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Assessment of Corneal Thickness and Keratocyte Density in a Rabbit Model of LASIK using Scanning Laser Confocal Microscopy

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INTRODUCTION

Corneal thickness is one of the primary parameters that determine the structural integrity of the cornea. It is reduced in keratoconus and other ectatic degenerations and has been shown to decrease with prostaglandin analog use for the treatment of glaucoma.¹ Keratocytes, the resident fibroblastic cells of the cornea, are responsible for maintenance and integrity of corneal tissue and their physiology, morphology and numbers are altered by clinical treatments such as corneal refractive surgery,² UV corneal crosslinking,^{3, 4} mitomycin C use,⁵ and other common interventions. In this research, we evaluate the use of in vivo scanning laser confocal microscopy to evaluate the accuracy and repeatability of in vivo measurements of corneal thickness and keratocyte density to determine the suitability of these measurements for laboratory and clinical studies of these structural parameters.

There are several clinical instrument designs available for in vivo confocal microscopy that provide en face optical tomographic sections of the cornea and other anterior segment tissues based on backscattered illumination principles that do not rely on exogenous contrast agents. McLaren and others have compared corneal thickness and cell density measurements using different in vivo confocal microscope designs and have shown that these parameters differ based upon the design principles of the confocal microscope.⁶ Likewise, others have shown that measurements of cell density and corneal thickness differ from other standard measurement techniques, e.g. specular microscopy⁷ or ultrasonic pachymetry.⁸ Recent reviews of this clinical imaging technology are available.⁹

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Nipkow disc-based tandem scanning instruments were one of the first designs capable of providing in vivo microscopic images with micron-level resolution.¹⁰ The optical inefficiency of these instruments requires relatively high illumination levels and longer image acquisition times, which has limited their clinical use. A scanning-slit microscope design was introduced in the 1980's (Nidek, Inc., Freemont, CA) and although light levels remain somewhat high, this design is optically more efficient and provides improved image contrast and reduced acquisition times.¹¹ As with tandem scanning confocal microscopes, the scanning-slit confocal objective lens is coupled to the ocular surface with a viscous fluid interface during imaging. Ocular pulsations, patient movements, or instrument movements relative to the objective lens are some of the factors that add to the variability and uncertainty of lateral position and axial imaging plane (z-axis). An ocular fixation device, the Z-ring adapter, was developed to help stabilize the axial position of the eye relative to the confocal microscope objective lens.⁸

More recently, a corneal confocal lens module was developed to adapt a retinal confocal scanning laser microscopy system for corneal imaging (Rostock corneal Module, Heidelberg Engineering, GmbH Vista, CA).^{12, 13} This laser-illuminated confocal imaging system provides greater image contrast relative to scanning-slit or spinning disc confocal designs and provides high contrast images of corneal microstructure with micron level resolution.^{9, 14} This imaging modality affords both en face and transverse views of the tissue (Figure 1). This laser confocal system is designed to contact tissue when imaging. While this does not prevent lateral movements of the eye during imaging, this contact design effectively minimizes axial movements of the eye reducing uncertainty associated with scan depth.

The purpose of this study was to assess the ability to estimate keratocyte density and central corneal thickness using a rabbit model of corneal refractive surgery. The specific objectives of this study were to (1) assess the agreement of corneal thickness measurements between ultrasonic pachymetry and laser confocal microscopy, (2) evaluate the depth plane resolution of the laser confocal microscope, (3) assess the repeatability of keratocyte density estimates by this imaging method, and (4) determine the effects of a lamellar incision on measured keratocyte density.

METHODS

This was a prospective experimental study design. The subjects for this study were five adult New Zealand white rabbits (2 male and 3 female). This research was reviewed and approved by the local Institutional Animal Care and Use Committee and all procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. Animals were housed singly and were watered and fed a standard chow diet *ad libitum*; their average weight was 4.5 kg.

Corneal thickness measurements, and corneal confocal microscopy imaging were performed before and six weeks after a lamellar corneal incisions was made. These procedures were performed on animals under general anesthesia which was achieved by intramuscular injection of ketamine and xylazine. Anesthesia was maintained using isoflurane gas. Ultrasonic corneal thickness measurements were determined as the average of 10 measurements recorded for each eye using a solid contact ultrasonic probe (DGH-550 Pachette II; DGH Technologies Inc., Exeter, PA). Our methods for determination of corneal thickness from confocal microscopy imaging are described below.

