# Mapping of the Normal Human Corneal Sub-Basal Nerve Plexus by In Vivo Laser Scanning Confocal Microscopy

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**Purpose.** To produce a two-dimensional reconstruction map of the living human sub-basal corneal nerve plexus using in vivo confocal microscopy.

**Methods.** Laser scanning in vivo confocal microscopy was performed on three normal eyes of three healthy human subjects. Subjects were asked to fixate on targets arranged in a grid to enable examination of the cornea in a wide range of positions. Using the section mode, a mean of 573 ± 176 images of the sub-basal plexus were obtained for each subject. The data were arranged and images were mapped for each subject into confluent montages.

**Results.** Mean dimensions of the corneal areas mapped were 4.95 ± 0.53 mm horizontally and 5.14 ± 0.53 mm vertically. In all subjects, the sub-basal nerve plexus appeared to radiate toward a whorl-like complex centered 1 to 2 mm inferior to the corneal apex. Outside this area, the nerve fiber bundles in the remainder of the cornea were arranged in a radiating pattern. Mean nerve density was significantly higher in the inferocentral whorl region (25,249 ± 616 $\mu m/mm^2$) compared with the central cornea (21,668 ± 1411 $\mu m/mm^2$) (Mann-Whitney $U$ test; $P = 0.05$).

**Conclusions.** This is the first study to elucidate the overall distribution of sub-basal nerves in the healthy, live central to mid-peripheral human cornea by laser scanning in vivo confocal microscopy. The whorl pattern of the sub-basal nerves is similar to that seen in the epithelium in corneal verticillata and may lend support to the theory that epithelial cells and nerves migrate centripetally in tandem. (*Invest Ophthalmol Vis Sci.* 2005;46:4485–4488) DOI:10.1167/iovs.05-0794

The cornea is one of the most densely innervated tissues in the human body, its innervation originating from the ophthalmic branch of the trigeminal nerve. In addition to their protective function, corneal nerves play important roles in regulating corneal epithelial integrity, proliferation, and wound healing.[1]

Corneal nerve architecture in humans has been studied using light and electron microscopy.[2–6] However, these studies have the intrinsic limitation that they were performed on corneal tissue obtained from cadavers or enucleated eyes and, therefore, were likely to be affected by artifacts resulting from tissue processing and postmortem, or ex vivo, nerve degeneration. The latter has been elegantly demonstrated by Muller et al.[2] in a transmission electron microscopy study of human cadaver eyes in which sub-basal nerve bundles showed signs of significant degeneration within 13.5 hours of death.

Although ex vivo studies have provided a wealth of ultrastructural data on human corneal nerves,[2–6] the architecture and distribution of nerves in the sub-basal plexus in vivo remains to be fully elucidated.

In vivo confocal microscopy provides a unique opportunity to examine 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, and the induction of artifacts observed with ex-vivo methods of examination are avoided. However, staining techniques may not be used in conjunction with in vivo confocal microscopy of human corneas.

The purpose of this study was to produce a two-dimensional reconstruction of the living human corneal sub-basal nerve plexus using laser in vivo confocal microscopy.

## Subjects and Methods

After informed consent, three subjects were recruited. The subjects had no personal or family history of eye disease and no history of contact lens wear, ocular trauma/surgery, or systemic diseases that might have affected the cornea. Two of the subjects (subject 1) and (subject 2) were women aged 42 and 47 years, respectively, and one (subject 3) was a man aged 46 years. The left eye of each subject was examined by slit lamp biomicroscopy, and all corneas were confirmed to be clinically normal.

The research adhered to the tenets of the Declaration of Helsinki. Informed, written consent was obtained from all subjects 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 on all subjects with the use of a corneal module (Heidelberg Retina Tomograph II Rostock Corneal Module [RCM]; Heidelberg Engineering GmbH, Heidelberg, Germany). This microscope uses a 670-nm red wavelength diode laser source. This 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 the subjects, the manufacturers have imposed a limit on the maximum period of exposure of 3000 seconds (50 minutes) for patient and operator 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, relative to the applanating cap, of 0.0 to 3.0 mm was used. The dimensions of each image produced using this lens are 400 $\mu m \times 400\ \mu m$, and the manufacturer quotes transverse resolution and optical section thickness as 2 $\mu m$ and 4 $\mu m$, respectively. The RCM uses an entirely digital image capture system.

