marked with a blue arrow in Fig. 6(B)). For this reason, this image does not show the typical hexagonally-shaped endothelial cells as seen in the IVCM image (Fig. 6(D)), or as generated by FF-OCT [21]. Both the IVCM and the FF-OCT [21] images are generated with axial resolution of ~10 µm and ~8 µm respectively, which is ~2x larger than the thickness of the corneal endothelial cells. Therefore, IVCM and FF-OCT integrate the light scattered from the nuclei and other cellular organelles, as well as some light scattered from the collagen fibrilae in the Descemet’s membrane. Thus, these imaging technologies are able to generate enface images that show the hexagonal shape of the corneal endothelial cells. As shown in Fig. 6(G), SD-OCT technology with 1 µm axial resolution is also able to generate images of the corneal endothelial cells of similar appearance, by utilizing maximum intensity projection over the entire thickness of the endothelial layer. Note that the image in Fig. 6(G) was acquired ex-vivo from a post-mortem corneal tissue sample. Therefore, the lack of eye motion artefacts allowed for generation of high resolution, high contrast images of the corneal endothelial cells.

In general, similar maximum projection approach can be applied to SD-OCT images of the corneal endothelium acquired in-vivo. Figure 7(A) shows a maximum intensity projection image of the healthy corneal endothelium over the entire thickness of the layer, that was acquired in-vivo with the 250 kHz OCT system. A small area in the image marked with the red rectangle shows hexagonally shaped endothelial cells. A magnified view of that area is presented in Fig. 7(B). Lateral saccades are clearly visible in the enface image (7A). B-scans (Fig. 7(C) and 7(D)) obtained from different locations of the 3D imaging stack (marked with the blue and orange arrows in Fig. 7(A)), demonstrate the loss of contrast due defocus resulting from fast axial eye motion. In this case, the combination of high spatial resolution (1.5 µm), limited depth-of-focus (~20 µm) and limited camera speed (250 kHz) offered by the current design of the SD-OCT system, limited severely the area over which hexagonal endothelial cells can be observed. Future improvements of the optical design of the SD-OCT system, or the use of faster cameras, could make this task feasible.

One interesting fact of note is that the nuclei of corneal endothelial cells appear as low reflective dots on a highly reflective background in IVCM (Fig. 6(D)) and FF-OCT images. However, the same nuclei appear as highly reflective dots on a low reflective background in the OCT enface images generated by the 250 kHz SD-OCT (Fig. 6(E)). Some factors that may contribute to the apparent inversion of the image contrast are:

a) the 3D shape of the light scattering probability profile, which is dependent on the size, shape and refractive index of the cellular nuclei and other organelles, relative to the wavelength range of the optical imaging beam;

b) the numerical aperture and the spatial resolution of the respective imaging system.
These factors will define the relative intensity of the IVCM / FF-OCT / SD-OCT signals generated by light scattered from the cellular nuclei and other cellular organelles. Since corneal endothelial cells are metabolically very active, they contain an abundance of mitochondria that are smaller in size relative to the central wavelengths of the IVCM and FF-OCT imaging beams and therefore will scatter light more isotropically. In contrast, the axial size of corneal endothelial nuclei is approximately equal to the thickness of the cells (~5 µm), which is ~3x larger than the central wavelength of the IVCM, FF-OCT and the SD-OCT instruments described here.

Because the SD-OCT axial resolution is smaller than the endothelial cells’ thickness by a factor of ~3x, and the effective NA of the microscope objective we used is only 5x, the SD-OCT images acquired from a depth location inside the endothelial cells emphasizes light back-reflected from the nuclei and suppresses detection of light scattered by the other cellular organelles. Therefore, the cellular nuclei in the SD-OCT images (Fig. 6(E)) appear as hyper-reflective structures on low reflective background.

Because the axial resolution of clinical IVCM is in the order of ~10 µm (depending on magnification), IVCM integrates the scattering signal over the entire thickness of the endothelial cells and may include some scattering signal from the Descemet’s membrane. As the cytoplasm of corneal endothelial cells has an abundance of sub-wavelength sized cellular organelles such as mitochondria, ribosomes, Golgi apparatus, etc., the integrated scattered signal from them is larger in magnitude compared to the light backscattered from the nucleus. For this reason the, cytoplasm appears “bright”, while the nuclei and the cellular membrane appear “dark” in IVCM images. The FF-OCT images of corneal endothelial cell published in [21] were acquired with axial resolution of 7.7 µm. Therefore, those images are generated by integrating the scattered light over the entire thickness of the endothelial cells, similar to the case of IVCM.