Surgical Procedures and Postoperative Care

A nominal 130 μm corneal flap was cut with a new blade (OASIS Medical, Inc. Glendora, CA) in the right eye of each subject using the ACS microkeratome (Bausch & Lomb, Rochester, NY). A nasal hinge position was used to avoid flap displacement by the nictitating membrane. After cutting the flap, a spatula was used to reflect the tissue. The stromal bed was irrigated with sterile saline and then the flap was repositioned and allowed to adhere to the stromal bed. Postoperatively, one drop of 0.3% ciprofloxacin was applied to the eye, and 0.03 mg/Kg buprenorphine was given via sub-cutaneous injection and animals were fitted with a cervical collar for the first three days after corneal incision to prevent them from physically disturbing the corneal lamellar incision, e.g. while grooming.

Animals received antibiotic prophylaxis (one drop of 0.3% ciprofloxacin hydrochloride) ophthalmic solution four times daily for one week and one drop of 1% prednisolone acetate ophthalmic suspension four times daily for one week to mitigate ocular inflammation. Animals were monitored daily using slit-lamp biomicroscopy for the first week to confirm that there were no visible signs of postoperative inflammation. Animals were then monitored weekly until examined again using confocal microscopy six weeks after the lamellar incision.

Confocal Microscopy Imaging

Calibration—The Rostock Corneal Module is comprised of a lens system fitted over the existing retinal confocal objective of the Heidelberg Retinal Tomography instrument (Heidelberg Engineering GmbH, Vista, CA). This lens system includes an immersion microscope lens objective (Achromplan 63x/0.95, 1.45 mm, Carl Zeiss GmbH, Göttingen, Germany) coupled with an internal exchangeable user-selected field lens that determines the lateral magnification and field of view. The immersion microscope objective lens is optically coupled to a flat disposable polymethylmethacrylate (PMMA; $n = 1.491$) appplanation lens using a viscous ophthalmic coupling solution (GenTeal Gel; Novartis Ophthalmics, East Hanover, NJ; $n = 1.339$). A small amount of this same coupling solution is applied to the outer surface of the PMMA lens and the lens is then applied to the surface of the cornea (Figure 2). The reported index of refraction for the PMMA lenses and coupling solution were measured using a calibrated Abbe refractometer (Abbe-3L, Spectronics Instruments, Inc., Thermo Instrument co., Rochester, NY).

The two-dimensional images captured with our system had a nominal $400 \times 400 \mu\text{m}$ field of view with a sampling resolution of $1 \mu\text{m} / \text{pixel}$. We verified the lateral resolution using a calibrated $5 \times 5 \text{ mm}$ photo-etched silicon wafer (Electron Microscopy Sciences, Hatfield, PA; part # 79502-01). This wafer has etchings that are $1.9 \mu\text{m}$ wide with $10 \mu\text{m}$ total separation between each division. This target was aligned to the system, coupled with the viscous gel, and imaged to verify the lateral magnification of our system and determine the uniformity of illumination across the image field (Figure 3).

The axial scan position of our confocal microscope was calibrated in a series of measurements similar to those previously described by Ivarsen and colleagues¹⁵ and McLaren and colleagues.^{8, 16} A series of 10 single-cut PMMA contact lenses ($n = 1.491$) of uniform thickness were made at our own custom contact lens manufacturing facility. Two lenses for each of 5 different thicknesses were made: 400, 450, 500, 550, and 600 μm . The anterior radius of curvature was specified as 7.75 mm and the posterior radius was 7.80 mm. The curvature of each lens was measured in triplicate using a radiuscope (American Optical) and the median value was selected to represent the true value. Thickness was measured using a high resolution micrometer with certified precision of $\pm 0.5 \mu\text{m}$ (MT12, Heidenhain Corp., Schaumburg, IL).

To measure these lenses with the confocal microscope, the lenses were mounted on an optical positioning stage with the back surface of the lens filled with saline. The front surface of the lens was centered on the confocal system optics and positioned axially so that the lens apex was touching the applanation lens. This was confirmed by observing a small circular region of touch and the formation of concentric Airy rings. Once aligned, the confocal microscope objective was advanced until the posterior lens surface was visible and then the lens position was recorded. As before, the median of three measures was selected as the measured lens thickness. Because the microscope does not observe the lens surface directly, but rather the image of the lens surface, we adjusted these measurements for the optical effects of refraction induced by surface curvature of the lens and differences in the index of refraction at each interface to provide a corrected estimate of the lens thickness using methods described by McLaren and colleagues.^{8, 16} These estimates were adjusted further to account for differences between the contact lens and the curvature (7.3 mm¹⁷) and index of refraction ($n = 1.38$) of the rabbit cornea.^{8, 16} For the interested reader, this method is described in detail in the references.^{8, 16} In addition to computational adjustments for the optical effects of the viscous coupling solution used with scanning-slit confocal microscope as described by McLaren and colleagues, our calibration calculations included additional compensation for the PMMA applanation lens used with this confocal imaging system. Our adjustment methods otherwise conformed to these previously described methods.

