Cornea

An Accurate Method to Determine Bowman's Layer Thickness In Vivo in the Human Cornea

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Purpose. To determine an accurate value for Bowman's layer (BL) thickness in vivo in humans.

Methods. Seventeen corneal transplant patients were examined preoperatively by laser-scanning in vivo confocal microscopy (IVCM), and corneal buttons were removed postoperatively and sectioned for light microscopy (LM). Nine corneas with uniformly thick BL by LM were used for thickness measurement. In the uniformly thick samples, probable overestimation of BL thickness in vivo by a first in vivo method (Method 1) led to the development of a revised in vivo method (Method 2). Method 2 was used to measure BL thickness in 20 healthy volunteers.

Results. In nine patients, mean BL thickness prior to transplantation was 13.7 ± 1.6 μm by IVCM (Method 1) while BL thickness of the removed corneal button was 9.7 ± 1.7 μm by LM ($P < 0.001$). The correlation of BL thickness between IVCM (Method 1) and LM was poor ($P = 0.226$). In 20 right eyes of 20 normal corneas, both in vivo methods were used to determine BL thickness. Mean BL thickness by Method 1 was 13.2 ± 1.6 μm and by Method 2 was 9.1 ± 1.4 μm ($P < 0.001$). BL thickness measurements by both in vivo methods were highly correlated ($P < 0.001$).

Conclusion. BL thickness by a revised in vivo method was close to LM values in this study and to values reported in fixed tissue in other studies. The authors believe this revised method provides the most accurate estimates of BL thickness in vivo to date. (*Invest Ophthalmol Vis Sci.* 2012;53:2354–2359) DOI:10.1167/iovs.11-8565

Bowman's layer (BL) is an acellular layer in the anterior cornea that consists of randomly oriented collagen fibrils. The anterior surface of BL is smooth and faces the epithelial basement membrane, while the posterior surface merges into the more organized anterior corneal stroma. Notably, BL does not regenerate after injury.[1–3]

Previous studies have found that BL is important as a physical barrier for the corneal stroma, positively influencing wound healing and nerve regeneration.[4,5] Consideration of BL is also required in the surgical management of conditions such as recurrent corneal erosions or epithelial basement membrane dystrophy (EBMD), where partial or total BL removal by laser ablation can influence the recurrence rate.[5,6] In such cases, the thickness of BL should be known accurately for planning precision ablation surgery; however, there is very little data available concerning the thickness of BL in vivo in humans.

Earlier studies attempting to measure human BL thickness have had varying results, with thickness ranging from 8 to 17.7 μm (Table 1), depending on the conditions of measurement (in vivo or ex vivo), tissue preparation method, and the measurement technique used. The techniques used to date include light microscopy (LM),[7] transmission electron microscopy (TEM),[8,9] tandem scanning confocal microscopy (TSCM),[10] and spectral domain optical coherence tomography (SD-OCT).[11]

Interestingly, there have been no studies using laser-scanning in vivo confocal microscopy (IVCM) to determine BL thickness, despite the superior resolution and magnification of this technique compared to other in vivo methods.[12] Moreover, no studies could be found directly comparing BL thickness in vivo to values obtained using ex vivo methods in the same cornea.

The purpose of this study was, therefore, to develop an accurate in vivo method of measuring BL thickness for eventual use in the treatment of EBMD by phototherapeutic keratectomy (PTK). To assist in this task, a direct comparison of high resolution in vivo measurements with a benchmark ex vivo method of BL thickness measurement was made using the same corneal samples.

Methods

Human Subjects

Following ethical approval obtained from the Linköping Human Research Ethics Committee and after obtaining informed consent, 17 consecutive corneal transplant patients were recruited preoperatively. Additionally, a group of 20 healthy volunteers was recruited. In transplant patients, preoperative examination of pathologic corneas using IVCM (HRT3-RCM, Heidelberg Engineering, Heidelberg, Germany) was conducted by a single operator. Details of the IVCM procedure have been described by Eckhard et al.[13] In healthy volunteers, examination included a thorough medical history using a standardized form, best spectacle-corrected visual acuity (BSCVA), and slit lamp examination, to rule out ocular pathology. IVCM was then performed (Heidelberg Engineering). The study adhered to ethical principles for research involving human subjects as stated in the Declaration of Helsinki.

