

***In vitro* measurement of rabbit corneal epithelial thickness using ultrahigh resolution optical coherence tomography**

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Abstract

The objective of this study was to reproducibly measure corneal epithelial thickness centrally and at the limbus in the rabbit cornea using ultrahigh resolution optical coherence tomography (OCT). Twelve freshly enucleated New Zealand white rabbit eyes were kept in a moist chamber at 4 °C. An ultrahigh resolution OCT system with a spatial resolution of 1.3 μm was used to image the cornea and its component layers. The central and peripheral (limbal) regions of all the samples were scanned within 6 h of harvest in order to minimize the post-mortem degradation of the corneal epithelium. The thickness of the corneal epithelium was determined by measuring the pixel equivalents of the obtained image. Unpaired Student's *t*-test was used to evaluate differences. The epithelial thickness centrally was found to be $45.8 \pm 2.2 \mu\text{m}$, and $37.6 \pm 1.4 \mu\text{m}$ at the limbus ($P < 0.001$). Rabbit corneal epithelium is thicker centrally than at the limbus when measured by ultrahigh resolution OCT. This technique will aid in delineating the pathophysiology of diseases of the anterior cornea.

Key Words: cornea, epithelial thickness, optical coherence tomography, rabbit

INTRODUCTION

Corneal thickness has been measured in a variety of ways with most requiring some type of fixation. In reports using light microscopy, formalin-fixed rabbit corneal epithelium was measured to be an average of 30–45 μm thick.^{1,2} An ultrasonic pachymetry study reported a thicker limbal epithelium ($40 \pm 4 \mu\text{m}$) compared with the central part ($35 \pm 6 \mu\text{m}$), similar to the human corneal epithelium distribution.³ More recently, confocal microscopy through focusing (CMTF) has demonstrated that the rabbit central corneal epithelium is thicker than the limbal epithelium.⁴ However, in that study, resolution of the image depended on submersion of the tissue in methyl cellulose, which may lead to a slight but significant swelling of both the stroma (0.5 $\mu\text{m}/\text{min}$) and epithelium (0.1 $\mu\text{m}/\text{min}$), which in turn, may have affected the accuracy of the reading.

Optical coherence tomography (OCT) offers a direct method for defining anatomy in living tissue samples unaltered by fixation and tissue preparation. It was first used in ophthalmology to study posterior structures.^{5–9} However, work in the last few years has extended the application of OCT to corneal and anterior segment evaluation with a high degree of repeatability and reproducibility.^{10–12}

This technology is analogous to an optical laser ultrasound that provides high-resolution imaging to the level of single cells without fixation, compression, or alteration of the tissue from its natural *in vivo* state.⁹ The principle of optical coherence tomography has been previously described in detail.^{11,13,14} It is primarily based on the Michelson interferometer where lateral scanning of the sample produces a two-dimensional cross-sectional image. Optical coherence tomography can be used to perform *in vivo* high-resolution cross-sectional imaging of biological tissues comparable with a histopathologic section observed under low-power microscopy.¹⁰ Early application of OCT in the cornea of humans reported central epithelial thickness to be $57.8 \pm 1.7 \mu\text{m}$.¹⁵

Basically, there are two types of OCT systems: fiber based and open air. In the fiber-based system, the two arms of the interferometer are constructed by means of optical fibers. Although most of the commercially available OCT systems are fiber based, the system has several disadvantages. These include the difficult task of constructing a 2×2 coupler with a bandwidth of $> 200 \text{ nm}$, the presence of chromatic aberration, a multimode fiber which introduces ghost lines into the OCT image and, lastly, polarization mode dispersion in a single mode fiber which may limit longitudinal resolution.¹⁶ Because the open air system does not utilize optical fibers,

there is no limitation on system bandwidth while maintaining high lateral resolution and increased image depth.¹⁶

Commercially available OCT devices (Humphrey Systems, Zeiss-Humphrey, Dublin, CA, USA), employing the fiber-based system, have a precision of 2.5 μm in measurements of human corneal epithelial thickness after hydrogel and contact lens wear.¹⁷ Even with currently improved OCT resolution, dispersion of the sampling medium is still a limitation when scanning across a long distance such as the eye. Generally, OCT systems require adjustment of the dispersion balance between the two arms of the interferometer as previously described.¹⁸ However, this adjustment works for only one plane, which prevents high axial resolution over the entire scanning depth.