The corneal confocal adapter has a total axial depth scanning range of approximately 1500 μm . The depth plane of the scan can be set manually using a rotating lens controller. This controller is electronically linked to a confocal lens position encoder providing a continuous readout of the axial depth plane. The scan lens position is set to zero prior to scanning by using the lens controller to advance the objective lens until the outer surface of the lens cap is located, providing a maximally bright specular reflection. The applanation lens surface is then placed against the cornea and the scan position adjusted to the users scan plane of interest. When applanated, the anterior-most corneal surface is fixed relative to the zero position and sequential scans are therefore registered in axial depth relative to the original zero position.

At any fixed position, an internal scanning lens is capable of providing automated depth scanning over a 60 μm depth interval; 30 images are captured over each interval, Figure 4. The nominal plane of separation between each image was, therefore 2 μm . We evaluated the nominal axial resolution of our system in two ways. First, by examination of common landmarks (e.g., keratocyte nuclei) across sequential tomographic planes with the automatic gain control of this system turned off. Previous studies of normal keratocyte morphology have established the average cell thickness as 1.34 μm .¹⁸ Second, we defined axial resolution by the full-width half-maximum height of a Gaussian fit to pixel intensity for an isolated region of interest (e.g. keratocyte nucleus) across sequential frames. For each method, mean depth of field was reported as the average for multiple locations ($n=15$; 3 different locations for each of 5 eyes).

In vivo imaging—Anesthetized animals were placed on their side and the eyes directed in a vertical position. The confocal imaging system was aligned to the apex of the cornea and images of the corneal tissue were captured over the full thickness of the cornea (from the superficial epithelium, to the endothelium) as described below. All eyes were measured twice during a single session both before and six weeks after a lamellar corneal incision.

We achieved full corneal thickness imaging by performing a sequential series of adjacent automated scans. To accomplish this, the position of the objective lens was first set to zero on the surface of the PMMA applanation lens. The cornea was contacted with the applanation lens and the capture mode set to automatic volume: 30 images/60 μm . After the

scan sequence was complete, the lens controller was advanced to 60 μm and another scan sequence was captured. This procedure was repeated over the full thickness of the cornea. Every image of each sequence was encoded with the axial depth relative to the initial zero position. Care was taken to avoid any lateral or axial displacement of the camera position during sequential scans. Corneal thickness was calculated as the difference in axial scan position from the first frame of the anterior-most layer of superficial epithelial cells to the endothelium. A comparison of ultrasonic and confocal corneal thickness measurements are reported in the results.

Keratocyte density was estimated in five distinct stromal regions that we defined similar to the methods of Patel and colleagues.¹⁹ Contiguous volumetric scans were performed in each of the different stromal regions. The anterior-most region, defined as the *First Countable Frame*, was located at a depth of 30–39 μm . This first countable frame was defined as the first image, deep to the epithelium, where no portion of the frame was obscured by the overlying acellular basement membrane.²⁰ The *Anterior Stromal* region was located at a depth ranging from 40–75 μm . The *Incisional Zone* was defined as 76–150 μm deep. *Mid- and Deep-Stromal Zones* were between 151–250 μm and 251–400 μm deep respectively. Keratocyte density was estimated as the number of nuclei visible within a rectangular sub-region of interest for each tomographic section. These regions were defined as the largest area that did not contain obscuring features, e.g. post-operative tissue folds. Cells touching either of the two adjacent sides of the upper right corner of the sub-region were included in counts and any cells touching the remaining sides were not.²¹ Section volume was calculated by multiplying the total section area by the calibrated depth of field. At least three individual frames were counted within each stromal region and the mean cell density was calculated. Differences in keratocyte density by stromal region were compared using a two-way repeated measure ANOVA model that included treatment, stromal region, and measurement order as factors.

We analyzed the within-session repeatability of cell density estimation and report the mean residual difference along with the 95% limits of agreement using the methods of Bland and Altman.²² We defined within-session repeatability as the difference in keratocyte density estimated by a single examiner from two different images acquired during the same session. These limits of agreement are reported for each stromal region before and six weeks after the lamellar incision.

