In Vivo Cross-Sectional Observation and Thickness Measurement of Bulbar Conjunctiva Using Optical Coherence Tomography

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PURPOSE. To observe in vivo bulbar conjunctival structures, and determine the reproducibility of thickness measurements of normal human conjunctiva through optical coherence tomography (OCT) images.

METHODS. The bulbar conjunctivas of three study groups were observed: five enucleated porcine eyes with 20 um thickness aluminum foils inserted beneath conjunctival stroma; five glaucoma patients with 6-0 thread placed beneath conjunctival stroma during trabeculectomy; and 18 normal subjects. The cross-sectional conjunctival images were acquired using the scanning protocol from an optical coherence tomography device (Anterior Segment 5 Line Raster scanning protocol, Cirrus HD-OCT; Carl Zeiss Meditec, Inc., Dublin, CA). The thickness of different conjunctival layers was measured using central corneal thickness measurement software in the 18 normal subjects. Two reproducible parameters—interobserver (within-subject between-examiners) and intraobserver (within-subject between-date)—were estimated with intraclass correlation coefficients (ICC).

RESULTS. A thin hyporeflective surface layer, corresponding to the conjunctival epithelium layer, was observed. Beneath it, the conjunctival stroma appeared hyperreflective and was separated by a demarcation from the underlying progressively lower reflective Tenon's capsule. The anatomic location of the demarcation, which represents the loose connection between the conjunctival stroma and Tenon's capsule, was confirmed by inserting aluminum foil in porcine eyes, and by a 6-0 thread mark in glaucoma patients' eyes. The average conjunctival epithelium, stroma, and full thickness of the 18 normal subjects was 47.3 ± 8.4 um, 190.8 ± 47.5 um, and 238.8 ± 51.1 um, respectively. The intraobserver and interobserver intraclass correlation coefficients ranged from 0.918 to 0.985.

CONCLUSIONS. It is possible to accurately image and measure the cross-sectional structures of the bulbar conjunctival tissue with high resolution OCT. (*Invest Ophthalmol Vis Sci.* 2011;52: 7787–7791) DOI:10.1167/iovs.11-7749

The conjunctiva not only forms a physiologic barrier to outside pathogenic factors, but also secretes mucus that forms an integral part of the preocular tear film to maintain a moist, hydrophilic ocular surface. The conjunctiva is composed of two main structures: a superficial epithelial layer and an underlying conjunctival stroma.[1] An accurate, in vivo observation of alterations in these two structures will benefit the early diagnosis and more precise monitoring of conjunctival diseases. An understanding of the microscopic anatomy of the conjunctiva has traditionally been derived from light and electron microscopic studies of excised tissue samples, impression cytology of the conjunctival surface, and laser scanning confocal microscopy examination of the conjunctival cell.[2] In a clinical environment, the gross appearance of conjunctival tissue is commonly observed using a slit-lamp biomicroscope. However, none of these aforementioned instruments can provide in vivo, cross-sectional observation of conjunctival epithelium and stroma.