We have developed a high-resolution, open-air OCT system that eliminates the influence of depth-dependent dispersion of water in tissue by using an ultra-broadband light source with a center wavelength near 1.0 μm .¹⁸

The general function of the corneal epithelium as a protective barrier has long been recognized. Understanding the normal anatomy of the corneal epithelium using this ultrahigh resolution imaging system will help to delineate the pathophysiology of diseases when abnormal epithelial surfaces are examined. Here we describe the principle and technique of using a novel ultrahigh resolution OCT system to reproducibly quantitate the rabbit central and limbal corneal epithelial thickness *in vitro*.

MATERIALS AND METHODS

Rabbit globe harvest

Twelve New Zealand white rabbit heads were obtained from an abattoir and kept at 4 °C until one eye was enucleated from each rabbit within 3 h of death. All rabbits were 8–10 weeks of age when killed. The globes were stored in a moist chamber at 4 °C until time of use. The central and random peripheral (limbal) regions of the 12 eyes were sequentially scanned within 6 h of harvest in order to maximize preservation of the corneal epithelium.

Optical coherence tomography

An open-air ultrahigh resolution OCT system was designed and constructed as shown in Fig. 1.¹⁶ The objective lens (L_3) and rectangular prism (P) are both mounted on a voice-coil translation stage for longitudinal scanning. The base plate is mounted on a stage for lateral scanning. Dispersion in this OCT system is balanced by variable-thickness fused silica prisms (FS). A neutral density filter (F_2) is inserted in the reference arm to decrease the intensity from the reference arm and reduce background noise. A dynamic focusing tracking method was used to keep a zero path-length difference between the reference and sampling beams in the focus region during longitudinal scanning.^{16,18} This makes it possible to increase imaging depth with constant high lateral resolution.

The interferometer light source is a broadband continuum generated from an air–silica microstructure fiber. The

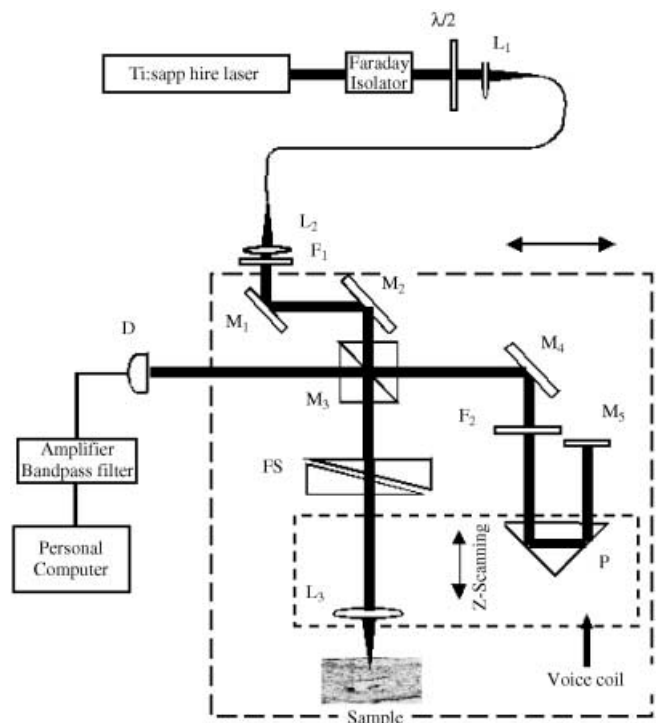


Figure 1. Schematic diagram of the ultrahigh resolution OCT system configuration. M_3 is a wide-band cube beam splitter at wavelengths from 900 to 1400 nm. M_1 , M_2 , M_4 , and M_5 are silver mirrors. F_1 is the long-pass filter and F_2 is the neutral density filter. FS are fused silica prisms. L_1 is the coupling lens, and L_2 is the collimated lens. L_3 is the objective lens. P is the rectangular prism. D is the detector. The interferometer light source is Ti/sapphire laser with wavelength of 780 nm.