Each eye was anesthetized with a drop of 0.4% benoxinate hydrochloride (Chauvin Pharmaceuticals, Surrey, UK). Viscotears (Carbomer 980, 0.2%; Novartis, North Ryde, NSW, Australia) was used as a coupling agent between the applanating lens cap and the cornea. During the examination, all subjects were asked to fixate on distance targets arranged in a grid pattern to enable examination of the cornea over a wide central to mid-peripheral area of approximately 5-mm diameter. The center of the grid (Fig. 1a) was aligned vertically and horizontally

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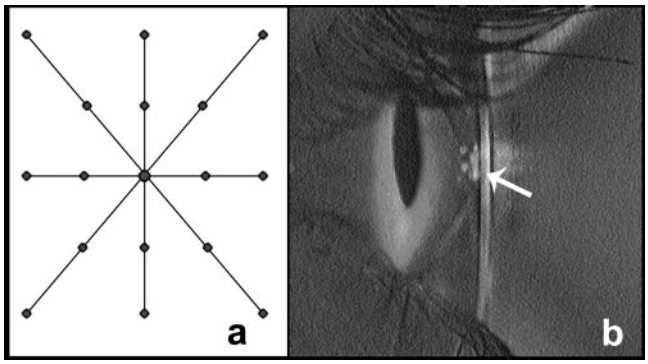


**FIGURE 1.** The fixation grid (**A**), consisting of 17 spot targets. Spots are separated from each other by 6 cm horizontally and 7 cm vertically. This grid was viewed by the subject at a distance of 1.1 m. A CCD camera "real time" or live image of the cornea from the temporal aspect (**B**) demonstrating how the laser beam is aligned with the corneal apex (*arrow*). Original magnification, 1×.

at 1.1 m from the contralateral eye and consisted of 17 printed spot targets (central spot, 1.5 cm; all other spots, 1 cm in diameter), each separated by 6 cm horizontally and 7 cm vertically (overall grid dimensions 28 cm × 24 cm wide). All the subjects had emmetropia, and none required correction of presbyopia; hence, they were able to clearly visualize the targets with the contralateral eye. 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. For subjects 1, 2, and 3, 640, 373, and 706 images were acquired for each cornea, respectively.

The overall examination took approximately 40 minutes to perform for each patient, including breaks every few minutes and a total confocal exposure time of <20 minutes. None of the subjects experienced any visual symptoms or corneal complications as a result of examination. A computer program (Macromedia Freehand 10; Macromedia Inc., San Francisco, CA) was used to arrange images for each eye into wide-field montages of the sub-basal nerve plexus.

Sub-basal nerve density measurements were performed on the montages using a caliper tool (analySIS 3.1; Soft Imaging System, Münster, Germany). In all cases, a standard frame size of 0.8 mm × 0.8 mm (area, 0.64 $mm^2$) was selected. Nerve density was assessed in the apical region of the central cornea and in the region of the inferocentral whorl by measuring the total length of nerves per defined frame.

## RESULTS

A mean of 573 ± 176 images was obtained from three left eyes of three subjects. All blurred, oblique, or duplicate images were discarded. Montages were thus created using 315, 301, and 366 images for subjects 1, 2, and 3, respectively. The mean dimensions of the corneal areas mapped were 4.95 ± 0.53 mm horizontally and 5.14 ± 0.53 mm vertically centered on the corneal apex.

In all three subjects, nerve fiber bundles were arranged in a radiating pattern, converging toward an area approximately 1 to 2 mm inferior to the central cornea (Fig. 2). A clockwise whorl or vortex pattern (whorl-like complex) was observed in the area of convergence of each subject (Fig. 3). Using a touch-pad drawing screen, electronic tracings of nerve fiber bundles were performed at high magnification on the confocal montage images. This enabled the production of schematics of the whorl-like complex in all three subjects (Fig. 4). The mean dimensions of these whorl-like complexes were 898 ± 201 $\mu$m horizontally and 671 ± 155 $\mu$m vertically.

The sub-basal corneal nerves appeared to form a complex series of anastomoses, with most nerves forming branches that subsequently joined adjacent nerve fiber bundles or rejoined the same nerve fiber bundle further along its course. The mean central nerve density was 21,668 ± 1411 $\mu m/mm^2$, and the mean nerve density in the whorl region was 25,249 ± 616 $\mu m/mm^2$. The difference in nerve density between the two areas reached statistical significance (Mann-Whitney $U$ test; $P = 0.05$).

Bright, irregularly shaped areas 20 to 40 $\mu$m in diameter were observed, usually in the mid-peripheral cornea at the sites of apparent termination of nerve fibers, and these were occasionally noted more centrally (Fig. 5a). These areas were observed at the level of the sub-basal nerve plexus and appeared to extend posteriorly.