Volumetric visualization of the cellular structure of the human cornea in UHR-OCT images could eventually lead to identification, counting and sizing of epithelial cells, keratocytes and endothelial cells by developing novel automatic OCT image processing algorithms. Previously corneal epithelial cells and keratocytes have only been visualized in histological sections or in confocal microscopy images acquired in-vivo through physical contact with the corneal epithelium. Although SD-OCT systems with < 1 µm [19], 1.1 µm [17] and 1.2 µm [26] were previously used to image the healthy and KC cornea, as well as measure the thickness of the tear film, none of them were able to image volumetrically the
cellular structure of the corneal epithelium most likely due to insufficient lateral resolution and limited image acquisition rate.

The micrometer scale axial resolution of the proposed high speed UHR-OCT system will also permit more precise thickness measurement of the thin layers of the human cornea, such as the BM and Descemet’s membranes and the corneal endothelium, since the precision of the layer thickness assessment is strongly dependent on the OCT axial resolution. This advantage of high axial resolution OCT has already been demonstrated by other research groups that used OCT technology with ~1.2 µm axial resolution in tissue to measure the thickness of the corneal epithelium and BM [17], and the tear film [26]. Our research group used a 0.95 µm axial resolution SD-OCT system to image in-vivo and quantify with high precision the thickness of the corneal endothelium, which is only ~4 µm thick, as well as the Descemet’s membrane and the pre-Descemet’s layer in the posterior healthy human cornea [27].

Although the current optical design of the fast scanning UHR-OCT system allows for in-vivo imaging of the cellular and sub-cellular structure of biological tissues, it has some limitations that offer opportunity for future optimization of the system’s performance. For example, the 250 kHz data acquisition rate certainly helps with suppressing significantly eye motion related artefacts. However, as shown in the images presented in Figs. 3(H), 4(A), 4(B) and 4(D), fast axial motion in the range of 10 µm to 100 µm, as well as fast lateral saccades, can introduce image artefacts in the volumetric UHR-OCT images even in the case when those are acquired from relatively small areas of the cornea (250 µm x 250 µm) with a total image acquisition time of ~300 ms. One approach to minimizing the effect of axial eye motion artefacts in the volumetric UHR-OCT images is to extend the depth-of-focus, for example by redesigning the core of the interferometer to incorporate axicon lenses [37]. Significant suppression or elimination of OCT image artefacts arising from fast lateral saccades in the eye, would require either increasing the camera readout rate in a SD-OCT design of a system, introducing eye tracking in the system, or the use of FF-OCT or line scanning (LS-OCT) [38] designs of the OCT technology. All of these approaches offer both advantages and limitations: increasing the camera readout rate will result in reduction of the SNR; parallel scanning OCT technology such as FF-OCT is highly susceptible to directly reflected or strongly scattered light in biological tissue, which would prevent imaging of certain regions or layers of the tissue; the optical design of LS-OCT, the number of camera pixels, as well as the cost and availability of ultrahigh speed area cameras with sufficient number of camera pixels, will limit the lateral and axial resolution, the scanning range and the SNR roll-off of the LS-OCT technology and as such affect adversely the affordability of such system.

A simple and fairly low-cost modification of the imaging probe of the current high speed UHR-OCT system would allow its use for non-invasive cellular resolution imaging of the human retina. In that case, the use of hardware or software adaptive optics will be required to visualize in-vivo individual retinal cells, as the lateral resolution of the unprocessed OCT retinal images is mainly determined by the optics of the eye and the diameter of the optical imaging beam. In the past, both hardware and computational adaptive optics (AO-OCT) [39] have been applied successfully to in-vivo retinal images and in those cases allowed for visualization of individual photoreceptors. Imaging of other parts of the human body with the same UHR-OCT system are also feasible, however, the optical density and light scattering of the imaged tissue will pose a limit to the penetration depth at which individual cells can be resolved in the OCT images.

In conclusion, we have developed a high speed (250 kHz) UHR-OCT system that offers theoretical isotropic imaging resolution of ~1.5 µm in corneal tissue. Volumetric images acquired in-vivo with the system from different depth locations in the healthy human cornea, demonstrate the ability of the system to image without contact with the corneal tissue, the cellular structure of the corneal epithelium, stroma and endothelium. The corneal images
presented here demonstrate the potential clinical value of the new high speed, UHR-OCT system for non-invasive diagnostics and monitoring the treatment of corneal diseases.

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Disclosures
The authors declare that there are no conflicts of interest related to this article.

