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# An accurate method to determine Bowman's layer thickness in vivo in the human cornea

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## ABSTRACT

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

**Methods:** 17 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). 9 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 9 patients, mean BL thickness prior to transplantation was  $13.7 \pm 1.6\mu\text{m}$  by IVCM (Method 1) while BL thickness of the removed corneal button was  $9.7 \pm 1.7\mu\text{m}$  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  $13.2 \pm 1.6\mu\text{m}$  and by Method 2 was  $9.1 \pm 1.4\mu\text{m}$  ( $P < 0.001$ ). 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. We believe this revised method provides the most accurate estimates of BL thickness in vivo to date.

**Key words:** Bowman's layer, confocal microscopy, light microscopy, corneal transplantation

## **Introduction:**

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.<sup>1-3</sup>

Previous studies have found that BL is important as a physical barrier for the corneal stroma, positively influencing wound healing and nerve regeneration<sup>4,5</sup>. 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<sup>5,6</sup>. 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 $\mu\text{m}$  (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)<sup>7</sup>, transmission electron microscopy (TEM)<sup>8,9</sup>, tandem scanning confocal microscopy (TSCM)<sup>10</sup>, and spectral domain optical coherence tomography (SD-OCT)<sup>11</sup>.

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<sup>12</sup>. 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 elsewhere<sup>13</sup>. 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 (HRT3-RCM, Heidelberg Engineering, Heidelberg, Germany). 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 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 2mm 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 Epon 812 resin (TAAB, Reading, England). 4µm-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, after sectioning for LM it was found 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. 9 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, 5-7 separate image sequences of the central cornea were taken by IVCN in section scan mode (2-7 $\mu$ m 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. IVCN 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 (Figure 1). Each measurement was performed by two independent observers. BL thickness for a given subject was reported as the mean thickness value across both observers, with each observer value representing the mean thickness across 5-7 image sequences.

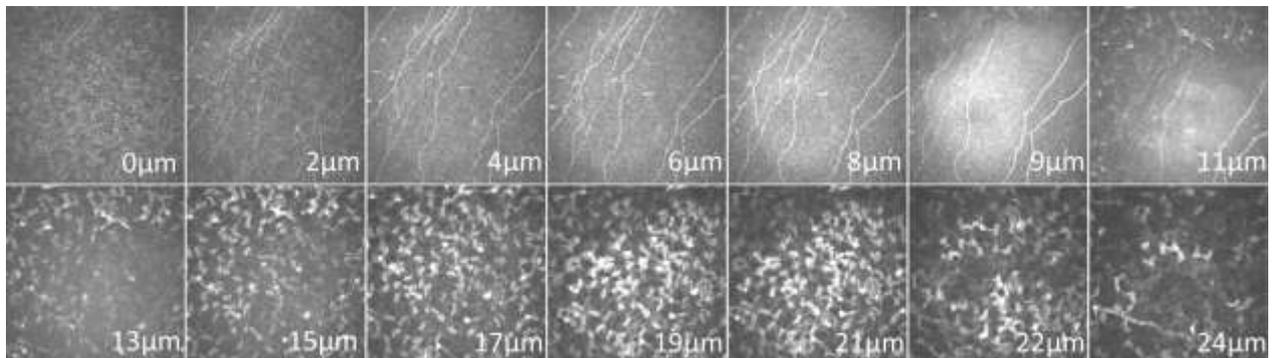
BL thickness by LM was determined using a Zeiss® Axiophot light microscope with a 100x 1.30 NA oil immersion objective lens. 4-12 separate sections for each central cornea were imaged in the light microscope, and three measurements per section were taken (Figure 2). Distances were calibrated by imaging a standard etched glass ruler microscope slide (Leitz Wetzlar, Germany), and Image J software<sup>14</sup> was used to take measurements. The value of central BL thickness for the patient was taken as the mean of all measurements from the various sections, and across two independent observers.

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 JEOL JEM-1230 (Tokyo, Japan) transmission electron microscope at 80kV was used.

