Corneal Fibroblast Migration Patterns During Intrastromal Wound Healing Correlate With ECM Structure and Alignment

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PURPOSE. To assess keratocyte backscattering, alignment, morphology, and connectivity in vivo following a full-thickness corneal injury using the Heidelberg Retina Tomograph Rostock Cornea Module (HRT-RCM), and to correlate these findings with en bloc three-dimensional (3-D) confocal fluorescence and second harmonic generation (SHG) imaging.

METHODS. Rabbit corneas were scanned in vivo both before and 3, 7, 14, and 28 days after transcorneal freeze injury (FI), which damages all corneal cell layers. Corneal tissue was also fixed and labeled for f-actin and nuclei en bloc, and imaged using 3-D confocal fluorescence microscopy and SHG imaging.

RESULTS. Using the modified HRT-RCM, full-thickness scans of all cell layers were consistently obtained. Following FI, stromal cells repopulating the damaged tissue assumed an elongated fibroblastic morphology, and a significant increase in cellular light scattering was measured. This stromal haze gradually decreased as wound healing progressed. Parallel, interconnected streams of aligned corneal fibroblasts were observed both in vivo (from HRT-RCM reflection images) and ex vivo (from f-actin and nuclear labeling) during wound healing, particularly in the posterior cornea. Second harmonic generation imaging demonstrated that these cells were aligned parallel to the collagen lamellae.

CONCLUSIONS. The modified HRT-RCM allows in vivo measurements of sublayer thickness, assessment of cell morphology, alignment and connectivity, and estimation of stromal backscatter during wound healing. In this study, these in vivo observations led to the novel finding that the pattern of corneal fibroblast alignment is highly correlated with lamellar organization, suggesting contact guidance of intrastromal migration that may facilitate more rapid wound repopulation.

Keywords: confocal microscopy, corneal wound healing, extracellular matrix, SHG imaging

Stromal keratocytes play a central role in mediating the corneal response to lacerating injury or refractive surgery.¹ During wound healing, quiescent corneal keratocytes surrounding the area of injury generally become activated, and transform into a fibroblastic repair phenotype.^{2,3} In certain wound types, fibroblasts further differentiate into myofibroblasts, which generate even stronger forces and synthesize a disorganized fibrotic extracellular matrix (ECM).^{4,5} Following lacerating injury, contractile force generation facilitates wound closure and helps preserve the mechanical integrity of the cornea.^{2,6} However, following vision correction procedures, such as photorefractive keratectomy (PRK) or LASIK, cellular force generation and fibrosis can alter corneal shape and reduce corneal transparency. Both of these procedures result in a region of keratocyte death beneath the laser-treated area.⁷⁻⁹ Stromal cell death also can be induced by toxic injury,^{10,11} as well as UV cross linking of the cornea in keratoconus patients.¹²⁻¹⁴ Ideally, repopulation of damaged stromal tissue following these insults should occur via intrastromal migration of keratocytes from the surrounding stromal tissue, without the generation of strong contractile forces that could disrupt the normal collagen architecture or the production of fibrotic ECM, which can reduce transparency. Although the factors affecting corneal myofibroblast transforma-

tion of corneal keratocytes have been studied extensively,^{2,4,15-19} less is known about the biochemical and biophysical signals that regulate intrastromal keratocyte migration.

In vivo confocal microscopy has been used in a variety of corneal research and clinical applications since its development more than 25 years ago (for reviews see Refs. 20-30), and is ideally suited to monitoring the cellular events of wound healing.^{2,26,29,31-33} Three main confocal systems have been developed for in vivo corneal imaging: the Tandem Scanning Confocal Microscope (TSCM; Tandem Scanning Corp., Reston, VA, USA),³⁴⁻³⁶ the Confoscan 4 (Nidek, Inc., Fremont, CA, USA),^{37,38} and the Heidelberg Retinal Tomograph with Rostock Corneal Module (HRT-RCM; Heidelberg Engineering, GmbH, Dossenheim, Germany).²⁶ The HRT-RCM is a laser scanning confocal microscope that operates by scanning a 670-nm laser beam in a raster pattern over the field of view.³⁹ The system uses a high numerical aperture $\times 63$ objective lens (0.95 NA), and produces images with high contrast and better axial resolution (7.6 μm) than other in vivo confocal systems (9 μm for the TSCM and 24 μm for the Confoscan).^{36,40,41} This has led to the expansion of its development and use in recent years.³⁰