Optical coherence tomography (OCT) is now well recognized as a noninvasive technique for high-resolution, cross-sectional tomographic imaging of tissue by measuring backscattered light.[3] The anterior segment OCT always allows near video rate imaging acquisition, greater penetration through tissues with high light scatter such as sclera and limbus, and visualization of the cornea, iris, ciliary body, ciliary sulcus, anterior lens, and retro-iris lens.[4–11] A few reports described conjunctival observations with OCT. Feng and Simpson used a modified retinal OCT (Stratus OCT, model II, Carl Zeiss Meditec, Inc., Dublin, CA) to visualize and measure thickness of the human conjunctival epithelial layer.[12] Gumus and associates evaluated the ability of another OCT (Fourier-Domain RTVue-100; Optovue, Inc., Fremont, CA) with anterior segment adaptor for the diagnosis and monitoring of conjunctivochalasis.[13] Several authors considered anterior segment OCT as a promising tool to image trabeculectomy blebs.[10,11] However, to the best of our knowledge, no study has examined the in vivo, cross-sectional, full thickness structures of human conjunctiva. The purpose of the present study was to determine whether different bulbar conjunctival structures can be clearly visualized through OCT images and, if so, to measure the thickness of normal human conjunctival epithelium, stroma, and full layers. The repeatability and reproducibility of thickness measurements were also analyzed in the study.
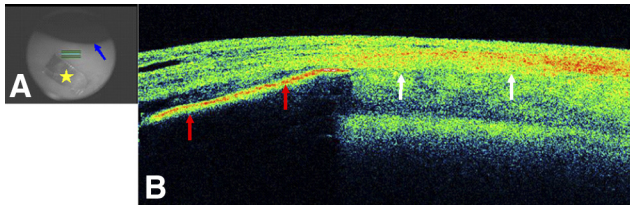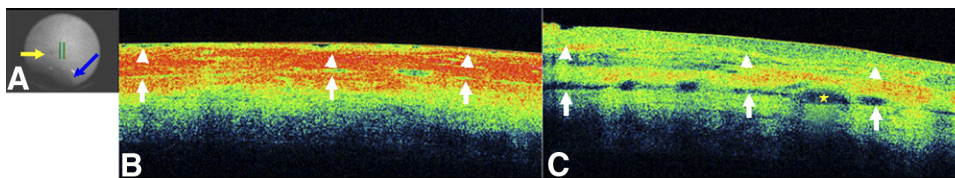
FIGURE 1.   The OCT images (Cirrus HD-OCT; Carl Zeiss Meditec Inc.) of an enucleated porcine eye. (**A**) Image from iris viewpoint. The Anterior Segment 5 Line Raster scan (*green lines*) was performed at the margin of a 2.5 × 3.0 mm aluminum foil (*yellow star*), which had been inserted beneath the conjunctival stroma 3 mm away from the corneal limbus (*blue arrow*). The conventional pseudocolor (**B**) OCT images corresponded to a selected individual scan of local bulbar conjunctiva. A discontinuous narrow low reflective surface layer was observed. The cross-section of aluminum foil appeared as a straight hyperreflective band (*red arrows*) just beneath the middle high reflective layer (conjunctival stroma). There was a narrow demarcation (*white arrows*) separating the high reflective layer and underlying progressively lower reflective layer in the conjunctival area without aluminum foil.

METHODS

The study had the approval of the Institutional Review Board of Putuo hospital, affiliated Shanghai Traditional Medicine University, and was carried out in accordance with the World Medical Association's Declaration of Helsinki. Written consent was obtained from each participant. The animal study was in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmology and Vision Research.

The OCT images were obtained using a Cirrus HD-OCT 4000 (Carl Zeiss Meditec Inc., Dublin, CA). The instrument uses optical interferometry to generate a log reflectivity profile describing the different layers of the tissue and allowing the separation of these reflective structures to be measured. This system is a spectral domain OCT platform that takes 27,000 axial scans per second and has a 5-μm axial resolution. The cross-sectional conjunctival images were acquired using the Anterior Segment 5 Line Raster scanning protocol, which is commonly used to view high resolution images of the anterior chamber angle and cornea. This mode scans through five parallel lines of equal length at 3 mm and separated by 250 μm. Each line is composed of 4096 A-scans and each 5 Line Raster scan was taken approximately 0.75 seconds (Carl Zeiss Meditec, Inc.; the scan mode can be found at www.meditec.zeiss.com). After image capture, the individual line with greatest clarity of detail was selected for conjunctival structure analysis by the high definition image analysis protocol. The conjunctival image should have had well-defined anterior and posterior surfaces and the least motion artifacts, especially within the area where the next measurement caliper was to be placed. If the images obtained after scanning were not satisfactory because of eye movement, the scan was repeated.

The conjunctival images of three study groups were evaluated in sequence. First, five fresh enucleated porcine eyes with the surrounding extraocular tissues (conjunctiva, Tenon's capsule, and extrabulbar muscles) were obtained from a nearby slaughterhouse and transported to the Putuo hospital, affiliated Shanghai Traditional Medicine University, in ice-cold modified Krebs-Ringer physiologic solution. In each of the animal eyes, a prepared approximate 2.5 × 3.0 mm, 20 um thickness of aluminum foil, which was originally used as the outer package of commercially available nimodipine tablets (Bayer Schering Pharmacy, Berlin-Wedding, Germany), was immediately inserted beneath conjunctival stroma to mark the anatomic location. OCT examination was performed at the margin of the aluminum foil, approximately 3 mm away from the corneal limbus, as shown in Figure 1. The OCT images were captured within 3 hours of the animal's death.