Two of the subjects exhibited multiple bright particles up to 15 $\mu$m in diameter that were separate from nerve structures (Fig. 5b). These particles were only observed at the level of the sub-basal nerve plexus, and dynamic imaging confirmed that they did not extend anteriorly or posteriorly to this level.

## DISCUSSION

Most corneal nerve fibers are derived from the ophthalmic branch of the trigeminal nerve. Nerve bundles enter the peripheral mid-stroma in a radial pattern. These nerves lose their perineurium and myelin sheath within 1 mm of limbus, aiding the maintenance of corneal transparency, and are subsequently surrounded by only Schwann cell sheaths.[3] The nerves course anteriorly, giving rise to multiple branches innervating the anterior and mid-stroma.[1] Branches form the subepithelial nerve plexus that lies at the interface between Bowman's layer and the anterior stroma. This plexus is sparse and patchy in distribution, largely limited to the mid-peripheral cornea, and may not be present in the central cornea.[7] Fifty percent of these nerves exhibit varicosities or beads.[1] Nerve bundles penetrate Bowman's membrane throughout the central and peripheral cornea[4,5] at approximately 400 sites.[8] Bundles then divide and run parallel to the corneal surface between Bowman's layer and the basal epithelium, forming the sub-basal nerve plexus.[4,5] These nerve bundles consist of straight and beaded fibers, with the beaded fibers located in the periphery of the bundle. The beads have been identified as axonal efferent and sensory terminals[6,8] and have been shown to consist of accumulations of mitochondria and glycogen.[2] Only the beaded fibers subsequently form branches that enter the corneal epithelium, where they terminate.[3,4,8]

Because early studies of corneal nerves were unable to clearly identify the corneal position, the orientation of the sub-basal nerves was uncertain.[4,5] The introduction of the clinical in vivo confocal microscope enabled imaging of the living human sub-basal nerve plexus.[9] On the basis of data from serial section light and electron microscopy studies, in vivo confocal microscopy, and immunohistochemistry, it has been postulated that nerve bundles are preferentially orientated in a superior-inferior direction at the corneal apex and in a nasal-temporal direction in surrounding areas, with no defined focus.[3]

The two-dimensional reconstructions we report reveal the architecture of the sub-basal nerve plexus over the central 5 to 6 mm of the cornea. Sub-basal corneal nerve architecture in vivo has not previously been elucidated to this extent because most in vivo confocal microscopy studies of the sub-basal nerves have restricted examination to the corneal apex. This is mainly because of the relative ease of obtaining good-quality tangential images in this region. The observation that the sub-basal nerves form a complex series of anastomoses is consistent with the pattern seen in the human dermal nerve plexus, though the latter shows a less regular arrangement.[10]

Human histologic studies have shown that epithelial nerve branches are orientated perpendicularly to the corneal surface.[5] This suggests that if there is centripetal epithelial slide,