Penetrating keratoplasty was performed by one of two surgeons at the Linköping University Hospital from May to September 2009. In all cases, the removed corneal button was obtained at surgery for further microscopic analysis. Since subsequent analysis of BL thickness in transplant patients by an in vivo method (Method 1, described below) revealed limitations of this in vivo technique, the in vivo method was

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Supported by a European Union Marie Curie Research Fellowship (NL).

Submitted for publication September 9, 2011; revised December 21, 2011; accepted February 26, 2012.

Disclosure: **J. Germundsson**, None; **P. Fagerholm**, None; **M. Koulikovska**, None; **N. Lagali**, None

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Investigative Ophthalmology & Visual Science, April 2012, Vol. 53, No. 4
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TABLE 1. Summary of Studies Reporting the Thickness of BL in Humans

Author/Year	Method	In Vivo?	N	Thickness (μm)	Reference
Ehlers N (2010)	LM	No	82	8.56 ± 2.76	[7]
Hayashi S (2002)	TEM	No	2	10 approx.	[8]
Komai Y (1991)	TEM	No	8	8–12	[9]
Li H (1997)	TSCM	Yes	7	16.6 ± 1.1	[10]
Tao et al. (2011)	SD-OCT	Yes	44	17.7 ± 1.6	[11]
This study	LM	No	9	9.7 ± 1.7	
	IVCM Method 1	Yes	9	13.7 ± 1.6	
	IVCM Method 2	Yes	20	9.1 ± 1.4	

N, number of samples.

revised (Method 2, see below) and validated using a separate group of healthy volunteers.

Corneal Sample Preparation

Samples of the central part of corneal buttons were taken using a 2 mm sterile dermal biopsy punch (Kai Sterile Dermal Biopsy Punch; Kai Industries Ltd.; Seki City, Japan), and stored in 2% glutaraldehyde solution for no more than one week. The samples were then imbedded in resin (Epon 812; TAAB, Reading, England). Four-micrometer thick sections were made and stained with toluidine blue dye for LM. Care was taken to section the tissue in a plane perpendicular to the corneal surface. One cornea was later prepared for TEM by placement of ultrathin 60 nm sections on Cu150 mesh grids. Ultrathin sections were then prepared with equal parts of 4% uranyl acetate solution and 100% ethanol, followed by lead citrate staining.

Technique of In Vivo and LM Measurement of BL

Of the 17 patient corneas, sectioning for LM disclosed that BL was absent in four cases. In a fifth case, it was not possible to perform IVCM examination prior to surgery. The remaining 12 corneas had suitable images of BL for in vivo and ex vivo comparison. Nine of these had a uniformly thick BL centrally in LM (based on absence of visible gross thickness variation or breaks in BL) and were selected for in vivo/ex vivo thickness comparison.

In vivo measurement of BL thickness was done as follows (Method 1). Preoperatively, five to seven separate image sequences of the central cornea were taken by IVCM in section scan mode (2–7 μm axial spacing) with images acquired at 8 frames/second while the focal plane was adjusted manually from the corneal surface through the epithelium, BL, and into the anterior stroma. Care was taken to avoid oblique images by adjusting microscope manual controls to ensure the presence of only one morphology (BL, keratocytes, subbasal nerves, or epithelium) in the field of view. IVCM scans were used to determine BL thickness as follows for Method 1: The top of BL (anterior surface of BL) was considered as the most posterior in-focus epithelial layer without haze (the normal, characteristic diffuse haze was taken to represent BL). The bottom of BL (posterior surface of BL) was considered as the most anterior layer of keratocytes that were in focus without haze present (Fig. 1). Each measurement was performed by two independent observers. Each subject's reported BL thickness was the mean of the values reported by the two observers, and each observer's value was the mean of five to seven image sequence measurements.