The second study group consisted of five randomly selected glaucoma patients scheduled for trabeculectomy surgery combined with releasable sutures for scleral flap. The suture technique followed Wilson's protocol using nylon 6-0 nonabsorbent thread.[14] During surgery, a minor anatomic gap close to the distal edge of the scleral flap (approximately 4 mm away from the corneal limbus), which separated the conjunctival stroma and underlying Tenon's capsule, was created by ophthalmic viscoelastic device injection through a blunt 27-gauge needle. A short part of the continuous thread was carefully placed inside the gap during suturing to mark the anatomic location. In each of the patients, OCT examinations were performed 1 day before and 5 days after trabeculectomy surgery. The cross-sectional conjunctival images were acquired after a blink without any topical anesthesia or lubricating drops. One experienced examiner (BL) positioned the patient's chin on the chin rest, and asked participants to press their heads firmly against the forehead rest and not to blink, to minimize movement during image capture. After turning off the room light, as recommended, the examiner positioned the external fixation target appropriately to direct the scanned eye's down gaze. This moved the eye position to expose the upper bulbar conjunctiva optimally. If necessary, the fellow eye was covered, to allow the participant to fixate with the scanned eye. The examiner adjusted the area of the eye visible in the iris viewpoint with chinrest control arrows until he was able to view the upper conjunctiva. The pre- and postoperative OCT images on the same region of upper conjunctiva, which included the thread in the anatomic gap, were compared, as shown in Figure 2.

Finally, 18 normal subjects were enrolled in the measurement reproducibility study group. These subjects were recruited from the staff at the Putuo hospital, affiliated Shanghai Traditional Medicine University. Only those subjects with no history or evidence of eyelid, ocular surface, or intraocular surgery, no lid abnormalities, no ocular surface diseases, or trauma history, and normal-appearing conjunctiva qualified as normal subjects. None were current contact lens wearers, or used any eye drops up to 2 hours before imaging. All patients had a complete anterior segment examination at each visit. OCT examination procedures were performed in this group similarly to the glaucoma patient group, except for directing the scanned eye's upper nasal

FIGURE 2.   OCT images (Cirrus HD-OCT; Carl Zeiss Meditec Inc.) of a randomly selected glaucoma patient's eye before trabeculectomy surgery and at 5 days after surgery. (**A**) Image from iris viewpoint. The Anterior Segment 5 Line Raster scan (*green lines*) was performed at the upper conjunctiva 4 mm away from the corneal limbus (*blue arrow*). After surgery, the scan lines ran across the nylon 6-0 nonabsorbent thread (*yellow arrow*), which was placed in a surgically-induced anatomic gap between conjunctival stroma and Tenon's capsule. The conventional pseudocolor (**B**) OCT images correspond to a selected individual scan of local bulbar conjunctiva before surgery. There was a continuous, narrow, low reflective surface layer (conjunctival epithelium, *superior to the white arrow heads*). Below the conjunctival epithelial layer, there was a high reflective layer (conjunctival stroma), separated from the underlying progressively lower reflective layer (Tenon's capsule) with a narrow demarcation (*white arrows*). The conventional pseudocolor (**C**) OCT images correspond to an individual scan of the same bulbar conjunctiva 5 days after surgery. The conjunctival epithelial layer (*superior to the white arrow heads*) and the conjunctival stroma layer (*between the arrow heads and arrows*) widened irregularly, and the reflectivity of them both decreased unevenly. The cross-sections of 6-0 nonabsorbent thread (*yellow star*) was located in the widened demarcation between the middle high reflective layer and underlying lower reflective layer.
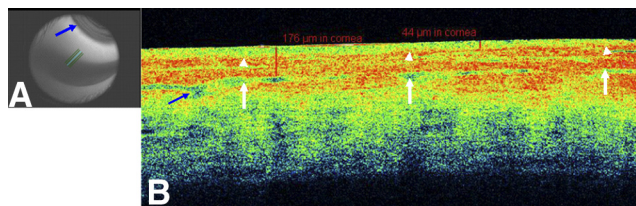
FIGURE 3. The OCT images (Cirrus HD-OCT; Carl Zeiss Meditec Inc.) of randomly selected normal subjects. (**A**) Image from iris viewpoint. The Anterior Segment 5 Line Raster scan (*green lines*) was performed at the lower temporal conjunctiva 5 mm away from the corneal limbus (*blue arrow*). The conventional pseudocolor (**B**) OCT images correspond to a selected individual scan of local bulbar conjunctiva. Thickness of the continuous, narrow, low reflective surface layer (conjunctival epithelium, *superior to the white arrow heads*) was approximately consistent. With central corneal thickness measurement software, the conjunctival epithelial layer thickness was measured as 44 μm. The thickness of conjunctival stroma (*between arrow heads and arrows*) was also consistent. The conjunctival full thickness was measured as 176 μm. A large branching capillary was incidentally observed (*blue arrow*).