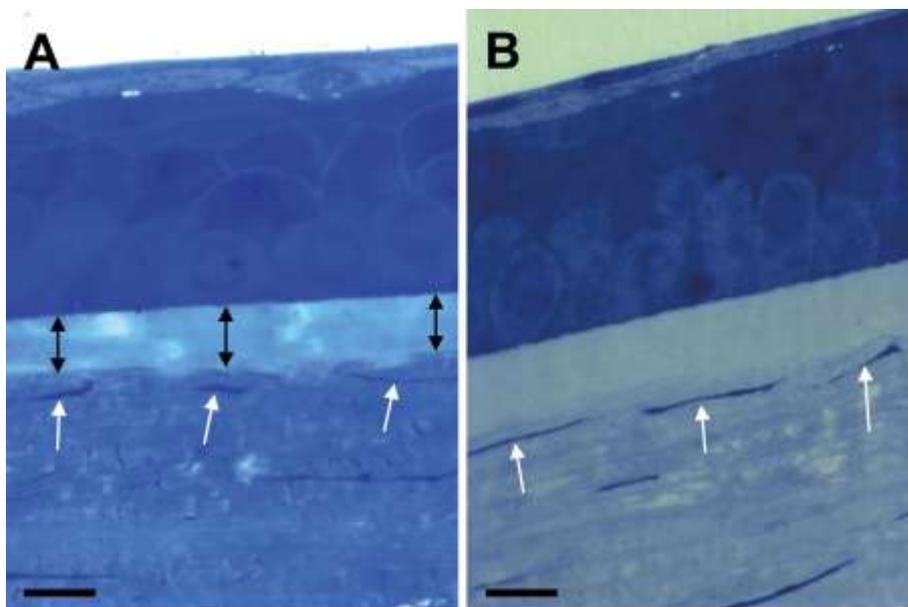
### **In-vivo and ex-vivo comparison of transplant samples**

In the 9 corneal transplants with uniform BL, a consistent difference in BL thickness between LM and IVCN 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 (Figure

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.



**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 Bowman's layer thickness. **Method 1:** Criteria used with the transplanted corneas. From the most posterior in-focus epithelium layer without haze ( $0\ \mu\text{m}$ ), to the most anterior layer where central keratocytes are in focus without haze ( $15\ \mu\text{m}$ ). Bowman's layer thickness by this method is  $15\ \mu\text{m}$ . **Method 2:** Measurement starts at the most posterior epithelium layer ( $2\ \mu\text{m}$ ) 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 ( $13\ \mu\text{m}$ ). Bowman's layer thickness by this method is  $11\ \mu\text{m}$ . All images are  $400 \times 400\ \mu\text{m}$ .



**Figure 2.** (A) illustration of the technique used for the measurement of Bowman's layer thickness by light microscopy from toluidine blue stained sections. Black arrows indicate location and extent of independent measurements of Bowman's layer as determined by a trained observer. (A, B) white arrows indicate the most anterior keratocytes, which are separated from the posterior aspect of Bowman's layer by a thin layer of stroma. Bar =  $10\ \mu\text{m}$ .

The method for measuring BL in vivo was therefore revised and used in the in vivo study of 20 healthy volunteers. We termed this Method 2: the IVCN volume scan mode was chosen (2 $\mu$ m axial spacing between images) to give a more consistent indication of BL borders. 5-7 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 with some haze present, and where indistinct keratocytes are visible (Figure 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, BL thickness for a given subject was reported as the mean thickness value across both observers, with each observer value representing the mean thickness across 5-7 distinct image sequences.

### **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 mean of the values from both observers. The Bland-Altman method was used to compare inter-observer differences using the 95% limits of agreement (LOA) <sup>15</sup>.

Comparison of BL thickness between LM and IVCN 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, we examined the correlations by regression analysis, using the built-in linear regression function in an Excel 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 two-tailed level of  $\alpha = 0.05$  was considered significant.