BL thickness by LM was determined using a light microscope (Axiophot; Zeiss, Oberkochen, Germany) with a 100× 1.30 NA oil immersion objective lens. Four to twelve separate sections for each central cornea were imaged in the light microscope, and three measurements per section were taken (Fig. 2). Distances were calibrated by imaging a standard etched glass ruler microscope slide (2 mm, 0.01 mm interval; Ernst Leitz, Wetzlar, Germany), and image processing software (ImageJ; public domain, http://rsbweb.nih.gov/ij/download.html)[14] was used to take measurements. Each patient's reported central BL thickness value was a mean of the measurements made by two independent observers, and each observer's value was the mean of all measurements from the various sections.

Additionally, it was noted in the LM sections that the posterior border of BL was often indistinct, so one cornea was selected for ultrastructural characterization to examine the BL to stromal transition region in greater detail. A transmission electron microscope at 80 kV (JEM-1230; JEOL, Tokyo, Japan) was used.

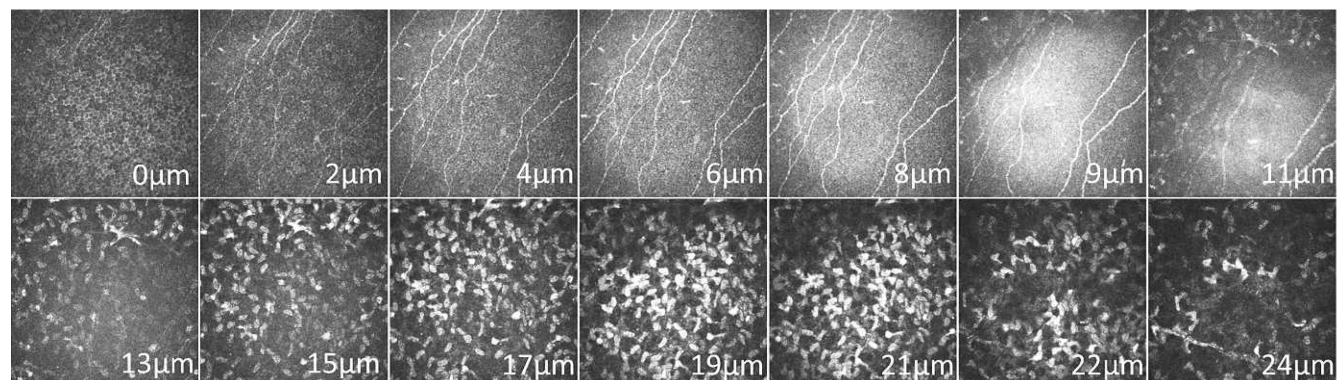

FIGURE 1. Series of consecutive image frames obtained by laser-scanning IVCM in a healthy volunteer, using the volume scan method. The series is used to illustrate two methods of in vivo measurement of BL thickness. Method 1: (Criteria used with the transplanted corneas.) Measurement starts at the most posterior in-focus epithelium layer without haze (0 μm) and stops at the most anterior layer where central keratocytes are in focus without haze (15 μm). BL thickness by this method is 15 μm. Method 2: Measurement starts at the most posterior epithelium layer (2 μm) with haze (often first layer with slight haze and subbasal nerves visible). The measurement stops at the most anterior layer with haze and where indistinct keratocytes are visible (13 μm). BL thickness by this method is 11 μm. All images are 400 × 400 μm.

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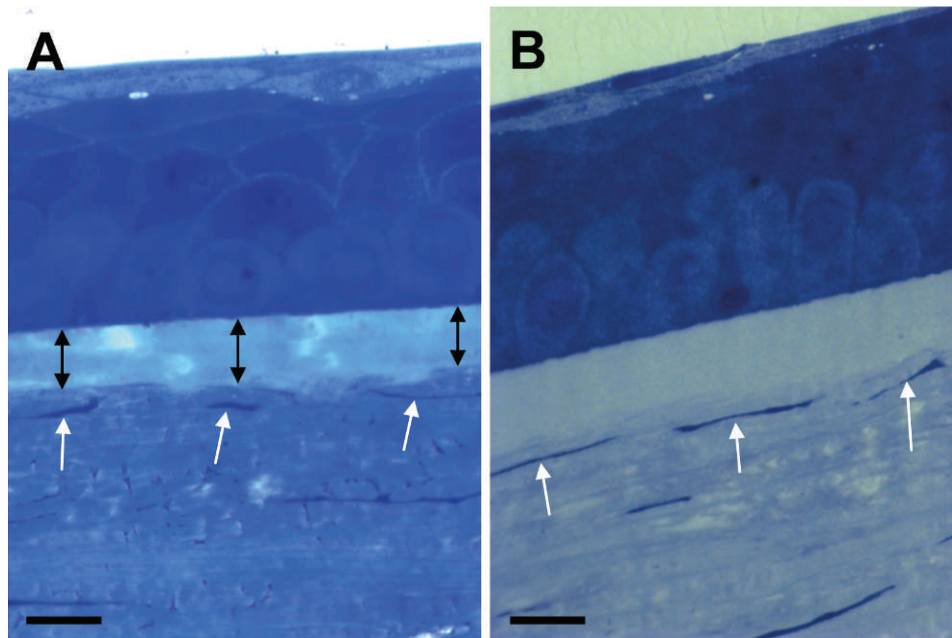