gaze and imaging the lower temporal conjunctiva, to the 7:30 o'clock position in right eye or 4:30 o'clock position in left eye, approximately 3 mm to 5 mm away from the corneal limbus, as shown in Figure 3. We chose this location for the reproducibility study for four reasons: (1) as is well-recognized, the conjunctiva and underlying Tenon's capsule are merged together within 3 mm of the limbus; (2) a pinguecula, which often occurs as a normal aging variation, occurs more frequently in the nasal conjunctiva than the temporal side;[15] (3) the lower temporal conjunctival area can be easily exposed; and (4) the interference in conjunctival structure observation from muscle tendons of the lateral rectus and inferior rectus can be minimized. The Anterior Segment 5 Line Raster scan lines were rotated to image a cross-section perpendicular to the corneoscleral limbus at the scanned location (Fig. 3). Three conjunctival thickness parameters, the conjunctival epithelial thickness, the conjunctival stroma thickness, and the conjunctival full thickness, were measured using central corneal thickness (CCT) measurement software on the Cirrus OCT device (Cirrus HD-OCT 4000; Carl Zeiss Meditec Inc.). On the cross-sectional image of conjunctiva, the measurements were made at positions with apparent tissue landmarks, which were identified by differences in brightness between tissues.[13] At each conjunctival thickness measurement session, five independent measurements of the temporal conjunctiva from both eyes were taken and the mean value used for statistical analysis. Two forms of different reproducibility were assessed. First is the interobserver reproducibility, which evaluates the consistency of two consecutive conjunctival measurements in each of the subjects made by two examiners (BL and HZ) in the same day. The instrument was realigned after each scan. The two measurements were made in a masked fashion, in as much as each of the examiners did not know the other examiner's measurement results. The second is the intraobserver reproducibility, which evaluates the consistency of two conjunctival measurements in each of the subjects made by the same examiner (BL) from two different scans (1 day apart). For each subject, scans were completed at approximately the same time on each day.

For the analysis of reproducibility, when the conjunctival thickness measurements were completed in each eye, the right and left eye measurements were analyzed. As there were no significant differences between eyes, the measurement results from one randomly chosen eye were used for data presentation and reproducibility analyses. The conjunctival thickness parameters of the 18 subjects were presented as mean, minimum and maximum values, standard deviations (SDs), and 95% confidence intervals (CIs), based on one of the examiners' (BL) initial measurement results. The intraclass correlation coefficient (ICC) with a two-way random effects model, which measures the association between two replicates of the same sample (in this case, two conjunctival thickness values from the same patient), was calculated to deter-

mine the intraobserver and interobserver reproducibility.[16] An ICC between 0.4 and 0.75 indicates fair to good reproducibility, and an ICC of 0.75 or greater indicates excellent reproducibility.[16] Statistical software (SPSS V10.0; Chicago, IL) was used for database setup and statistical analyses. The level of statistical significance was set at $P <$ 0.05.

RESULTS

During the aluminum foil insertion surgery of the five enucleated porcine eyes, a loose connection between conjunctival stroma and underlying Tenon's capsule was observed and then easily broken within the area for insertion. When the incident light was at a right angle to the bulbar conjunctival surface, the OCT image appeared approximately horizontal and a specular reflection was present primarily off the front surface. The bulbar conjunctival OCT image contains a large range of reflectivity. A discontinuous narrow low reflective surface layer was observed, below which lay a relatively wider layer with high reflectivity. In the OCT image, the cross-section of aluminum foil appeared as a straight hyperreflective band just beneath the high reflective layer. Therefore, the high reflective layer was regarded as conjunctival stroma. In the conjunctival area without aluminum foil, a narrow demarcation, although not always apparent, separated the conjunctival stroma and the underlying layer with progressively lower reflectivity, the latter of which is supposed to be Tenon's capsule, as shown in Figure 1.