References
1. M. S. Oliva, T. Schottman, and M. Gulati, “Turning the tide of corneal blindness,” Indian J. Ophthalmol. 60(5), 423–427 (2012).
2. D. Pascolini and S. P. Mariotti, “Global estimates of visual impairment: 2010,” Br. J. Ophthalmol. 96(5), 614–618 (2012).
3. J. P. Whitcher, M. Srinivasan, and M. P. Upadhyay, “Corneal blindness: a global perspective,” Bull. World Health Organ. 79(3), 214–221 (2001).
4. M. Burton, “Corneal blindness: prevention, treatment and rehabilitation,” Community Eye Health 22(71), 33–35 (2009).
5. E. Villani, C. Baudouin, N. Efron, P. Hamrah, T. Kojima, S. V. Patel, S. C. Pflugfelder, A. Zhivov, and M. Dogru, “In vivo confocal microscopy of the ocular surface: from bench to bedside,” Curr. Eye Res. 39(3), 213–231 (2014).
6. R. L. Niederer and C. N. J. McGhee, “Clinical in vivo confocal microscopy of the human cornea in health and disease,” Prog. Retin. Eye Res. 29(1), 30–58 (2010).
7. J. Jaibert, F. Stapleton, E. Papas, D. F. Sweeney, and M. Coroneo, “In vivo confocal microscopy of the human cornea,” Br. J. Ophthalmol. 87(2), 225–236 (2003).
8. D. Huang, E. A. Swanson, C. P. Lin, J. S. Schuman, W. G. Stinson, W. Chang, M. R. Hee, T. Flotte, K. Gregory, C. A. Puliafito, and J. G. Fujimoto, “Optical coherence tomography,” Science 254(5035), 1178–1181 (1991).
9. A. F. Fercher, “Optical coherence tomography,” J. Biomed. Opt. 1(2), 157–173 (1996).
10. Optical Coherence Tomography: Technology and Applications, W. Drexler and J.G. Fujimoto, eds. (Springer, 2015).
11. J. L. Ramos, Y. Li, and D. Huang, “Clinical and research applications of anterior segment optical coherence tomography - a review,” Clin. Exp. Ophthalmol. 37(1), 81–89 (2009).
12. J. Wang, M. Abou Shousha, V. L. Perez, C. L. Karp, S. H. Yoo, M. Shen, L. Cui, V. Humrecic, C. Du, D. Zhu, Q. Chen, and M. Li, “Ultra-high resolution optical coherence tomography for imaging the anterior segment of the eye,” Ophthalmic Surg. Lasers Imaging 42(4 Suppl), S15–S27 (2011).
13. B. Povazay, K. Bizheva, A. Unterhuber, B. Hermann, H. Sattmann, A. F. Fercher, W. Drexler, A. Apolonski, W. J. Wadsworth, J. C. Knight, P. S. Russell, M. Vetterlein, and E. Scherzer, “Submicrometer axial resolution optical coherence tomography,” Opt. Lett. 27(20), 1800–1802 (2002).
14. A. Dubois, K. Grieve, G. Moneron, R. Lecaque, L. Vabre, and C. Boccara, “Ultrahigh-resolution full-field optical coherence tomography,” Appl. Opt. 43(14), 2874–2883 (2004).
15. D. Cui, X. Liu, J. Zhang, X. Yu, S. Ding, Y. Luo, J. Gu, P. Shum, and L. Liu, “Dual spectrometer system with spectral compounding for 1-μm optical coherence tomography in vivo,” Opt. Lett. 39(23), 6672–6730 (2014).
16. L. Liu, J. A. Gardecki, S. K. Nadkarni, J. D. Toussaint, Y. Yagi, B. E. Bouna, and G. J. Tearney, “Imaging the subcellular structure of human coronary atherosclerosis using micro-optical coherence tomography,” Nat. Med. 17(8), 1010–1014 (2011).
17. R. Yadav, K. S. Lee, J. P. Rolland, J. M. Zavislan, J. V. Aquavella, and G. Yoon, “Micrometer axial resolution OCT for corneal imaging,” Biomed. Opt. Express 2(11), 3037–3046 (2011).
18. R. Yadav, R. Kottaiyan, K. Ahmad, and G. Yoon, “Epithelium and Bowman’s layer thickness and light scatter in keratoconic cornea evaluated using ultrahigh resolution optical coherence tomography,” J. Biomed. Opt. 17(11), 116010 (2012).
19. K. Bizheva, B. Tan, B. MacLelan, O. Kralj, M. Hajialamdar, D. Hileeto, and L. Sorbara, “Sub-micrometer axial