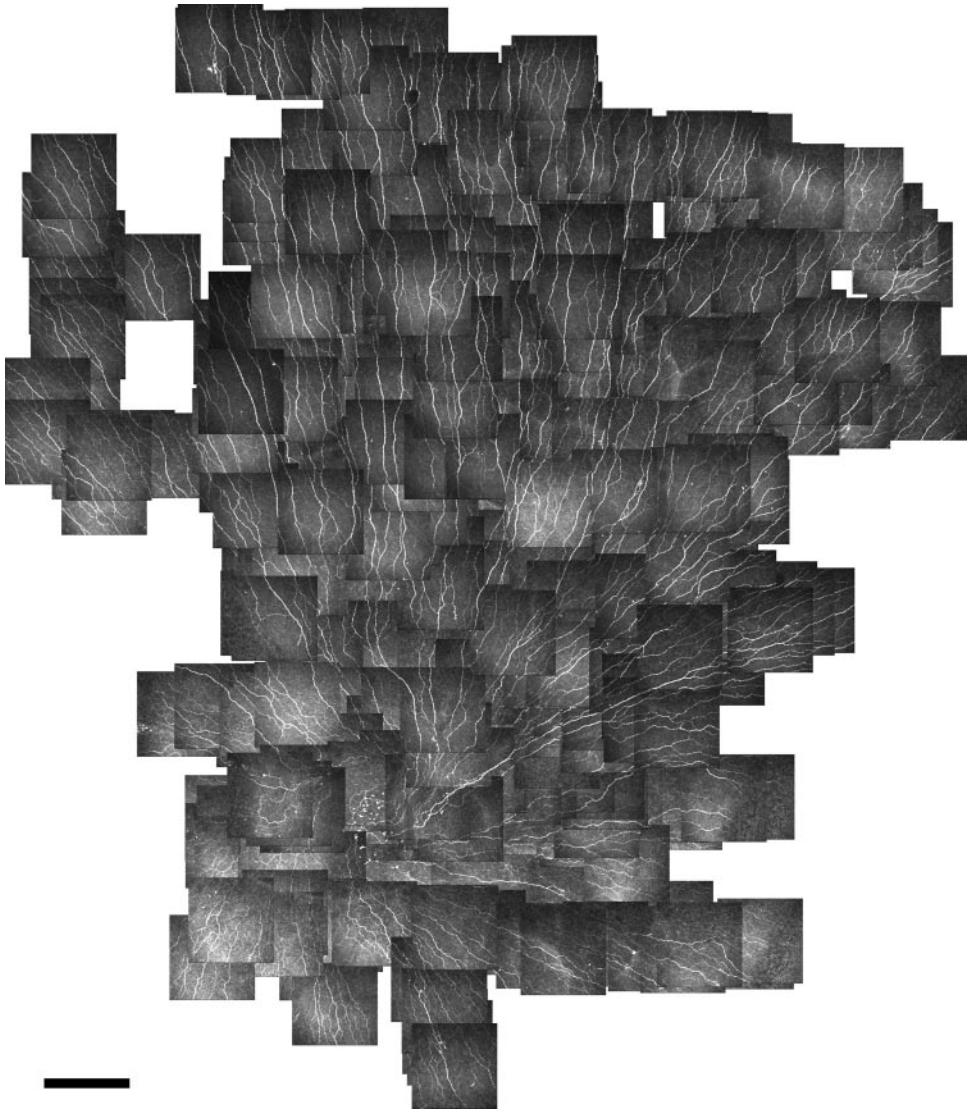

**FIGURE 2.** A montage of 315 images depicting the architecture of the sub-basal nerve plexus of subject 1. Scale bar, 400 $\mu$m.

the epithelial cells and epithelial nerves must be moving in the same direction and at the same velocity. Using in vivo confocal microscopy, Auran et al.[7] provided evidence that corneal epithelial nerves participate in centripetal migration. Sub-basal nerves were demonstrated to shift centripetally toward an area just inferior to the corneal apex, with velocities ranging from 1.7 to 32.0 $\mu$m a day. Shapes and lengths of nerve segments varied slightly as axons slid centripetally. Studies have also shown that, unlike the sub-basal plexus, the stromal nerves, subepithelial plexus, and nerve perforation points through Bowman's layer remain stationary over extended periods of time and may be used as landmarks for study.[7,11]

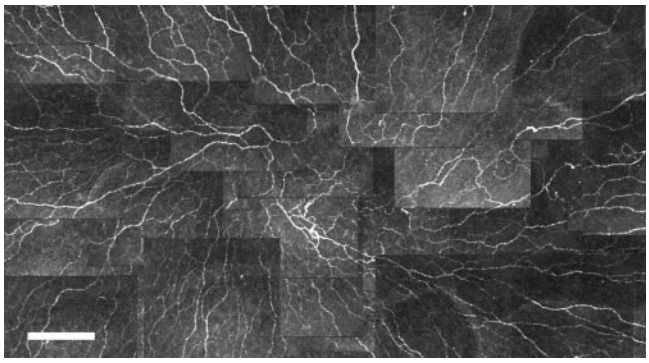

**FIGURE 3.** The vortex or whorl-like pattern of the sub-basal nerve plexus in the infero-central cornea of subject 2. This montage was constructed using 32 images. Scale bar, 200 m.

The radiating pattern of nerve fibers converging on a whorl-like complex reported here echoes the pattern seen in the epithelium in corneal verticillata and toxic keratopathies.[12] This suggests that epithelial cells and nerves may migrate centripetally in tandem. The patterns identified in the latter two conditions are typically clockwise.[13] Dua et al.[13] postulated that the combined effect of the electric and magnetic fields on centripetally migrating epithelial cells would result in a clockwise whorled pattern. Other theories regarding the driving force for centripetal movement of epithelial cells include: (1) preferential desquamation of the central corneal epithelium, drawing peripheral cells toward the central cornea; (2) population pressure from limbus and peripheral cornea because of proliferation and migration of cells; (3) gradient of chemical signals emanating from limbal capillary vessels; and (4) stimulation by epithelial sympathetic nerves.[14]

A possible explanation for the location of the whorl in the inferocentral cornea is that cell and nerve migration may be affected by shearing forces exerted by the eyelids on blinking,