## **Results**

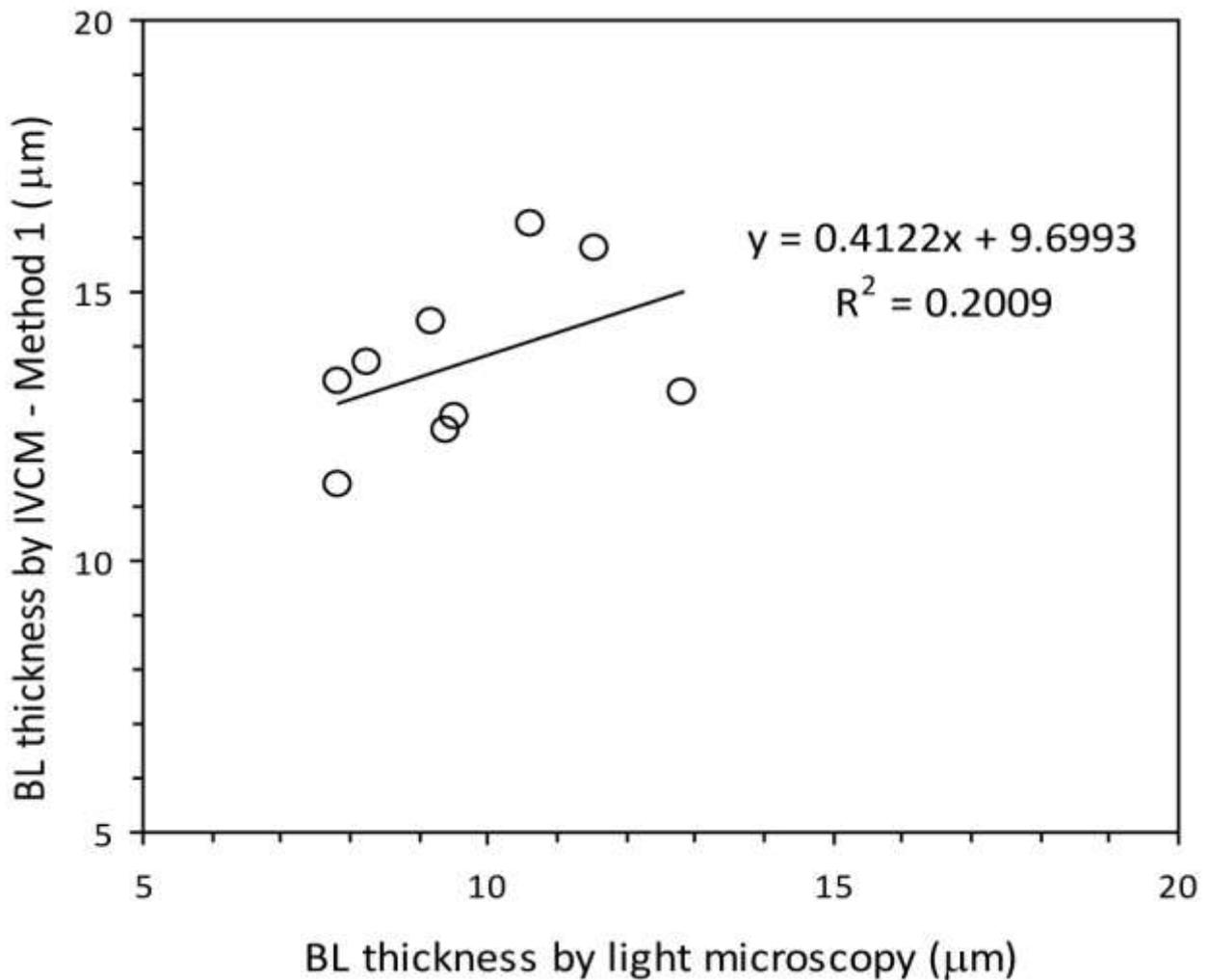
### **Patients and Healthy Volunteers**

Of the 9 transplant patients with uniform BL in LM sections, 8 were primary penetrating keratoplasties and 1 was a re-transplantation. Indications for PK included endothelial decompensation, keratoconus, and keloids with an uneven corneal surface (Table 2). 78% of

patients were females, and the mean age of patients was 64y (range 23-89y). In the healthy volunteer group 45% were females, and the mean age was 54y (range 15-88y).

### BL thickness by LM and IVC Method 1

In the 9 transplant patients, mean BL thickness was  $13.7 \pm 1.6\mu\text{m}$  by IVC (Method 1). Mean BL thickness in the same corneas by LM was  $9.7 \pm 1.7\mu\text{m}$ . BL thickness in vivo by IVC was significantly greater than ex vivo by LM ( $P < 0.001$ ; Table 3). The correlation between BL thickness by LM and IVC was tested (Figure 3), and found to be poor ( $R^2 = 0.20$ ,  $P = 0.226$ ). BL thickness varied by 4-5 $\mu\text{m}$  among the 9 transplant patients.