pump source for continuum generation is a self-mode-locked KLM Ti/sapphire laser. The total output laser power is > 700 mW, with pulse duration of 110 fs and a repetition rate of 76 MHz. The laser output wavelength is 780 nm. The laser beam is then coupled into a microstructure fiber after a Faraday isolator to avoid interference of back-reflected light with the mode locking and $\lambda/2$ wave plate. Nitrogen gas is slowly blown onto the coupling part to purge the fiber tip and avoid damage. The $\lambda/2$ wave plate after the Faraday isolator is used to adjust the polarization state of the light input to the fiber to optimize the spectrum. Continuum light generation from 400 to 1400 nm is observed from the fiber. A log pass filter (F_1) is used to block all light with wavelengths < 800 nm. The total output power from the microstructure fiber can be as high as 100 mW and the remaining power is \approx 50 mW after a long pass filter. The ultrahigh resolution OCT system was optimized to support a 1.8- μm longitudinal resolution in free space at a center wavelength of 1.1 μm with a silver mirror as the sample. Considering the refractive index of biological tissue, the corresponding tissue resolution is 1.3 μm . The lateral resolution of the system was 7 μm , which was determined by the achromatic objective lens.

The system was calibrated using a silver mirror as the sample. For the mirror surface, there is a bright line on the

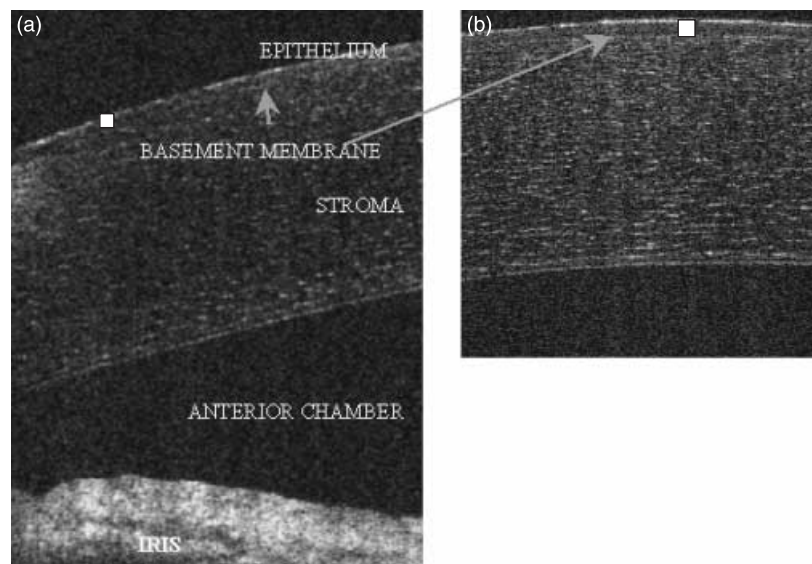


Figure 2. Ultrahigh resolution OCT image of rabbit cornea from two samples. (a) Rabbit cornea at the limbus, (b) central rabbit cornea. White squares indicate where thickness measurements were taken. These figures are not contiguous and only placed proximately to indicate anatomical relation.

OCT image. Movement, Δz , of the silver mirror will lead to a variation, Δm , in the position of this bright line on the image. The ratio $k = \Delta z / \Delta m$ represents the distance for one pixel on the image. In biological tissue, this factor should be divided by 1.35, which is the group refractive index of biological tissue. Thus, the calibration factor is $k/1.35$ to calculate structure thickness inside biological tissue.

The scanning rate of the moving stage was 2 Hz. It took ≈ 3 min to obtain one image. The signal-to-noise ratio is 40 dB. The light power input onto the sample was 20 mW. Because this was not an *in vivo* experiment, ANSI limits and safety issues were not a concern.