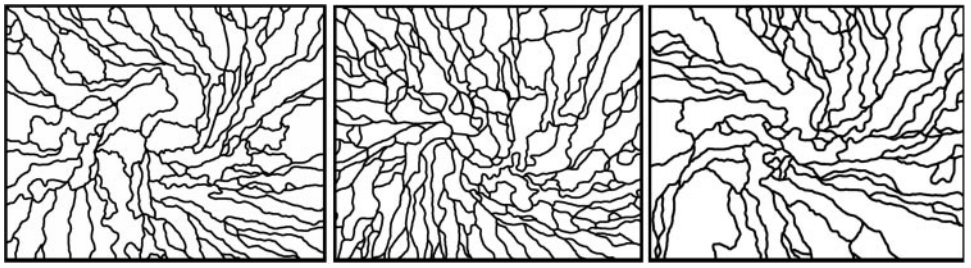

**FIGURE 4.** Electronic tracings of nerve fiber bundles provide schematics devoid of background data that highlight the infero-central whorl-like complex in three corneas of three subjects.

and the focus of the whorl is at the site of the end of upper and lower lid excursion.

The mean central nerve density reported here (21,668 $\mu$m/mm$^2$) is significantly higher than values previously reported in studies using white light in vivo confocal microscopy ([11,110 $\mu$m/mm$^2$][1] [8404 $\mu$m/mm$^2$][15]), probably because of differences in image quality and contrast. Images obtained using slit-scanning in vivo confocal microscopy are brightest along a central vertical strip and become darker laterally. Thus, nerve fibers at the edges of the image may not be clearly visible. Images obtained using the RCM are relatively uniform in contrast and brightness throughout the image. Additionally, the light source for this modality is coherent and brighter. This appears to enable visualization of the thinner nerve fiber bundles, which are not otherwise visible with slit-scanning in vivo confocal microscopy (authors' unpublished data, May 2005). The recent observation that there appears to be no correlation between age and sub-basal nerve density[15] suggests that differences in the ages of the subjects in this study, compared with other in vivo confocal studies of nerve density, are unlikely to explain the observed differences in sub-basal nerve density.

The central corneal location was determined by use of a charge-coupled device (CCD) camera attachment enabling live imaging of the cornea from the temporal side during examination (Fig. 1b). Although this technique is useful for localizing the approximate area of the cornea under examination, it is not sufficiently accurate to enable determination of the exact point location of the corneal apex.

The use of a fixation grid is a novel method of facilitating in vivo confocal examination of different corneal locations. Theoretically, a precise relationship between points on the grid and locations on the cornea could be developed; however, factors such as disconjugate eye movements, microsaccades, involuntary patient movements, and difficulty aligning the center of the grid with the corneal apex make such topographic measurements inaccurate at present.

The multiple bright particles observed in two of the subjects resemble those recently reported by Zhivov et al.,[16] who postulated that these represent Langerhans cells. In the latter study, these cells were present in the central cornea and in the inferior periphery of 85.7% of healthy subjects.

We report the first study to elucidate the distribution of sub-basal nerves in the living human cornea. Although this pilot study is limited by the small number of subjects and although the power of the study was low (50% on post hoc power analysis), it provides interesting new data regarding the architecture of the corneal sub-basal nerve plexus. Knowledge of the orientation of these nerves in various regions of the cornea may be helpful in aiding localization during in vivo confocal microscopy, when only small areas of the cornea are examined. Future studies may be directed at analyzing nerve architecture over time or in disease states known to affect corneal nerve structure and corneal sensation.

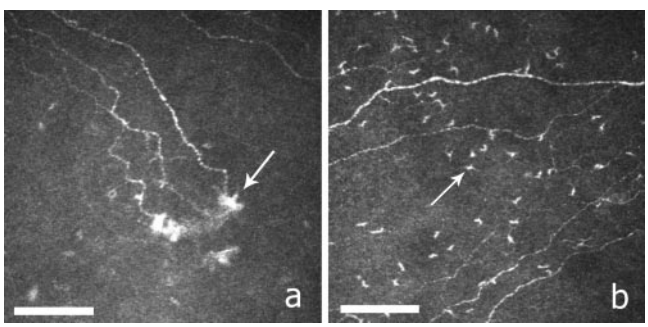


**FIGURE 5.** (**a**) Probable sites of perforation of nerves through Bowman's layer (*arrow*) in the infero-temporal mid-periphery of subject 3. (**b**) Multiple nonepithelial cells, possibly Langerhans cells (*arrow*), observed anterior to Bowman's layer, as determined by dynamic imaging, in the nasal mid-periphery of subject 3. Scale bar, 100 $\mu$m.

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