In Vivo Laser Scanning Confocal Microscopy Confirms that the Human Corneal Sub-basal Nerve Plexus Is a Highly Dynamic Structure

Dipika V. Patel and Charles N. J. McGhee

PURPOSE. To add to findings in a prior study on the two-dimensional arrangement of the living human sub-basal corneal nerve plexus and determine whether it is a dynamic structure.

METHODS. Laser scanning in vivo confocal microscopy was performed on the left cornea of a healthy subject who had been examined with the same methodology 2 years earlier. Examinations were performed once a week for 6 weeks with the purpose of producing a two-dimensional reconstruction map of the living human sub-basal corneal nerve plexus at each session. A two-dimensional graphics program was used to arrange and map images obtained at each session into confluent montages.

RESULTS. The mean dimensions of the corneal areas mapped were 4.80 ± 0.45 mm horizontally and 4.60 ± 0.52 mm vertically. The nerve branching patterns observed 2 years earlier did not correspond with those in any recent maps. Over the 6-week period, the sub-basal nerve pattern appeared to migrate centripetally from the corneal periphery toward an inferocentral whorl. In the region of the whorl the nerves altered their generally centripetal direction of migration, undergoing clockwise rotation. The centripetal rate of migration decreased with proximity to the center of the whorl (5.6 ± 3.4 μm/wk at 13μm from the whorl, 13.9 ± 5.5 μm/wk at 333 μm from the whorl, and 25.9 ± 8.6 μm/wk at 698 μm from the whorl).

CONCLUSIONS. This study provides strong evidence that the living human sub-basal corneal nerve plexus is a highly dynamic structure, with continuous centripetal movement of identifiable branch points of up to 26 μm/wk, creating dramatic pattern changes in the plexus over a 6-week period. (*Invest Ophthalmol Vis Sci.* 2008;49:3409–3412) DOI:10.1167/iovs.08-1951

The in vivo confocal microscope provides a unique opportunity for examination of the living human cornea at the cellular level. The noninvasive nature of this technique means that multiple examinations may be performed on the same tissue over time while avoiding the induction of artifacts.[1–3] Two years ago, Patel et al.[4] used this technology to elucidate the two-dimensional architecture of the corneal sub-basal nerve plexus in the living human eye, demonstrating a radiating pattern of nerve fiber bundles converging toward an area approximately 1 to 2 mm inferior to the corneal apex in a whorl-like pattern.

The purpose of this study was to produce multiple two-dimensional reconstructions of the living human corneal sub-basal nerve plexus over time, to determine whether the sub-basal nerve plexus is a dynamic structure.

SUBJECTS AND METHODS

A 44-year-old female was recruited for the study and provided informed consent. The subject had no personal or family history of eye disease and no history of contact lens wear, ocular trauma/surgery, or systemic diseases that may affect the cornea. The left eye cornea was confirmed to be clinically normal on slit lamp biomicroscopy.

The subject had been examined 2 years previously (named subject (i)) on a single occasion with a methodology similar to that presented herein.[4]

The research adhered to the tenets of the Declaration of Helsinki. Informed, written consent was obtained from the subject after explanation of the nature and possible consequences of the study. The protocol used was approved by the Auckland ethics committee.

Laser scanning in vivo confocal microscopy was subsequently performed with a retinal tomographer (Heidelberg Retina Tomograph II Rostock Corneal Module [RCM], Heidelberg Engineering GmbH, Heidelberg, Germany). This microscope utilizes a 670-nm red wavelength diode laser source. It is a class 1 laser system and so, by definition, does not pose any ocular safety hazard. However, to guarantee the safety of the operator and subjects, the manufacturers have imposed a limit on the maximum period of exposure for patient and operator of 3000 seconds (50 minutes) in any single examination period. A 60× objective water immersion lens with a numerical aperture of 0.9 (Olympus, Tokyo, Japan) and a working distance of 0.0 to 3.0 mm, relative to the applanating cap, was used. The dimensions of each image produced using this lens are 400×400 μm, and the manufacturers quote transverse resolution and optical section thickness as 2 and 4 μm, respectively. The RCM uses an entirely digital image capture system.