RESULTS

Calibration

Lateral magnification of our confocal microscopy system using the photo-etched silicon target shows a total horizontal field of $400 \times 390 \mu\text{m}$ that is in agreement with the nominal specifications of 1 μm /pixel. The calibration target image (Figure 3) is 40 divisions wide (400 μm) by 39 divisions high (390 μm). This area was digitally sampled with 384×384 pixels providing a lateral resolution of 1.04 μm /pixel horizontally and 1.01 μm /pixel vertically. The mean deviation in intensity over the entire field for this target was less than 6% of the 8-bit dynamic range of the camera.

Image thicknesses of the PMMA calibration lenses were corrected for changes in index of refraction at each surface (objective lens, coupling solution, PMMA cap, and cornea) and were then compared against measured thickness determined by scanning laser confocal microscopy in Figure 5. A regression through these calibration points shows that there was good correlation between the image thickness of the contact lenses and thickness measured by the confocal microscope ($R^2 = 0.96$). This regression line provides the calibration function needed to adjust confocal microscopy measurements for real thickness ($y = 1.32x$

–145). Thus, the separation of each confocal tomographic plane was 2.6µm. Mean thickness of the contact lenses when measured with the confocal microscope were, on average $4.1 \pm 22.5 \mu\text{m}$ thinner than the thickness measured with the micrometer after adjustment for differences in the refractive index of the contact lens and the cornea.

On average, stromal keratocytes were visible over 3.5 sequential frames, where each frame was separated by 2.6 µm Figure 4. The calculated depth of field by this method was 9.1 µm (95% CI: 7.7 to 10.6 µm). Using a second approach, we fitted a Gaussian function to pixel intensity plotted as a function of axial depth position (Figure 6). Using the measure of full width at half-maximum height we calculated the mean depth of field as 7.8µm 95% CI: 7.1 – 8.6µm (n = 15; 3 estimates × 5 eyes).

Corneal Thickness

The measured mean and distribution of corneal thickness in this sample by ultrasonic pachymetry was 447 µm; 95% CI: 421 to 473 µm. Corneal thickness by confocal microscopy was 445 µm; 95% CI: 418 to 472 µm. Within-session repeatability of corneal thickness measurements was estimated by calculating the intra-class correlation coefficient (ICC) for ultrasonic pachymetry (ICC= 0.92; 95% CI: 0.80 to 1.0), and for confocal microscopy was (ICC= 0.94; 95% CI: 0.84 to 1.0). The average difference between paired measurements of total corneal thickness (Ultrasound – Confocal microscopy) on the same eye, during the same measurement session, was 2.1 µm (95% CI: –7.0 to +11.2 µm; $P = .61$). Residual differences of these paired comparisons are plotted in Figure 7. Six weeks after the incision, the average corneal thickness by confocal microscopy was decreased: 424 µm; 95% CI: 393 to 454 µm. This 5% reduction in thickness was not statistically significant ($P = .16$).

Keratocyte Density

Preoperatively, keratocyte cell density was greater in the anterior stroma and decreased throughout the deeper layers of the stroma (Figure 8). By two-way repeated measure ANOVA, keratocyte density was significantly different by stromal region (Greenhouse-Geisser adjusted $P = .006$). Mean keratocyte density in the first countable frame was 53,800 cells/mm³ (95% CI: 35,500 – 72,000). This was significantly greater than the cell density in each of the other defined regions (all $P < .05$). Detailed comparisons of cell density by region are provided in the Table.

Keratocyte density six weeks after the lamellar incision remained highest in the first countable frame and decreased gradually with increasing stromal depth (Figure 9). Cell density in the first countable frame was significantly greater than the deep stromal region Table 1 ($P = .026$), but was not significantly greater than any of the remaining regions.

The average keratocyte cell density of the first countable frame six weeks after the incision (43,700 cells/mm³; 95% CI: 31,800 – 55,500) was not significantly different from the corresponding preoperative cell density (53,800 cells/mm³; 95% CI: 35,500 – 72,000; Figure 9). Similarly, there was no significant change observed in keratocyte cell density six weeks after the lamellar incision in any of the deeper stromal regions (Table).

Repeatability of cell density measurements

The 95% limits of agreement for repeated measurements of keratocyte density at baseline (before the lamellar incision) are plotted as a function of stromal region in Figure 10 (*top*). The mean residual difference between repeated measurements of keratocyte cell density was 4,300 cells/mm³ (95% limits of agreement: $\pm 20,900$ cells/mm³).

Six weeks after the lamellar incision the 95% limits of agreement for repeated measurements of keratocyte density are plotted as a function of stromal region in Figure 10 (*bottom*). Following the lamellar incision, the mean residual difference and limits of agreement between repeated measurements of keratocyte cell density were decreased: $-2,800$ cells/ mm^3 (95% limits of agreement: $\pm 10,300$ cells/ mm^3).