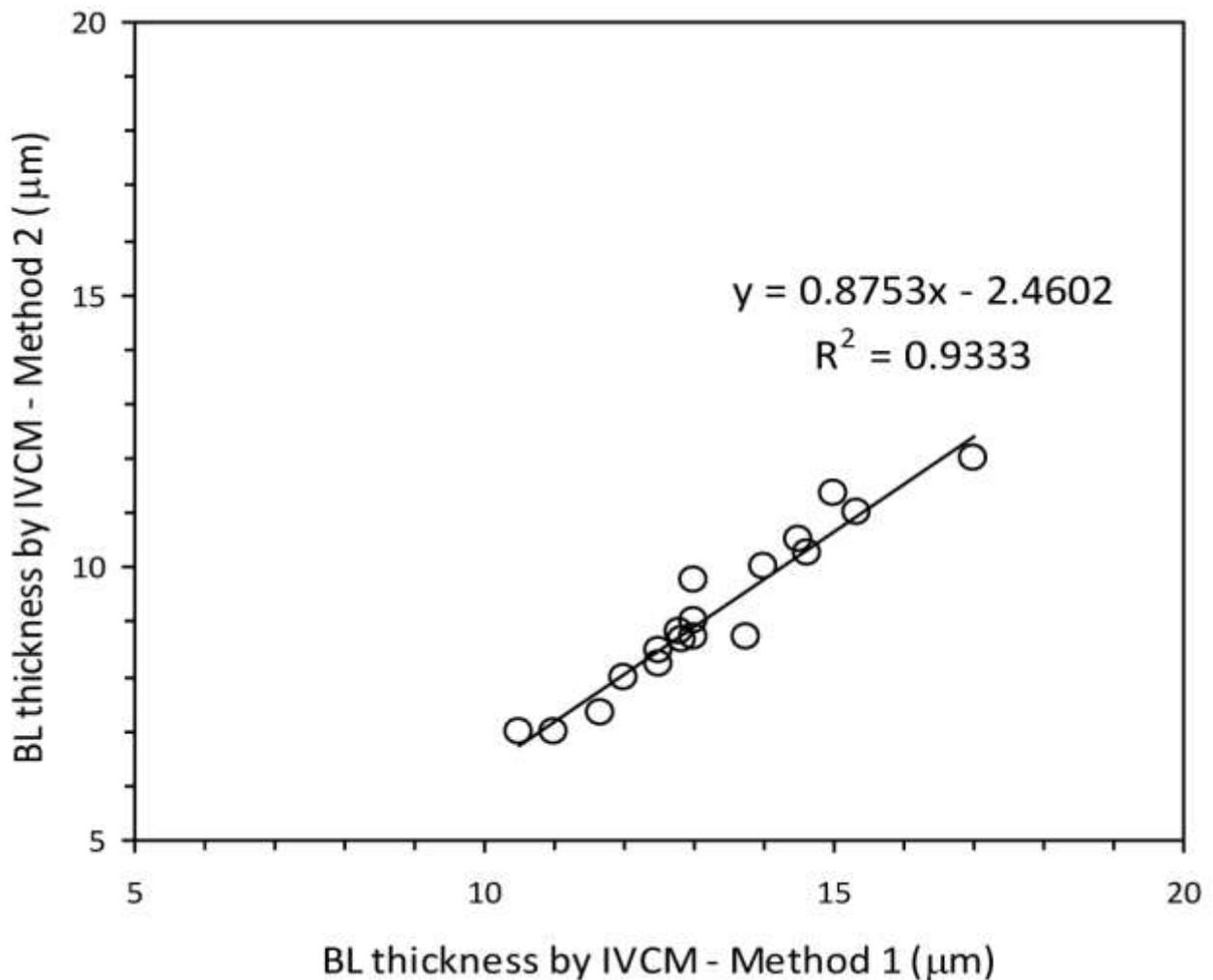


**Figure 3.** Comparison of Bowman’s layer thickness in nine corneal transplant patients by light microscopy and in vivo confocal microscopy methods. Thickness values were poorly correlated and thickness among patients varied by up to 5 $\mu\text{m}$  independent of the method used.

### BL thickness by two IVC 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  $13.2 \pm 1.6\mu\text{m}$ , while by Method