The subject's eye was anesthetized with a drop of 0.4% benoxinate hydrochloride (Chauvin Pharmaceuticals). Carbomer 980 (0.2% Viscotears; Novartis, North Ryde, NSW, Australia) was used as a coupling agent between the applanating lens cap and the cornea. During the examination, the subject was asked to fixate on distance targets arranged in a grid pattern to enable examination of the cornea over a wide central to midperipheral area of approximately 5.00 mm diameter. The center of the grid was aligned vertically and horizontally at 1.1 m from the contralateral eye and consisted of 17 printed spot targets (central spot 1.5 cm and all other spots 1.0 cm in diameter), each separated by 6 cm horizontally and 7 cm vertically (overall grid dimensions 28×24 cm wide). The cornea was scanned using the device's "section mode" to obtain high-quality images of the sub-basal nerve plexus in each position. The section mode enables instantaneous imaging of a single area of the cornea at a desired depth.

At least 800 images were acquired at each examination session. The overall examination took approximately 50 minutes to perform for each session, including breaks every few minutes and a total confocal exposure time of less than 30 minutes. Midway through the second examination session, the subject experienced blurred vision in the right eye due to drying. This effect was prevented in subsequent examinations by increasing the frequency of breaks and regularly instilling synthetic tears into the right eye.
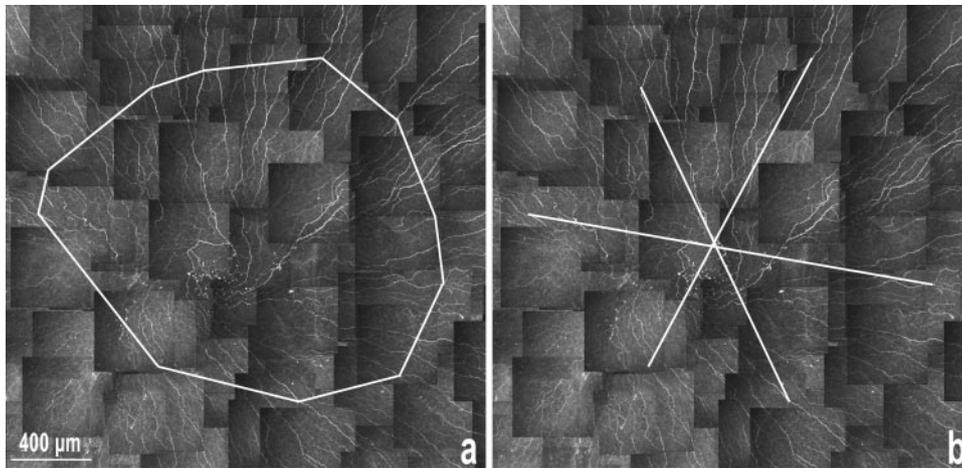
FIGURE 1. Adjacent identifiable branch points were joined by (**a**) *straight lines* such that concentric polygons were drawn centered around the region of the inferocentral whorl, and (**b**) opposing points were joined by straight lines passing through the inferocentral whorl.

A two-dimensional graphics program (Macromedia Freehand ver. 10; Adobe Systems, San Jose, CA) was used to arrange images for each eye into wide-field montages of the sub-basal nerve plexus.

Analysis

Measurements were performed on the montages with a caliper tool (analySIS 3.1; Soft Imaging System, Münster, Germany). Consistently identifiable branch points on successive montages were marked with dots. Adjacent dots were joined by straight lines such that concentric polygons were drawn centered around the region of the inferocentral whorl (Fig 1a), and the area of each polygon measured (in square millimeters per week). In addition, opposing dots were joined by straight lines passing through the inferocentral whorl (Fig 1b) and the lengths of these lines were measured (in micrometers).

RESULTS

A mean of 886 ± 29 images were obtained from the subject at each session (total, 5307 images). All blurred, oblique, or duplicate images were discarded. Montages were thus created with a mean of 408 ± 18 images for each session. The mean dimensions of the corneal areas mapped were 4.80 ± 0.45 mm horizontally and 4.60 ± 0.52 mm vertically.

When compared with the sub-basal plexus appearance identified 2 years before the current observations, the nerve branching patterns observed 2 years previously did not correspond with those in any recent maps (Fig. 2).

Comparing the sequence of maps over a 6-week period, the shapes of nerve branching patterns were noted to change each week (Figs. 3, 4, 5), with more dramatic changes in shape occurring in the peripheral cornea. It was therefore increasingly difficult to detect identifiable branch points in successive images with increasing distance from the whorl.