In the preoperative bulbar conjunctival OCT images of the five glaucoma patients' eyes, there were superior low reflective layers, which were continuous, narrow, and of approximately uniform thickness. As suggested by Feng and Simpson,[12,17] this layer corresponded to the thin bulbar conjunctival epithelium. Below the conjunctival epithelial layer, there was a high reflective layer, separated from the underlying progressively lower reflective layer with a narrow demarcation. Although the demarcation was inconspicuous in a few A-scans, the distance between epithelial layer and visible demarcation seemed consistent. During trabeculectomy surgery, the injected viscoelastic device broke the loose connection between the conjunctival stroma and underlying Tenon's capsule. In the 5-day postoperative OCT images (as shown in Fig. 2), the superior conjunctival epithelial layer and the higher reflective layer below widened irregularly, and the reflectivity of both decreased unevenly. The demarcation between the middle high reflective layer and underlying lower reflective layer still existed, and became more apparent and wider. Several typical ellipse nonreflective areas with slight to mild following shadow were only located in the demarcation, and were later confirmed as the cross-sections of nylon 6-0 nonabsorbent threads. Hence, it is considered that the demarcation can be used to separate the conjunctival stroma and Tenon's capsule.

In the measurement reproducibility study group, the 18 normal subjects consisted of 14 women and 4 men, aged 22 to 57, with an average age of 43.3 years (SD $\pm$ 11.6 years). All subjects were Chinese Han race. OCT was well tolerated by all subjects at all visits. In the lower temporal bulbar conjunctival OCT images of the 18 normal subjects, the superior low reflective epithelial layer, as well as the high reflective conjunctival stroma layer below was of approximately equal thickness, as shown in Figure 3. Several large branching capillaries, inside which there were moderate reflective light spots, were incidentally observed in the conjunctival stroma layer or the demarcation between stroma and Tenon's capsule. The bulbar conjunctival epithelial thickness and the conjunctival stroma thickness were measured between different tissue landmarks. The bulbar conjunctival full thickness is the sum of the conjunctival epithelial and stromal thickness. In all 18 subjects, the

TABLE 1. Bulbar Conjunctival Thickness Measurement Results (μm) in 18 Normal Subjects

	Mean	SD	Minimum	Maximum	95% CI
Bulbar conjunctival epithelial thickness	47.3	8.4	39	68	43.1-51.5
Bulbar conjunctival stroma thickness	190.8	47.5	116	284	167.2-214.5
Bulbar conjunctival full-thickness	238.8	51.1	160	336	213.4-264.2

conjunctival thickness measurements were accomplished successfully from the OCT image, regardless of when or by whom it was obtained. The initial bulbar conjunctival measurement results from examiner B.L. are presented in Table 1. A summary of the intraobserver and interobserver ICCs is presented in Table 2. The ICCs ranged from 0.918 to 0.985, indicating good agreement between measurements made by the examiners between dates or by two examiners within a date.

DISCUSSION

Histologically, bulbar conjunctival epithelium comprises several well-aligned cell layers[1]; thus, the incident light from OCT scatters less in this layer, which results in the hyporeflective feature of the conjunctival epithelial layer on the OCT image.[12,17] In the present study, hyporeflective surface layers in cross-sectional conjunctival OCT images were observed in all eyes examined, regardless of porcine, human, preoperative, or postoperative status. The thickness of the conjunctival epithelial layer seems consistent in normal human eyes. As we hypothesized, the conjunctival epithelial layer was discontinuous in the porcine eyes in vitro because of inevitable dehydration or injury during enucleation or transportation, and the widened conjunctival epithelial layer with subdued reflectivity corresponded to edematous epithelial cells after trabeculectomy surgery.

Under confocal microscopy, in vivo conjunctival stroma was observed as being composed of a network of mainly irregularly arranged delicate fibers, perfused blood vessels, cystic spaces often in close association with vessels, and single inflammatory cells.[18] In OCT imaging, the conjunctival stroma highly scatters incident light, and thus appears hyperreflective and clearly visible beneath conjunctival epithelium. Lawrenson described that in vitro conjunctival stroma can be resolved by light microscopy into two distinct layers: a superficial adenoid layer containing lymphocytes and mast cells, and a deeper and thicker fibrous layer containing the majority of conjunctival blood vessels and nerves.[19] Efron and associates described the different typical cellular morphology of the adenoid layer and the deeper fibrous layer of the conjunctival stroma with in vivo confocal microscopy.[2] However, according to the results of this study, it was hard to differentiate the two layers on the OCT image. It is plausible that the transition in tissue morphology from adenoid layer to fibrous layer is continuous. Another possibility is that these two layers may have histologic structure of similar refractive index, and the interface will not scatter and/or reflect light.