FIGURE 2. (A) Illustration of the technique used for the measurement of BL thickness by light microscopy from toluidine blue stained sections. Black arrows indicate the location and extent of independent measurements of BL as determined by a trained observer. (A, B) White arrows indicate the most anterior keratocytes, which are separated from the posterior aspect of BL by a thin layer of stroma. Bar = 10 μm.

In Vivo and Ex Vivo Comparison of Transplant Samples

In the nine corneal transplants with uniform BL, a consistent difference in BL thickness between LM and IVCM measurements was noted (see Results). It was also noted in LM sections that keratocytes were separated from the posterior border of BL by a thin region of stroma (Fig. 2). Taking into account this observation and the relatively coarse axial spacing of adjacent IVCM axial frames, it was determined that Method 1 likely overestimated BL thickness in vivo.

The method for measuring BL in vivo was therefore revised and used in the in vivo study of 20 healthy volunteers. The authors termed this Method 2: The IVCM volume scan mode was chosen (2 μm axial spacing between images) to give a more consistent indication of BL borders. Five to seven separate image sequences of the central cornea were taken with the volume scan mode. Interpretation of the borders was also revised, such that the top of BL was considered as the most anterior epithelial layer with haze (often with a slight haze and subbasal nerves visible). The bottom of BL was considered as the most anterior layer of stroma that showed some haze present and had visible indistinct keratocytes (Fig. 1). By including images with slight haze at the anterior and posterior limits of BL, Method 2 attempts to compensate for the overestimation of BL thickness by Method 1, which completely excludes out-of-focus light scattered by BL. For Method 2, each subject's reported BL thickness was the mean of the values reported by the two observers, and each observer's value was the mean of five to seven distinct image sequence measurements.

Quantitative Analysis and Statistics

All measurements of BL thickness were made by two independent, trained observers, and the values for BL thickness were taken as the mean of the values from both observers. The Bland-Altman method was used to compare interobserver differences using the 95% limits of agreement (LOA).[15]

Comparison of BL thickness between LM and IVCM was performed using the paired *t*-test and Pearson correlation. Thickness values in vivo by Method 1 and Method 2 were compared using the paired *t*-test and Pearson correlation. Additionally, the authors examined the correlations by regression analysis, using the built-in linear regression function in a spreadsheet (Excel 2007; Microsoft Inc., Redmond, WA). All statistical tests were performed using commercial software (SigmaStat 3.5; Systat Software Inc., Chicago, IL) where a 2-tailed level of $\alpha = 0.05$ was considered significant.

RESULTS

Patients and Healthy Volunteers

Of the nine transplant patients with uniform BL in LM sections, eight were primary penetrating keratoplasties (PK) and one was a retransplantation. Indications for PK included endothelial decompensation, keratoconus, and keloids with an uneven corneal surface (Table 2). Seventy-eight percent of patients were females, and the mean age of patients was 64 years (range 23–89). In the healthy volunteer group, 45% were females, and the mean age was 54 years (range 15–88).