resolution OCT for in-vivo imaging of the cellular structure of healthy and keratoconic human corneas,” Biomed. Opt. Express 8(2), 808–812 (2017).
20. R. M. Werkmeister, S. Sapeta, D. Schmidt, G. Garhöfer, G. Schmiding, V. Araña Dos Santos, G. C. Aschinger, I. Baumgartner, N. Pircher, F. Schwarzhans, A. Pantalon, H. Dua, and L. Schmetterer, “Ultrahigh-resolution OCT imaging of the human cornea,” Biomed. Opt. Express 8(2), 1221–1239 (2017).
21. V. Mazlin, P. Xiao, E. Dalimier, K. Grieve, K. Irsh, J. A. Sahel, M. Fink, and A. C. Boccara, “In vivo high resolution human corneal imaging using full-field OCT,” Biomed. Opt. Express 8(2), 557–568 (2018).
22. R. M. Werkmeister, S. Sapeta, D. Schmidl, G. Garhöfer, G. Schmidinger, V. Aranha Dos Santos, G. C. Aschinger, I. Baumgartner, N. Pircher, F. Schwarzhans, A. Pantalon, H. Dua, and L. Schmetterer, “Ultrahigh-resolution OCT imaging of the human cornea,” Biomed. Opt. Express 8(2), 1221–1239 (2017).
23. V. Mazlin, P. Xiao, E. Dalimier, K. Grieve, K. Irsh, J. A. Sahel, M. Fink, and A. C. Boccara, “Ocular tissue imaging using ultrahigh-resolution, full-field optical coherence tomography,” Invest. Ophthalmol. Vis. Sci. 45, 4126–4131 (2004).
24. A. H. Karimi, A. Wen, and K. Bizheva, “Automated detection and cell density assessment of keratocytes in the human corneal stroma from ultrahigh resolution optical coherence tomograms,” Biomed. Opt. Express 2(10), 2905–2916 (2011).
25. N. Hutchings, T. L. Simpson, C. Hyun, A. A. Moayed, S. Hariri, L. Sorbara, and K. Bizheva, “Swelling of the human cornea revealed by high-speed, ultrahigh-resolution optical coherence tomography,” Invest. Ophthalmol. Vis. Sci. 51(9), 4579–4584 (2010).
26. K. Grieve, C. Georgeon, F. Andreiuolo, M. Borderie, D. Ghoubay, J. Rault, and V. M. Borderie, “Imaging microscopic features of keratoconic corneal morphology,” Cornea 35(12), 1621–1630 (2016).
27. W. Wieser, B. R. Biedermann, T. Klein, C. M. Eigenwillig, and R. Huber, “Multi-megahertz OCT: High quality 3D imaging at 20 million A-scans and 4.5 GVoxels per second,” Opt. Express 18(14), 14685–14704 (2010).
28. B. Potsaid, I. Gorczynska, V. J. Srinivasan, Y. Chen, J. Jiang, A. Cable, and J. G. Fujimoto, “Ultrahigh speed spectral / Fourier domain OCT ophthalmic imaging at 70,000 to 312,500 axial scans per second,” Opt. Express 16(19), 15149–15169 (2008).
29. T. Schmoll, C. Kolbitsch, and R. A. Leitgeb, “Ultra-high-speed volumetric tomography of human retinal blood flow,” Opt. Express 17(5), 4166–4176 (2009).
30. L. An, P. Li, T. T. Shen, and R. Wang, “High speed spectral domain optical coherence tomography for retinal imaging at 500,000 A-lines per second,” Biomed. Opt. Express 2(10), 2770–2783 (2011).
31. Rostock Cornea Module - Quick Operation Notes. Heidelberg Engineering.
32. M. Tavakoli and R. A. Malik, “Corneal confocal microscopy: a novel non-invasive technique to quantify small fibre pathology in peripheral neuropathies,” J. Vis. Exp. 47, 47 (2011).
33. Z. Ding, H. Ren, Y. Zhao, J. S. Nelson, and Z. Chen, “High-resolution optical coherence tomography over a large depth range with an axicon lens,” Opt. Lett. 27(4), 243–245 (2002).
34. L. Ginner, T. Schmoll, A. Kumar, M. Salas, N. Pricoupenko, L. M. Wurster, and R. A. Leitgeb, “Holographic line field en-face OCT with digital adaptive optics in the retina in vivo,” Biomed. Opt. Express 9(2), 472–485 (2018).
35. M. Pircher and R. J. Zawadzki, “Review of adaptive optics OCT (AO-OCT): principles and applications for retinal imaging [Invited],” Biomed. Opt. Express 8(5), 2536–2562 (2017).