Over the 6-week period, the sub-basal nerve pattern appeared to migrate centripetally from the corneal periphery toward an inferocentral whorl. In the region of the whorl, the nerves altered their generally centripetal direction of migration, undergoing clockwise rotation. The centripetal rate of migration increased with increasing distance from the center of the whorl (Table 1). There was a corresponding increase in the rate of change of area with increasing distance from the center of the whorl (Table 2).

DISCUSSION

The 2-year data from this study conclusively prove that the normal human sub-basal nerve plexus is a dynamic structure. The weekly data confirm identifiable centripetal motion of nerves toward the inferocentral whorl, with substantial alterations in the shapes of nerve branching patterns that occur over a period of only 6 weeks.

Using a slit-scanning in vivo confocal microscope, Auran et al.[5] documented sub-basal nerve movement over 21 to 61 days in three human subjects. Postulated entry points of stromal nerves through Bowman's layer were used as reference points for serial imaging of small areas of the peripheral sub-basal nerve plexus. The shape and length of nerves were noted to vary as they slid centripetally at a speed of 5.5 to 17 μm/d, a higher rate than observed in the present study. This difference may be attributable in part to the different corneal locations
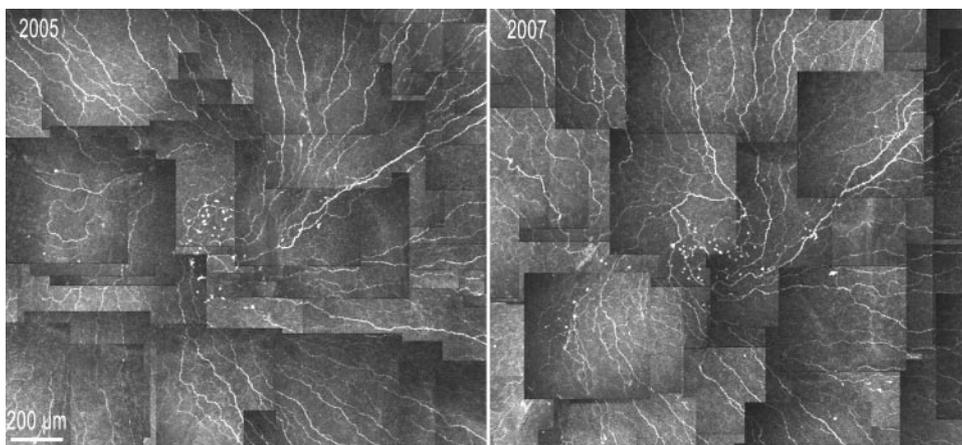

FIGURE 2. Montages depicting the architecture of the subbasal nerve plexus in the inferocentral region of the same cornea in 2005 and 2007. Dramatic alterations in nerve branching patterns are apparent.

FIGURE 3. Alterations in the shapes and branching patterns of sub-basal nerves occurred over the 6-week period, as demonstrated by this sequence of in vivo confocal microscopy images taken from a region superotemporal to the whorl. An identifiable branch point is followed (*arrows*).
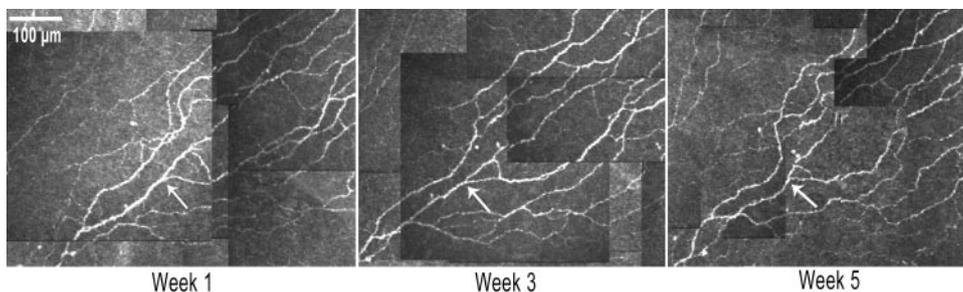