TABLE 2. Characteristics of the Nine Patients in This Study for Which In Vivo and Ex Vivo Comparison of BL in the Same Corneal Samples Was Possible

Patient Number	Sex	Age	Indication	Re-transplant
1	F	61	Endothelial decompensation	N
2	F	76	Endothelial decompensation	N
3	F	75	Endothelial decompensation	N
4	F	55	Endothelial decompensation	N
5	F	64	Endothelial decompensation	N
6	F	89	Endothelial decompensation	N
7	M	49	Surface uneven, keloid	Y
8	M	23	Keratoconus	N
9	F	87	Endothelial decompensation	N

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TABLE 3. Comparison of BL Thickness in Corneas by IVCM and LM

Group	Transplant		Normal	
N	9		20	
Method	LM	IVCM Method 1	IVCM Method 1	IVCM Method 2*
BL thickness (μm)	9.7 ± 1.7	13.7 ± 1.6	13.2 ± 1.6	9.1 ± 1.4
Range (μm)	7.8–12.8	11.4–15.8	10.5–17.0	7.0–13.0
95% LOA†	± 13%	± 14%	± 11%	± 17%

Values quoted are mean ± standard deviation. N, number of subjects.
* Two IVCM methods were used to determine thickness values, with the revised method (Method 2) applied in normal subjects only.
† Values quoted for the 95% LOA indicate the interobserver variation expressed as a percentage of the mean.

BL Thickness by LM and IVCM Method 1

In the nine transplant patients, mean BL thickness was 13.7 ± 1.6 μm by IVCM (Method 1). Mean BL thickness in the same corneas by LM was 9.7 ± 1.7 μm. BL thickness in vivo by IVCM was significantly greater than ex vivo by LM ($P < 0.001$; Table 3). The correlation between BL thickness by LM and IVCM was tested (Fig. 3) and found to be poor ($R^2 = 0.20$, $P = 0.226$). BL thickness varied by 4 to 5 μm among the nine transplant patients.

BL Thickness by Two IVCM Methods

In 20 right eyes of 20 normal corneas, both in vivo methods were used to determine BL thickness. Mean BL thickness by Method 1 was found to be 13.2 ± 1.6 μm, while by Method 2 BL thickness was 9.1 ± 1.4 μm. Method 1 gave significantly thicker values than Method 2 ($P < 0.001$; Table 3); however, the correlation between Method 1 and Method 2 was very strong (Fig. 4; $R^2 = 0.93$, $P < 0.001$). BL thickness varied by 6 μm among the 20 healthy subjects.

Ultrastructure of BL to Stromal Transition

Both LM and in vivo images demonstrated the lack of a distinct border between BL and the anterior stroma. Ultrastructural analysis by TEM revealed a gradual, indistinct transition of the disordered collagen fibrils of BL to the more ordered collagen lamellae of the anterior stroma (Fig. 5).

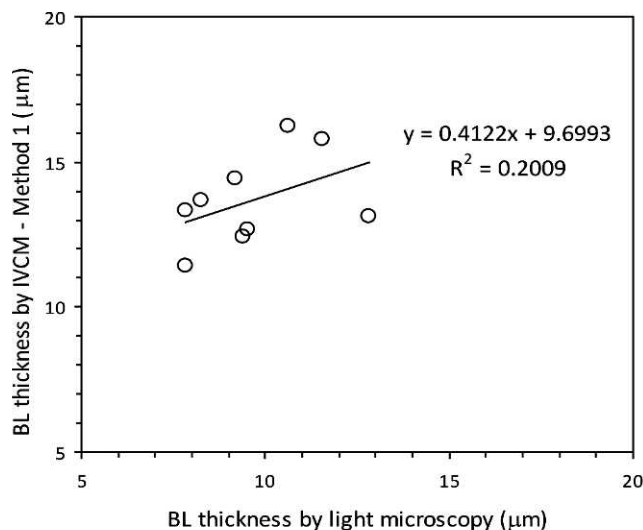

FIGURE 3. Comparison of BL thickness in nine corneal transplant patients by light microscopy and in vivo confocal microscopy methods. Thickness values were poorly correlated, and thickness among patients varied by up to 5 μm independent of the method used.