The Tenon's capsule (fascia bulbi) posterior to the conjunctiva is mainly composed of irregular fibers and its high reflectivity is similar to the conjunctival stroma in the OCT image. In the study performed by Feng and Simpson, the authors found that it is impossible to distinguish the limbal conjunctival stroma and its underlying connective tissue using modified Stratus OCT.[17] Although not observed, we suppose the intermingled conjunctival stroma and Tenon's capsule tissues at the limbal region cannot be differentiated from each other using the modified Cirrus OCT either. However, we observed that at least 3 mm away from the corneal limbus, there was a demarcation between bulbar conjunctival stroma and underlying Tenon's capsule in the Cirrus OCT image. We considered that this demarcation corresponds to the loose connection between conjunctival stroma and Tenon's capsule. In some areas where Tenon's capsule clings to the conjunctival stroma, the demarcation is not obvious. On the contrary, the demarcation becomes apparent after trabeculectomy surgery.

By using an OCT imaging protocol with clearly defined anatomic landmarks, the thickness of the bulbar conjunctival epithelium could be measured. In the study performed by Feng and Simpson, the mean thickness of bulbar conjunctival epithelial of 13 healthy subjects was 44.9 ± 3.4 μm,[12] which is slightly smaller than our measurement results. We believe there are at least two reasons for that difference. First, the subjects they observed were from a Canadian population with much younger age than the participants in the present study. Second, the Cirrus OCT is an evolution of the Stratus OCT (Carl Zeiss Meditec, Inc.), with higher axial resolution and faster image acquisition time. Thus, thickness measurement according to the Cirrus OCT is theoretically more precise. With confocal microscopy, the bulbar conjunctival epithelial thickness was reported as 32 to 32.9 μm,[2,18] much smaller than the measurement results from OCT. It is inferred that the entirely different thickness measurement method between OCT and confocal microscopy leads to the disparity.

The demarcation between conjunctival stroma and underlying Tenon's capsule enables our conjunctival thickness measurement. Based on our MedLine search, this report is the first to present data on conjunctival stroma thickness and full thickness values. An essential quality in determining the utility of a device for clinical use is its measurement of reproducibility. In the present study, even though slight variations were noted between a few measurements between two examiners, overall results demonstrated high intraobserver and interobserver reproducibility.

TABLE 2. Summary of Intraobserver and Interobserver Intraclass Correlation Coefficients

	ICC	95% CI	Statistic F	P
Intraobserver				
Bulbar conjunctival epithelial thickness	0.967	0.912-0.988	30.34	<0.01
Bulbar conjunctival stroma thickness	0.983	0.954-0.994	58.14	<0.01
Bulbar conjunctival full thickness	0.984	0.958-0.994	67.30	<0.01
Interobserver				
Bulbar conjunctival epithelial thickness	0.918	0.780-0.969	12.15	<0.01
Bulbar conjunctival stroma thickness	0.985	0.961-0.995	68.28	<0.01
Bulbar conjunctival full-thickness	0.982	0.952-0.993	55.47	<0.01

This suggests that conjunctival thickness measurements using OCT are immune to variability in measurements that occur over time both within and between sessions. This further emphasizes the ability of the Cirrus OCT instrument to measure small changes of conjunctival tissue accurately, potentially resulting in earlier detection of conjunctival disease or its progression before the onset of functional changes.

The limitations of the present study should not be neglected. First, the ruler we used in thickness measurement is calibrated for measuring corneal tissue, based on the refractive index of the cornea (approximately 1.38). Therefore, an inherent systematic bias on our conjunctival thickness values is inevitable. Nevertheless, there are no reported data describing the refractive index of conjunctival tissue. It is therefore impractical to adjust the obtained values in the equation. These measurement data should be considered as preliminary and subject to further refinement to improve accuracy. Second, our results were obtained on a very specific small group of the healthy Chinese population. Caution should be used when applying these data to other subjects. Third, the conjunctival thickness in the temporal region presented here might not be representative data for the whole conjunctiva. A future large scale population-based investigative study will generate more precise characterization of various regions of the conjunctiva.

In conclusion, observations of the porcine, human patient, and normal subject bulbar conjunctiva using the spectral domain Cirrus OCT are consistent with the histologic and clinical data. This novel, high axial resolution OCT makes it possible to image and measure the cross-sectional structures of bulbar conjunctival tissue, and that may aid in the specific differential diagnoses of inflammation, lesions, tumors, surgical, and drug- or contact lens-induced changes in conjunctiva.

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