FIGURE 4. A sequence of in vivo confocal microscopy images taken from the region of the whorl. Identifiable branch points of sub-basal nerves are followed over 6 weeks (*arrows* and *arrowheads*).
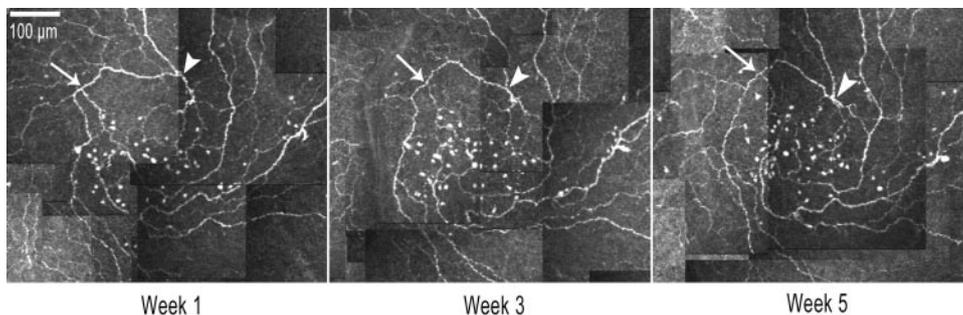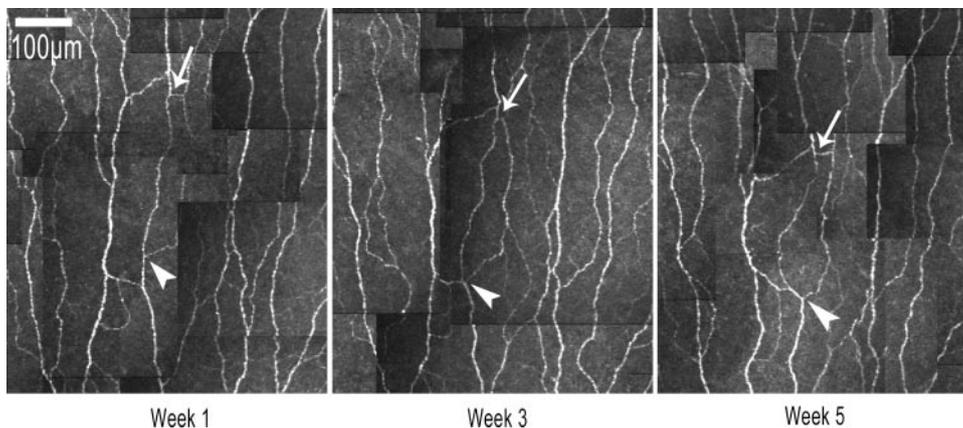

FIGURE 5. A sequence of in vivo confocal microscopy images taken from the central cornea, demonstrating dramatic changes in sub-basal nerve patterns. Identifiable branch points of sub-basal nerves were observed over 6 weeks (*arrows* and *arrowheads*).

imaged. The previous study imaged the peripheral cornea, approximately 2 mm from the limbus, whereas the present study imaged a large central area, including the region of the whorl, extending to the mid-periphery. Another factor may be differences in the method of measurement of nerve movement. Although both studies measured distances between two identifiable branch points, the prior study analyzed points within the same peripheral region, whereas the present study used points opposing each other on either side of the whorl.

The observed decrease in the centripetal rate of nerve movement with increasing proximity to the whorl may be explained by alterations in the direction of nerve movement, with a tendency to clockwise rotation. Therefore, although the velocity (rate of centripetal movement) decreases, the speed of nerve movement (rate of movement irrespective of direction) may be constant. In the present study, genuinely static structures could not be detected within successive montages. The presence of static structures such as scars involving Bowman's layer would allow accurate overlaying of the montages and enable detailed tracking of the speed and direction of nerve movements, as well as the production of a time-lapse sequence.

Although there are likely to have been alterations in the patient's head tilt and globe torsion at each examination session, this is unlikely to have affected the results of this study significantly because none of the measurements relied on over-

TABLE 1. Mean Centripetal Rates of Nerve Migration at Various Distances from the Center of the Sub-basal Whorl

Distance from Center of the Whorl (μm)	Mean Rate of Centripetal Migration (μm/wk)
13	5.6 ± 3.4
333	13.9 ± 5.5
698	25.9 ± 8.6

TABLE 2. Mean Rates of Change of Area of Three Polygons Centered around the Region of the Inferocentral Sub-basal Whorl

Area at Week 1 (mm^2)	Mean Rate of Change of Area (mm^2/week)
0.0389	0.0028 ± 0.0020
0.314	0.0221 ± 0.0118
1.417	0.0840 ± 0.0290

laying or registration of successive montages. Misalignment of the images while constructing the montages is a potential source of error when measuring nerve migration rates; however, each montage was manually constructed by a single experienced observer, thus excluding interobserver errors.