DISCUSSION

Calibration

Our axial calibration results show excellent agreement between known thickness measurements (caliper-based measurements) and measurements by confocal microscopy (Figure 5), with a mean difference of $4.0 \pm 22 \mu\text{m}$. McLaren and colleagues have shown similar calibration functions for tandem-scanning and slit-scanning confocal microscopes.^{8, 16} Despite their reported excellent correlation between measured caliper thickness and confocal thickness (Confoscan 4: mean difference was $4.0 \pm 3.9 \mu\text{m}$), they still observed a relatively large bias ($38 \pm 15 \mu\text{m}$) towards thicker ultrasonic measurements in vivo. We also found that our ultrasonic measurements were thicker on average, but not to the same degree ($2.1 \pm 11.8 \mu\text{m}$). Our smaller bias may be due in part to differences in confocal microscope design (e.g. applanation lens), measurement methodology and a different calibration function (Figure 5) that we discuss below. Additional studies making direct comparisons between in vivo measurements of corneal thickness by ultrasound and by scanning laser confocal microscopy would be desirable to confirm our results.

Our Lateral calibration results confirmed the nominal calibration specifications provided by the manufacturer, $1 \mu\text{m}/\text{pixel}$ over a $400 \times 400 \mu\text{m}$ field of view. Imaging with the calibration target also demonstrated uniform illumination over the entire field of view. Despite our observed uniformity of illumination using a test target, there were other causes for non-uniform illumination in vivo that prevented us from using the entire frame for counting cells for in vivo keratocyte density measurements. One such limitation was the presence of folds in the corneal tissue postoperatively. It is also possible to generate distortion of the cornea when imaging by compressing the tissue too much during applanation. When these conditions were absent we observed good contrast with uniform intensity across the entire field of view.

The reflective intensity of individual structural features (e.g., isolated keratocyte nuclei or corneal nerve axons) first appear dim, increase to maximum reflective intensity, and then fade as these features approach, enter, and then exit the confocal imaging plane. The minimum nominal separation between axial images is $2 \mu\text{m}$ (hardware constraint), which we determined was $2.6 \mu\text{m}$ for our system. With this setting, structures with an axial dimension of $1 \mu\text{m}$ (keratocytes¹⁸) were visible over 3.5 frames on average. By this technique, our results show that the observed axial confocal depth of field for the Rostock Cornea Module is approximately $9 \mu\text{m}$ in vivo—wider than the nominal specifications provided by the manufacturer ($4 \mu\text{m}$).⁹

As an attempt to resolve this discrepancy, we performed additional analysis to determine the axial resolution of our confocal microscope. We used post-processing image analysis techniques in an attempt to further refine our estimates of confocal depth resolution. In short, the reflectance profile (pixel intensity) of objects imaged over a series of axial tomographic planes should approximate a Gaussian function, where the best estimate of axial position at each pixel is the peak of the fitted Gaussian function.²³ By fitting the reflectance profile over sequential images, it is possible to interpolate between the discrete confocal planes and determine the true axial position of objects spanning multiple planes with better precision (Figure 6). Using this approach, we confirmed that the depth of field is

7.8 μm . Our in vivo results also agree well with those reported by Zhivov and colleagues (7.6 μm) who performed their measurements using an in vitro target.²⁴ It is possible that our data would show a narrower depth of field that may agree better with the manufacturer's nominal specifications if we were to use a more peaked fitting function as opposed to the Gaussian fit that we chose here. Nevertheless, both our results and those reported by Zhivov et al. differ greatly from the findings of De Benito Llopis and colleagues (15.6 μm). It is not clear from the description of the author's methods whether or not they disabled the automatic gain function of their camera. If they did not, the number of sequential frames over which keratocyte nuclei are visible—their criteria for calculating depth of field—would be increased and could account for this finding.