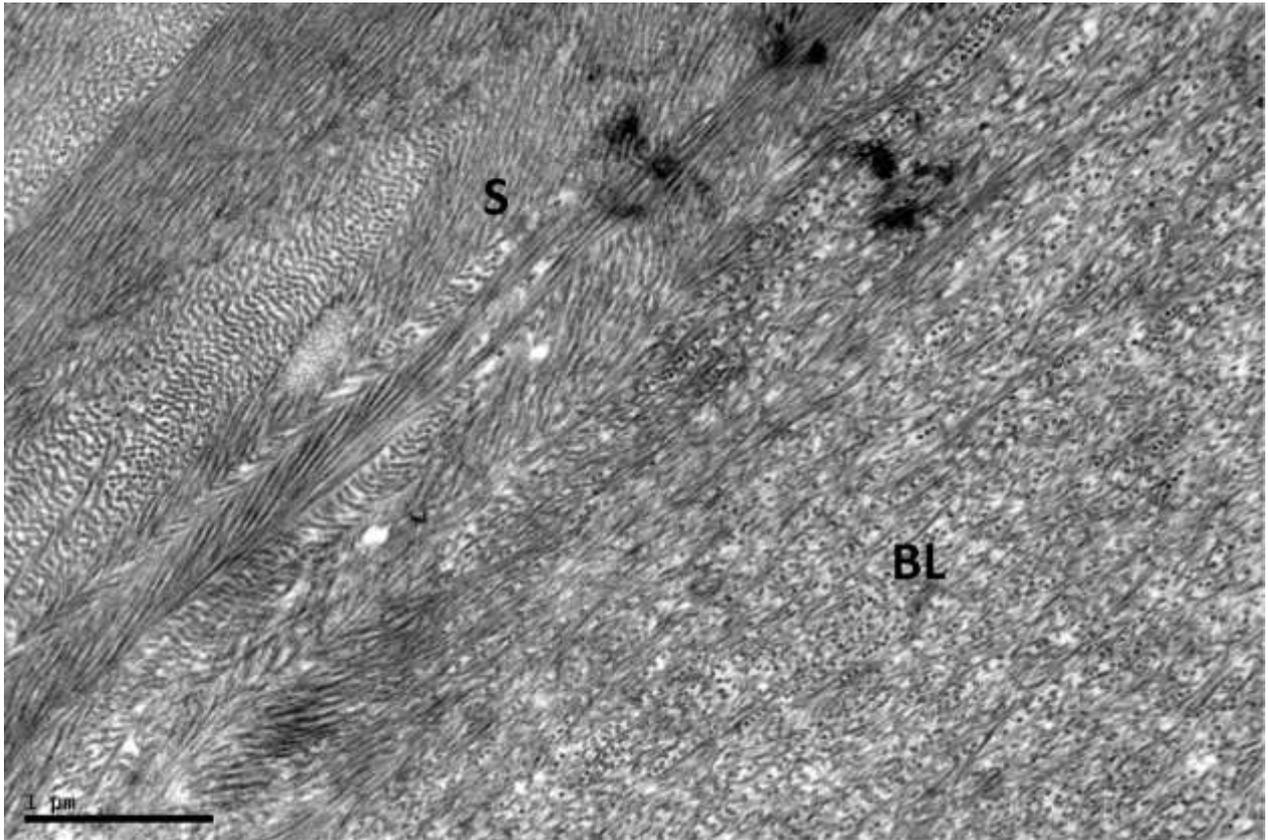
2 BL thickness was  $9.1 \pm 1.4 \mu\text{m}$ . Method 1 gave significantly thicker values than Method 2 ( $P < 0.001$ ; Table 3), however, the correlation between Method 1 and Method 2 was very strong (Figure 4,  $R^2 = 0.93$ ,  $P < 0.001$ ). BL thickness varied by  $6 \mu\text{m}$  among the 20 healthy subjects.



**Figure 4.** Comparison of Bowman’s layer thickness in 20 healthy corneas measured by two in vivo confocal microscopy methods. The values were highly correlated, with Method 1 yielding values  $4 \mu\text{m}$  greater than Method 2. With both methods, thickness varied by up to  $6 \mu\text{m}$  among subjects in the group.

#### **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 (Figure 5).



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

### **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 - 12 μm<sup>7-9</sup> while in vivo values using TSCM and OCT were significantly thicker, ranging from 13 - 21 μm<sup>10,11</sup>. This was the first study to attempt an accurate in-vivo measurement of BL thickness with laser-scanning IVCN. Direct comparison of BL thickness by LM and IVCN (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. We suspect that the 2-7 μm separation of adjacent axial images in the sequence scan mode in Method 1 were 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<sup>10,11</sup>. This could be due to the better image quality obtained with laser-scanning IVCN. The reported axial resolution of TSCM is 9 - 11

$\mu\text{m}$ <sup>16,17</sup>, SD-OCT is  $3 \mu\text{m}$ <sup>11</sup> and IVCN is  $4 \mu\text{m}$ <sup>18</sup>. Image quality, however, depends on both resolution and contrast<sup>19</sup>, and at a microscopic level, laser-scanning IVCN provides the best image contrast of these methods<sup>12</sup>. Of the in vivo studies in 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, we 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 IVCN, 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.<sup>10</sup> indicated that their use of pixel intensity values from TSCM may have resulted in thicker values for BL, as they measured BL thickness as the distance between peaks in image intensity corresponding to subbasal nerves and keratocyte nuclei, as an indicator of BL thickness. Similarly, high-resolution OCT measurements of BL rely on intensity of light scatter to locate BL. Additionally, as mentioned by Tao et al.<sup>11</sup>, the accuracy of 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.