Unfortunately, no other published studies have been conducted to evaluate such a large area of normal human nerves in vivo for comparison. It is particularly interesting in the present study that the sub-basal nerve architecture appeared to converge, with the direction of branching being opposite to the direction of movement. Some investigators have suggested that the nerves in the region of the whorl degenerate or slough into the tear film.[5]

A recent study of murine corneas showed a whorling sub-basal nerve pattern, although the spiraling of nerves in the mouse cornea was more pronounced than that in the human.[6] This spiral pattern is similar to the known migration pattern of corneal epithelial cells in mice as they move from the limbus to the central cornea.[7] In, humans a similar migratory path has been inferred by observations in cases of corneal verticillata and toxic epitheliopathies.[8,9] These observations suggest that corneal epithelial cells and sub-basal nerves may migrate centripetally in tandem, although the driving force for these movements remains unknown. Human histologic studies have shown that epithelial nerve branches are oriented perpendicular to the corneal surface.[10] This suggests that if there is centripetal epithelial slide, then the epithelial cells and epithelial nerves move in the same direction and at the same velocity. Nagasaki and Zhao[11] have shown that centripetal movement of corneal epithelial cells in mice occurs at a steady rate of approximately 26 μm/d in vivo. However, the rate of sub-basal nerve migration in normal mice has yet to be ascertained.

This study provides strong evidence that the living human sub-basal corneal nerve plexus is a highly dynamic structure, with weekly centripetal movement of identifiable branch points, of up to 26 μm per week, creating dramatic pattern changes over a 6 week period.

Future studies might examine nerve migration in diseases such as keratoconus in which sub-basal nerve architecture is known to be significantly altered.[12] It would also be of interest to investigate the rate and pattern of nerve regeneration after trauma or surgery.

References

1. Patel DV, McGhee CNJ. Contemporary in vivo confocal microscopy of the living human cornea using white light and laser scanning techniques: a major review. *Clin Exp Ophthalmol.* 2007; 35:71–88.

2. Chiou AGY, Kaufman SC, Kaufman HE, Beuerman RW. Clinical corneal confocal microscopy. *Surv Ophthalmol.* 2006;51:482–500.

3. Jalbert I, Stapleton F, Papas E, Sweeney DF, Coroneo M. In vivo confocal microscopy of the human cornea. *Br J Ophthalmol.* 2003;87:225–236.

4. Patel DV, McGhee CNJ. Mapping of the normal human corneal sub-basal nerve plexus by in vivo laser scanning confocal microscopy. *Invest Ophthalmol Vis Sci.* 2005;46:4485–4488.

5. Auran JD, Koester CJ, Kleiman NJ, et al. Scanning slit confocal microscopic observation of cell morphology and movement within the normal human anterior cornea. *Ophthalmology.* 1995; 102:33–41.

6. Yu CQ, Rosenblatt MI. Transgenic corneal neurofluorescence in mice: a new model for in vivo investigation of nerve structure and regeneration. *Invest Ophthalmol Vis Sci.* 2007;48:1535–1542.

7. Collinson JM, Chanas SA, Hill RE, West JD. Corneal development, limbal stem cell function, and corneal epithelial cell migration in the Pax6$^{+/-}$ mouse. *Invest Ophthalmol Vis Sci.* 2004;45:1101–1108.

8. Dua HS, Gomes JA. Clinical course of hurricane keratopathy. *Br J Ophthalmol.* 2000;84:285–288.

9. Dua HS, Watson NJ, Mathur RM, Forrester JV. Corneal epithelial cell migration in humans: 'hurricane and blizzard keratopathy'. *Eye.* 1993;7:53–58.

10. Schimmelpfennig B. Nerve structures in human central corneal epithelium. *Graefes Arch Clin Exp Ophthalmol.* 1982;218:14–20.

11. Nagasaki T, Zhao J. Centripetal movement of corneal epithelial cells in the normal adult mouse. *Invest Ophthalmol Vis Sci.* 2003; 44:558–566.

12. Patel DV, McGhee CNJ. Mapping the corneal sub-basal nerve plexus in keratoconus by in vivo laser scanning confocal microscopy. *Invest Ophthalmol Vis Sci.* 2006;47:1348–1351.