Corneal Thickness

There was no statistically significant difference observed between paired ultrasonic and confocal microscopy measurements of corneal thickness acquired within the same session (2.1 μm ; 95% CI: -7.0 to $+11.2$ μm , $P = .61$). Within-session repeatability for confocal microscopy measurements of total corneal thickness was very good (SD = 7.3 μm) and comparable to the repeatability of ultrasound measurements. These results demonstrate that scanning laser confocal microscopy provides repeatable measurements of corneal thickness that are comparable to conventional ultrasonic measurements. Furthermore, the observed 5% decrease in corneal thickness after a corneal lamellar incision was not statistically significant indicating that the lamellar incision did not change corneal thickness from baseline measurements. It is important to note that a significant change in corneal thickness would be an important confounding factor to consider in the interpretation of keratocyte density after the lamellar incision. McLaren and colleagues have reported that confocal measurements of corneal thickness are typically thinner than ultrasonic measurements by as much as 35–40 μm using tandem-scanning and slit-scanning confocal microscopes. Using scanning laser confocal microscopy and similar calibration techniques, we do not find such a large difference between these two methods of measurement. To reconcile this difference we consider our measured calibration function described above. This function intersects the 1:1 correlation line near the average thickness of the rabbit cornea. This reduces the difference between these two measurements on average over the range of thickness evaluated in this study (400–500 μm). Given this function, we would expect to find differences between confocal and ultrasonically measured corneal thickness similar to what has been reported by McLaren and others if we were measuring corneal tissues with typical human thickness values.

The Rostock Corneal Module is not designed to provide measurements of total corneal thickness in an automated fashion—automated depth scanning is limited to 60 μm increments and serial automated scans must be performed to scan the total thickness of the cornea when using maximum axial resolution scans settings. We chose to perform sequential automated scans to measure total corneal thickness in this study. This approach requires manually advancing the lens controller after each scan, which creates the possibility of altering the lateral or axial position of the camera, thereby introducing both systematic and random errors to the measurements. Despite this potential limitation, the excellent agreement of our data with ultrasonic measurements shows that this approach can produce comparable measurements in an anesthetized animal. Alternatively, large scan ranges can also be achieved manually by specifying a user-selected axial depth plane and scan interval. Nevertheless, this will not eliminate the possibility of the types of systematic and random errors that we discuss next.

The Rostock Corneal Module is a contact probe coupled to the eye with a flat PMMA lens. Unlike fluid-coupled measurement methods, contact measurements are influenced very little by axial motions of the subject (e.g., ocular pulsations and head motion) or operator (e.g.,

instrument movement). The Z-Ring adapter for the ConfoScan 4 serves a similar function and improves the stability of the eye relative to the microscope, albeit in a different way. Our results demonstrate that thickness estimates by this technique do not differ significantly from typical point contact probes used in ultrasonic pachymetry. Nevertheless, several potential sources of error, both systematic and random, persist. Applanation of the imaged tissue will physically compress the object. It is possible that the direction and magnitude of these errors are not very different from what is caused by applanation with the ultrasound probe tip. Zhivov and colleagues have described a novel modification to the PMMA applanation lens— a central cutout to relieve compression overlying the imaged tissue.²⁴ This design minimizes imaging artifacts due to tissue compression. We did not control or account for these effects in our study and they should be considered as a possible systematic source of error in our reported measurements.

Lateral displacements of the eye or the camera relative to one another during imaging will alter the measured position from the intended location. Depending upon the stability of this lateral displacement (e.g. a constant positional error vs. one that varies throughout the scan period), the induced error effects could be either systematic or variable. There is no way to fully prevent such movements during imaging in vivo. In our study we had the advantage of an anesthetized animal to minimize these potential errors.

Keratocyte density

Our results show differences in keratocyte density by stromal depth. As others have shown, cell density is greatest in the most superficial anterior region and declines with increasing stromal depth. While the anterior-most region had significantly greater cell density than all other regions, none of the deeper regions were significantly different from one another. These observed differences in regional cell density agree well with the findings of Patel and colleagues who used a Nipkow disc based confocal microscope to evaluate the New Zealand white rabbit.²¹ They reported 47,072 cells/mm³ in the anterior stroma compared to our 53,800 cells/mm³ and 27,873 cells/mm³ in the posterior stroma compared with our measurement of 27,100 cells/mm³. Using the Rostock Cornea Module, Hovakimyan and colleagues reported similar cell densities to what we report: 48,734 cells/mm³ anteriorly and 33,291 cells/mm³ posteriorly. Care must be taken when interpreting the results from different studies as definitions of each of the stromal regions may differ between studies and not all are precisely defined. Patel and colleagues¹⁹ and Niederer and colleagues²⁵ have also reported similar patterns of greater cell density in the anterior regions of the stroma in humans. Increased image contrast of the scanning laser confocal microscope did not change our observed pattern of cell density by stromal depth, nor were there substantial differences between our two studies in observed cell density by region. Most authors who report cell density with the Rostock Cornea Module report area measures (cells/mm²). Knowing the confocal depth of field permits one to convert these area measures to volumetric estimates of cell density by dividing the area results (in square millimeters) by .0078 mm, our calculated depth of field. McLaren and colleagues,⁶ and Patel and McGhee⁹ have reviewed a number of confocal microscopy studies and show that estimates of keratocyte density vary widely by study, by stromal region, and by the type of confocal microscope used. The widest range of reported cell density is in the anterior stroma. As others have reported,^{19, 26} we observed a rapid decline in cell density over the depth of the anterior stroma. This variation can be explained in part by inconsistent definitions of the anterior stroma between studies. Another potentially confounding variable, which we measured and controlled for this study is the confocal depth of field.