DISCUSSION

In the published literature, there is a large discrepancy in reported values of the thickness of BL (Table 1). Values in fixed specimens range from 8 to 12 μm[7–9] while in vivo values using TSCM and SD-OCT were significantly thicker, ranging from 13 to 21 μm.[10,11] This was the first study to attempt an accurate in vivo measurement of BL thickness with laser-scanning IVCM. Direct comparison of BL thickness by LM and IVCM (Method 1) in the same cornea tissue sample indicated that in vivo values were significantly thicker than by LM, but the values were poorly correlated. The authors suspected that the 2 to 7 μm separation between adjacent axial images in the sequence scan mode in Method 1 was too coarse to enable the boundaries of BL to be accurately determined, contributing to the poor correlation. Another possible contribution to the poor correlation is that samples fixed and prepared for LM may have undergone shrinkage to varying degrees.

The values for BL thickness by Method 1, although significantly greater than by LM, were still below those reported by other in vivo methods.[10,11] This could be due to the better image quality obtained with laser-scanning IVCM. The reported axial resolution of TSCM is 9 to 11 μm,[16,17] SD-OCT is 3 μm,[11] and IVCM is 4 μm.[18] Image quality, however, depends on both resolution and contrast,[19] and, at a microscopic level, laser-scanning IVCM provides the best image contrast of these methods.[12] Of the in vivo studies in

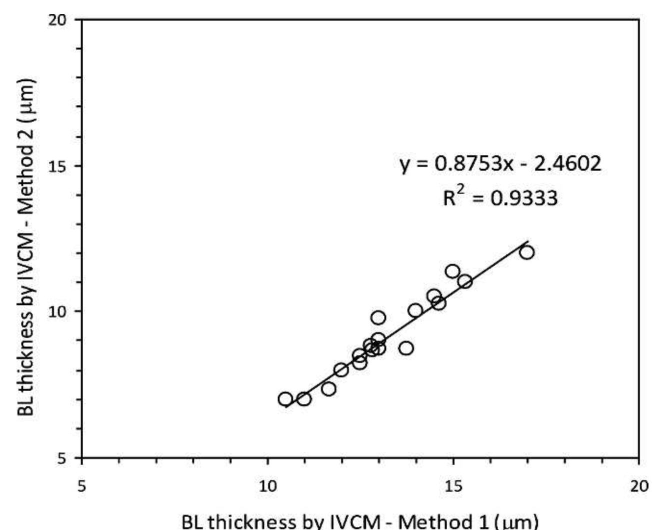

FIGURE 4. Comparison of BL thickness in 20 healthy corneas measured by two in vivo confocal microscopy methods. The values were highly correlated, with Method 1 yielding values 4 μm greater than Method 2. With both methods, thickness varied by up to 6 μm among subjects in the group.

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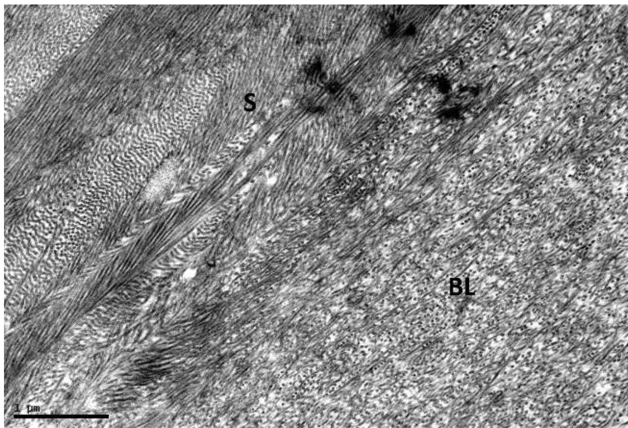

FIGURE 5. A gradual transition of the disordered collagen fibers of BL (BL) to the oriented collagen fibers arranged in lamellae within the stroma proper (S). No distinct or abrupt interface is visible. Scale bar: 1 μm.