Measurement and data analysis

To ensure orthogonality of the probe beam to the corneal surface at both the central cornea and limbus, multiple sets of measurements were obtained at each site on the surface of each cornea. Those measurements that displayed the lowest corneal epithelial thickness at each site were included in the data analysis. That is, the orthogonal angle was taken as the angle that produced the shortest measured distance between the front and back of the corneal epithelium. The Microsoft Office Draw program was used to determine the rabbit corneal epithelium thickness. The same observer performed all measurements. Each image represented a section $\approx 1.35 \times 1.5$ mm (vertical \times horizontal), which was converted into pixels. Pixels were correlated with the epithelium thickness and later converted to distance based on $2.7 \mu\text{m}$ per pixel value. Unpaired Student's *t*-tests were performed to compare differences between the measured regions. *P*-values < 0.05 were considered statistically significant.

RESULTS

In Fig. 2, a characteristic high-resolution image obtained using this system is shown. The basement membrane separating the corneal epithelium and the underlying stroma is

clearly seen. In these sections, the thickness variation between the thinner limbal and thicker central epithelium can be appreciated.

Using these digitally recorded images, limbal and central epithelial thickness measurements for 12 corneas were obtained. The limbal epithelial thickness ($37.6 \pm 1.4 \mu\text{m}$) was found to be significantly less than the central thickness ($45.8 \pm 2.2 \mu\text{m}$; $P < 0.001$).

DISCUSSION

Our noncontact high-resolution OCT measurements of fresh rabbit corneal epithelium reveal that the epithelial depth is significantly thicker at the center than at the limbus.

Ringvold *et al.*¹⁹ proposed that the epithelium plays a role in protection from ambient radiation and appears to have seasonal variation in thickness in response to atmospheric radiation as it thickens in the summer. Also, although not statistically significant, a pattern of thicker central epithelium was also described in their study. It is important to point out that, although rabbits did not show as much of a seasonal variation as other species in their study, the authors acknowledged that the light variability in the rabbit housing may not have been sufficient to detect the phenomenon as in wild rabbits. Regardless, this study suggests a possible protective function against ambient radiation for the epithelium. Because the central corneal epithelium is exposed to more light than the periphery owing to the dome-shaped globe in the rabbit, the epithelium may be thicker in the center, as determined in our study.

During our study, caution was taken to minimize spurious measurements resulting from post-mortem damage to the *ex vivo* eyes, especially corneal edema. It is well known that several factors, including hypoxia, can lead to corneal swelling.¹⁵ However, the ultrahigh resolution OCT system developed can address the issue of corneal edema. A study by Wang *et al.*¹⁸ showed that resonant molecular absorption of

common tissue constituents such as water may be eliminated by using a light source with a center wavelength near 1.0 μm , and achieved an ultrahigh resolution of 1.3 μm in ophthalmic imaging with a microstructure fiber as a light source. From this ultrahigh resolution OCT measurement itself, a source of error could result from variable oscillation times, which would also cause disturbances in the resolution of the imaging. Because this would manifest as image smear, we were able to immediately identify and discard those images and rescan the tissue until the oscillation stabilized. To minimize any changes that may result from slight changes of the configuration as depicted in Fig. 1, the machine was meticulously tuned and calibrated each time measurements were made, and all the measurements were performed in one day on the same group of eyes on the same apparatus.

Standard resolution OCT can achieve axial resolutions of 10–15 μm ,²⁰ whereas we have demonstrated that this ultrahigh resolution OCT can provide imaging with axial resolutions as fine as 1–2 μm . The image resolution that the ultrahigh resolution OCT can achieve is comparable with that of conventional histopathology and is up to 10–100 times finer than other clinical imaging techniques.²⁰

Overall, the data obtained from this experiment demonstrate that the ultrahigh resolution OCT system developed may penetrate deeply into biological tissues and reproducibly measure the central and limbal epithelial thickness of the rabbit corneal epithelium. Clinically, the importance of the epithelium cannot be overemphasized. A small change in thickness of the epithelium during wound healing can cause significant refractive change. This ultrahigh resolution OCT may prove useful to monitor changes in epithelial thickness during healing following corneal surgery or after injury. We acknowledge that although fresh tissue was used in our measurements, this was an *in vitro* experiment. The next phase of the planned study is to adapt the OCT instrument for *in vivo* observations.