Six weeks after the anterior lamellar corneal incision, we noted no significant change in keratocyte cell density in the anterior-most region of the corneal stroma. Our failure to detect a change in postoperative cell density is due in part to the wider distribution of

observed keratocyte density in the anterior stroma after surgery. We were therefore statistically underpowered to detect a significant change given the observed variability. Erie and colleagues evaluated keratocyte density after LASIK in humans and showed that cells decreased 22% in the stromal flap region six months after LASIK.² More recently, Ali Javadi and colleagues also found decreases in keratocyte density of 23% using a scanning-slit confocal microscope design.²⁷ The distribution of keratocyte density in our data before and after LASIK (Figure 9) suggest that there is little change in cell density at any stromal depth below the anterior-most region in the early postoperative period. Our findings are unique in that we report cell density by stromal depth in the early postoperative period. It is quite possible that the increases in cell density in some of the animals that we observed are the highly reflective cell bodies and cytoplasm of activated keratocytes present during the early postoperative time period.

The repeatability of our cell density measurements were calculated by comparing measurements from two different images captured within a single imaging session, performed by a single examiner. Our reported limits of agreement were wider in the stromal tissue anterior to the corneal lamellar incision. These wider limits of agreement were driven by greater variation in the measurements of anterior stromal regions that have higher cell density. Deep to the corneal lamellar incision, these measurements were more repeatable.

Keratocyte density is greatest in the anterior stroma in both rabbits²⁸ and in humans.²⁹ This may have implications for the development of keratoectasia after LASIK. Stromal keratocytes are necessary to the maintenance, transparency, and structural integrity of stromal collagen and their density is greatest in the anterior stroma where the tissue is normally incised with LASIK. At this point, there are too few comparisons of keratocyte density after LASIK or in patients who develop keratoectasia to say what role these observations may play in the etiology of this condition. Ali Javadi and colleagues have reported a difference in keratocyte distribution by stromal density among patients who develop keratoectasia suggesting a migration of deeper keratocytes to the anterior regions in the eyes studied.²⁷ Given the complexity of this condition and the numerous suspected etiologies, it would be surprising if a single measure such as keratocyte density could fully explain this enigmatic condition. Nevertheless, additional studies characterizing microscopic structural and cellular abnormalities using confocal microscopy may provide valuable insights to this disease mechanism. In conclusion, we have shown that confocal scanning laser microscopy of the cornea is a reliable technique for the measurement of corneal thickness and keratocyte density.

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Biography

Dr. Twa is an Assistant Professor at the University of Houston College of Optometry. He received the OD degree from the University of California at Berkeley, in 1990, practiced and conducted clinical research in corneal refractive surgery at the University of California San Diego from 1992 to 2001, and received the PhD degree in vision science from The Ohio State University in 2006. His research interests include biomedical imaging, quantitative image processing and tissue biomechanics.

Figure 1.

Cross sectional and en face in vivo confocal microscopy images of the rabbit cornea before and after lamellar incision. Axial cross section of the cornea showing the corneal epithelium (white arrow), basement membrane (black arrow), anterior stroma (*) and lamellar incision zone as the diffuse speckled region in the lower right portion of the image (*left*); Tangential (en face) section of the anterior corneal stroma before treatment (*middle*); six weeks after a 130 µm deep lamellar incision, keratocytes are more numerous and irregular in shape with highly reflective nuclei and visible cytoplasm revealing cell-cell junctions (*right*). Microscopic folds are also visible in the stroma. Scale bar = 50 µm.

Figure 2.

Configuration of the objective and applanation lenses of the Rostock cornea module. The objective lens is coupled to a PMMA cap using a viscous coupling solution. During confocal scanning, the outer surface of the PMMA cap applanates the corneal surface and may be moistened with additional coupling solution placed between the PMMA cap and cornea.

Figure 3.

Image of a lateral magnification calibration target acquired with the Rostock Corneal Module. Divisions are $10\mu\text{m}$ apart. Nominal scaling factor of $1\mu\text{m}/\text{pixel}$ was measured as $1.04\mu\text{m} / \text{pixel}$ horizontally and $1.01\mu\text{m}/\text{pixel}$ vertically. Illumination was uniform across the field. Scale bar= $50\mu\text{m}$.