Table 3, the present work is the only study to use actual images to determine the boundaries of BL in vivo. Other studies use averaged pixel intensity values to define the borders of BL, a method that assumes the boundaries of BL are abrupt, highly-reflective surfaces. When measuring BL thickness by LM, the authors noted that the posterior border of BL was often not sharp, and this was confirmed by TEM, where the posterior border of BL had a gradual transition from randomly-oriented collagen fibrils to the ordered collagen lamellae of the anterior stroma. By IVCM, both anterior and posterior borders of BL did not appear to be abrupt; instead a gradual diffuse light scatter indicated the disorganized BL collagen. Li et al.[10] indicated that their use of pixel intensity values from TSCM may have resulted in thicker values for BL, because they measured BL thickness as the distance between peaks in image intensity corresponding to subbasal nerves and keratocyte nuclei. Similarly, high-resolution SD-OCT measurements of BL rely on intensity of light scatter to locate BL. Additionally, as mentioned by Tao et al.,[11] the accuracy of SD-OCT measurements is limited by the value of refractive index used in the calculation algorithms, a parameter that is not directly measured for each corneal sample.

The authors noted that Method 1 likely also overestimated BL thickness, since the BL boundaries by this method were defined by basal epithelial cells and stromal keratocytes, both of which lie outside of BL, as was found in the LM tissue sections. For this reason, the authors revised the IVCM method to exclude the in-focus basal epithelial cells and anterior stromal keratocytes. Unfortunately, Method 2 could not be applied in the transplant patients, since the section scanning mode of IVCM (and not the volume scan) was used for the transplant patients prior to operation. To test the feasibility of Method 2, the authors instead examined a group of healthy volunteers, using a volume scan mode with a finer separation of adjacent axial images (1–2 μm). BL thickness values by Methods 1 and 2 were highly correlated, but the two values were offset by about 4 μm. Interestingly, BL thickness values by Method 2 were much closer to our LM values and values reported in ex vivo fixed tissue sections. The authors believe that Method 2 provides the most accurate estimates for BL thickness in vivo to date. BL thickness in vivo was determined to be 9.1 ± 1.4 μm in 20 healthy corneas, a value 6% greater than the value of 8.56 ± 2.76 μm reported by Ehlers et al. in ex vivo sections measured by LM in 82 corneas.[7] Tissue fixation and preparation for sectioning, however, are known to cause shrinkage artifacts,[20] and the shrinkage artifacts may partially account for this discrepancy.

An unexpected but significant finding of this study was that a large inter-individual variation exists in human BL thickness. In the relatively small number of samples examined in this study, the authors noted variation of BL thickness by 5 to 6 μm across subjects in both LM and IVCM measurements. In a larger sample of normal corneas, this range could be expected to be wider. This physiologic variability may be an additional source of the discrepancy in reported BL thickness values, especially in studies with smaller sample sizes. While the reasons for this variability are unknown, its existence should be acknowledged in situations where BL is to be surgically altered. Treatment planning for procedures such as refractive surgery or PTK, therefore, could be accompanied by individual in vivo BL thickness measurement to determine an appropriate ablation depth and to ensure proper postoperative epithelial adhesion.

Several methodological limitations became evident during the course of this study. It was noted that IVCM imaging of BL can be difficult due to patient and operator-induced motion artifacts, and an experienced operator performing multiple volume scans through BL is necessary to obtain quality image stacks for analysis. Also, as noted earlier, initial use of the section scan method in transplant patients precluded direct comparison of Method 2 thickness values with LM in the same corneas. Another limitation is the axial resolution of laser-scanning IVCM, which, at 4 μm presently, is almost half the thickness of BL in sections. To better locate the borders of BL, a finer axial resolution is required, combined with a finer axial spacing of adjacent confocal images. Also, as observed in LM sections in this study not included in the analysis, BL thickness may not be constant over the entire central cornea, particularly in pathologic cases. The assumption of constant BL thickness in the central cornea may therefore be incorrect, so, ideally, several central locations should be sampled in vivo. Knowledge of BL thickness variation could be important in planning ablation procedures such as PTK that typically involve a 7 mm diameter of the central cornea. Although in this study several in vivo scans were obtained per cornea—likely representing slightly different central locations—a more formal protocol for in vivo sampling could be employed. Another limitation of the in vivo technique is that determination of BL borders by IVCM is dependent on somewhat subjective criteria. Although Method 2 attempts to use anatomic features to define BL, analysis of images requires interpretation of features, and to compensate for individual variations in interpretation, the thickness value could be the mean of measurements by two observers. Despite these limitations, however, the authors believe that the method presented represents the most accurate means to date to determine BL thickness in vivo.

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