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REFERENCES

- Dierick HG, Van Mellaert CE, Missotten L. Histology of rabbit corneas after 10-diopter photorefractive keratectomy for hyperopia. *Journal of Refractive Surgery* 1999; **15**: 459–468.
- Lohmann CP, Patmore A, Reischl U *et al.* The importance of the corneal epithelium in excimer-laser photorefractive keratectomy. *German Journal of Ophthalmology* 1997; **5**: 368–372.
- Jester JV, Li HF, Petroll WM *et al.* Area and depth of surfactant-induced corneal injury correlates with all death. *Investigative Ophthalmology and Visual Science* 1998; **39**: 922–926.
- Ivarsen A, Stultiens BAT, Moller-Pedersen T. Validation of confocal microscopy through focusing for corneal sublayer pachymetry. *Cornea* 2002; **21**: 700–704.
- Ciardella AP, Borodoker N, Costa DL *et al.* Imaging the posterior segment in uveitis. *Ophthalmology Clinics of North America* 2002; **15**: 281–296.
- Ip M, Garza-Karren C, Duker JS *et al.* Differentiation of degenerative retinoschisis from retinal detachment using optical coherence tomography. *Ophthalmology* 1999; **106**: 600–605.
- Massin P, Vicaut E, Haouchine B *et al.* Reproducibility of retinal mapping using optical coherence tomography. *Archives of Ophthalmology* 2001; **119**: 1135–1142.
- Schuman JS, Kim J. Imaging of the optic nerve head and nerve fiber layer in glaucoma. *Ophthalmology Clinics of North America* 2000; **13**: 383–406.
- Tadrous PJ. Methods for imaging the structure and function of living tissues and cells: 1. Optical coherence tomography. *Journal of Pathology* 2000; **191**: 115–119.
- Hirano K, Ito Y, Suzuki T *et al.* Optical coherence tomography for the noninvasive evaluation of the cornea. *Cornea* 2001; **20**: 281–289.
- Hoerauf H, Wirbelauer C, Scholz C *et al.* Slit-lamp-adapted optical coherence tomography of the anterior segment. *Graefes Archive for Clinical and Experimental Ophthalmology* 2000; **238**: 8–18.
- Muscat S, McKay N, Parks S *et al.* Repeatability and reproducibility of corneal thickness measurements by optical coherence tomography. *Investigative Ophthalmology and Visual Science* 2002; **43**: 1791–1795.
- Fujimoto JG, Brezinski ME, Teraney GJ *et al.* Optical biopsy and imaging using optical coherence tomography. *Nature Medicine* 1995; **1**: 970–972.
- Huang D, Swanson EA, Lin CP *et al.* Optical coherence tomography. *Science* 1991; **254**: 1178–1181.
- Wang J, Fonn D, Simpson TL *et al.* The measurement of corneal epithelial thickness in response to hypoxia using optical coherence tomography. *American Journal of Ophthalmology* 2002; **133**: 315–319.
- Wang Y, Zhao Y, Nelson JS *et al.* Ultrahigh-resolution optical coherence tomography by broadband continuum generation from a photonic crystal fiber. *Optics Letters* 2003; **28**: 182–184.
- Wang J, Fonn D, Simpson TL. Topographical thickness of the epithelium and total cornea after hydrogel and PMMA contact lens wear with eye closure. *Investigative Ophthalmology and Visual Science* 2003; **44**: 1070–1074.
- Wang Y, Nelson S, Chen Z *et al.* Optimal wavelength for ultrahigh-resolution optical coherence tomography. *Optics Express* 2003; **11**: 1411–1417.
- Ringvold A, Anderssen E, Kjonniksen I. Impact of the environment on the mammalian corneal epithelium. *Investigative Ophthalmology and Visual Science* 2003; **44**: 10–15.
- Fujimoto J. Optical coherence tomography for ultrahigh resolution *in vivo* imaging. *Nature Biotechnology* 2003; **21**: 1361–1367.