Figure 4. Sequential tomographic sections from confocal microscopy of the rabbit cornea. Anterior (*top left*) to posterior (*bottom right*) sequential depth scan through the mid stromal region. A nerve fiber bundle is evident in the center of the image for reference. Keratocyte nuclei are bright, oval and circular spots scattered throughout the field. Scale bar = 50 μ m.

Figure 5.

Correlation between PMMA calibration lens image thickness and measured thickness using scanning laser confocal microscopy. Lens image thickness was adjusted for differences in the index of refraction at each interface—objective lens and coupling solution, coupling solution and PMMA cap, and PMMA cap and cornea (see Figure 2). Regression equation provides linear calibration parameters needed to adjust measured image values to object thickness. Dashed line is the reference 1:1 line.

Figure 6.

Pixel intensity as a function of axial position from corneal confocal microscopy in the rabbit. Axial depth of field is determined by the width of the fitted function at half the maximum height. Observations are shown as points and data is fitted with a Gaussian function ($R^2=0.85$; Full width at half maximum height = 7.8 μm).

Figure 7. Residual difference between total corneal thicknesses measured ultrasonically and by scanning laser confocal microscopy in the rabbit. Solid line is the identity line (perfect agreement); dashed line is the mean difference (2.1μm); dotted lines are the upper (27.3μm) and lower (-23.0μm) 95% limits of agreement.

Figure 8.

Measured keratocyte density in the rabbit as a function of tissue depth. Cell density as a function of tissue depth is plotted from the anterior to mid-stroma (top). The polynomial regression line is plotted along with the 95% prediction interval. Representative examples of keratocyte density by stromal region are shown beneath the plot by increasing depth from left to right: First countable frame (*left*, 35 μ m), anterior stroma (*middle left*, 70 μ m), incision zone (*middle right*, 130 μ m), and deep stroma (*bottom right*, 380 μ m). Scale bar = 50 μ m.

Figure 9.

Box-plot comparison of keratocyte density in the rabbit before and after the stromal lamellar incision. Keratocyte density is given by stromal region before and six weeks after a 130 μm deep lamellar incision. First is defined as the first countable frame (30–39 μm deep); Anterior stroma =40–75 μm ; Incision zone=76–150 μm ; Middle stroma=151–250; Deep stroma=251–400 μm .

Figure 10.

Within-session repeatability of keratocyte density measurements in the rabbit before and after lamellar corneal incision. Residual difference in keratocyte density measurements between two separate measurements obtained within a single examination session before a corneal lamellar incision (*top*) and 6 weeks following a corneal lamellar incision (*bottom*). The difference between repeated measurements are plotted a function of the average of paired measurements. Total residual differences are shown as the mean (dashed line) ± 1.96 SD interval (dash-dot lines). Measurements for each stromal region (e.g. anterior, middle, deep) are indicated by symbols in the legend. First is defined as the first countable frame (30–39 μ m deep); Anterior stroma =40–75 μ m; Incision zone=76–150 μ m; Middle stroma=151–250; Deep stroma=251–400 μ m.

Table

Keratocyte Density in the Rabbit by Stromal Region

Cell density before and after stromal lamellar incision by region is reported as the mean number of cells/mm³ in the table heading. Comparisons of cell density by region are reported in the table cells as the difference between corresponding regions. Sidak's adjusted *P* values (Student's *t*-test) shown in parenthesis below each comparison. First is defined as the first countable frame (30–39 μ m deep); Anterior stroma = 40–75 μ m; Incision zone=76–150 μ m; Mid stroma=151–250; Deep stroma=251–400 μ m.

Preop	First	Anterior	Incision	Mid	Deep
Anterior	53,800	39,100	36,200	31,200	27,100
	-14,700 (.044)				
Incision	-17,600 (.011)	-2,900 (.976)			
Mid	-22,600 (.001)	-7,900 (.536)	-5,00 (.859)		
Deep	-26,700 (<.001)	-1,200 (.163)	-9,100 (.397)	-4,100 (.919)	
Postop	First	Anterior	Incision	Mid	Deep
	43,700	41,200	36,900	32,500	30,100
Anterior	-2,500 (.999)				
Incision	-6,800 (.531)	-4,300 (.952)			
Mid	-11,200 (.089)	-8,700 (.290)	-4,400 (.951)		
Deep	-13,600 (.026)	-11,100 (.094)	-6,800 (.627)	-2,400 (>.999)	