We noted that our 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 we noted in LM sections. For this reason, we revised the IVCN 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 we only used the section scanning mode of IVCN for the transplant patients prior to operation. To test the feasibility of Method 2, we instead examined a group of healthy volunteers, using a volume scan mode with a finer separation of adjacent axial images ( $1\text{-}2 \mu\text{m}$ ). BL thickness values by Methods 1 and 2 were highly correlated, but offset by about  $4 \mu\text{m}$ . Interestingly, BL thickness values by Method 2 were much closer to our LM values and values reported in ex vivo fixed tissue sections. We believe that Method 2 provides the most accurate estimates for BL thickness in vivo to date. BL thickness in vivo was determined to be  $9.1 \pm 1.4 \mu\text{m}$  in 20 healthy corneas, a value 6% greater than the value of  $8.56 \pm 2.76 \mu\text{m}$  reported by Ehlers et al. in ex vivo sections measured by LM in 82 corneas<sup>7</sup>. Tissue fixation and preparation for sectioning, however, is known to cause shrinkage artifacts<sup>20</sup>, and 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 our relatively small samples, we noted variation of BL thickness by 5 – 6  $\mu\text{m}$  across subjects in both LM and IVCN 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 IVCN 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 IVCN, which at 4 $\mu\text{m}$  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 7mm 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 IVCN is dependent on somewhat subjective criteria. Although Method 2 attempts to use anatomic features to define BL, analysis of images requires interpretation of features, which could be partially compensated by using the mean thickness value between two observers. Despite these limitations, however, we believe that the method presented represents the most accurate means to date to determine BL thickness in vivo.

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Table 1. Summary of studies reporting the thickness of Bowman’s layer in humans. N = number of samples. LM = light microscopy; TEM = transmission electron microscopy; TSCM = tandem-scanning confocal microscopy; SD-OCT = spectral domain optical coherence tomography; IVCM = in vivo confocal microscopy (laser-scanning).

Author/Year	Method	In-vivo?	N	Thickness ( $\mu\text{m}$ )	Reference
Ehlers N (2010)	LM	No	82	$8.56 \pm 2.76$	[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	$16.6 \pm 1.1$	[10]
Tao et al. (2011)	SD-OCT	Yes	44	$17.7 \pm 1.6$	[11]
This study	LM	No	9	$9.7 \pm 1.7$	
	IVCM Method 1	Yes	9	$13.7 \pm 1.6$	
	IVCM Method 2	Yes	20	$9.1 \pm 1.4$	

Table 2. Characteristics of the 9 patients in this study for which in vivo and ex vivo comparison of Bowman's layer in the same corneal samples was possible.

Pat nr	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

Table 3. Comparison of Bowman’s layer (BL) thickness in corneas by in vivo confocal microscopy (IVCM) and light microscopy (LM). Values quoted are mean  $\pm$  standard deviation. Two IVCM methods were used to determine thickness values, with the revised method (Method 2) applied in normal subjects only. N = number of subjects. Values quoted for the 95% LOA (limits of agreement) indicate the inter-observer variation expressed as a percentage of the mean.

<b>Group</b>	<b>Transplant</b>		<b>Normal</b>	
<b>N</b>	9		20	
<b>Method</b>	LM	IVCM Method 1	IVCM Method 1	IVCM Method 2
<b>BL thickness (<math>\mu\text{m}</math>)</b>	9.7 $\pm$ 1.7	13.7 $\pm$ 1.6	13.2 $\pm$ 1.6	9.1 $\pm$ 1.4
<b>Range (<math>\mu\text{m}</math>)</b>	7.8 - 12.8	11.4 - 15.8	10.5 - 17.0	7.0 - 13.0
<b>95% LOA</b>	$\pm$ 13%	$\pm$ 14%	$\pm$ 11%	$\pm$ 17%