To collect and quantify three-dimensional (3-D) information from the cornea, a technique termed confocal microscopy

through-focusing (CMTF) was developed for the TSCM by Li et al.^{42,43} This technique is based on the observation that different corneal sublayers generate different reflective intensities when imaged using confocal microscopy.⁴⁴ The CMTF scans are obtained by scanning through the cornea from the epithelium to endothelium at a constant lens speed, while continuously acquiring images. One important limitation of the HRT-RCM is that although volume scans of approximately 80 μm can be generated using a motorized internal lens drive,^{30,44,45} a manual thumbscrew drive must be used to change the focal plane position over larger distances, which requires rotating the objective housing by hand. Because CMTF imaging requires continuous focal plane movement at a known speed, *quantitative* high-resolution 3-D imaging of the full-thickness cornea is not possible with the standard HRT-RCM system. In a recent study, however, the HRT-RCM hardware and software were modified to address this limitation and allow quantitative CMTF imaging of the normal rabbit cornea.^{30,45}

In this study, we use this modified HRT-RCM system to assess keratocyte backscattering, alignment, morphology, and connectivity during intrastromal wound healing in vivo, following a full-thickness corneal freeze injury (FI) in the rabbit model. We also correlate these findings with en bloc 3-D confocal fluorescence imaging of cellular patterning, and second harmonic generation imaging (SHG) of the corneal collagen lamellae. Using this combined approach, we identify a unique pattern of keratocyte alignment and connectivity during wound healing that is highly correlated with the structural organization of the lamellae, suggesting contact guidance of intrastromal cell migration. Although biophysical cues have been shown to impact fibroblast behavior in in vitro models,^{46–48} to our knowledge this is the first demonstration that ECM structure mediates the pattern of intrastromal corneal fibroblast migration during in vivo wound healing.

METHODS

Animal Model

Studies were performed using 22 New Zealand white rabbits (3–4 kg; Charles River Laboratories, Wilmington, MA, USA). All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For transcorneal freeze injury, a 3-mm-diameter stainless steel probe cooled with liquid nitrogen was applied to the anterior, central corneal surface of one eye per animal three times for 10 seconds each time. This creates a region of cell death through the full thickness of the central cornea (epithelium, stroma, and endothelium).^{49,50} After each surgery, one drop of gentamicin antibiotic solution was applied twice a day for 3 days.

In Vivo Confocal Microscopy

To collect and quantify 3-D information from the cornea, CMTF was performed with an HRT-RCM that was custom modified in our laboratory.^{42,43,45} This system uses a joystick-controlled lens drive system, and incorporates software from Heidelberg Engineering that allows real-time “streaming” of images to the hard drive during an examination. Rabbits were scanned 1 week before surgery, and at 1, 3, 7, 14, and/or 28 days postoperatively. Before confocal imaging, rabbits were anesthetized with 50 mg/kg intramuscular ketamine and 5.0 mg/kg xylazine. A drop of topical anesthetic (proparacaine) was also applied to each eye. For confocal imaging, a drop of Genteal (Novartis Pharmaceuticals Corporation, East Hanover, NJ, USA) was placed on the tip of the HRT-RCM objective lens. The objective lens was then positioned so that flat-field images were observed at a central region of the cornea (confirming

proper alignment). For CMTF, scans were made from the endothelium to the epithelium at a constant speed of 60 μm per second, while collecting images using the HRT streaming software function with the acquisition rate set to 30 frames per second. Scans were made with the “automatic brightness” function in the HRT II software turned off (by unchecking the corresponding box). The gain level was controlled using the horizontal slider above the “automatic brightness” box. Images were collected at gains of both 6 and 10 (corresponding to the number of mouse clicks the slider was moved to the right). A minimum of four CMTF scans were performed at each gain setting, in the central region of each cornea. Each scan had a step size of approximately 2 μm between images. The field of view for each 384×384 -pixel image was $400 \times 400 \mu\text{m}$, resulting in a voxel size of $1.04 \times 1.04 \times 2 \mu\text{m}$ (x, y, z).

For analysis of HRT-RCM data, we used our in-house CMTF software in which “.vol” files can be directly loaded for 3-D visualization and analysis. Confocal microscopy through-focusing intensity curves were generated by calculating the average pixel intensity of each image and plotting versus z -depth.^{42,43} Using intensity peaks corresponding to basal lamina and endothelium, measurements of stromal thickness were made as previously described.⁴² A relative estimate of stromal cell and ECM backscattering was obtained by measuring the area under the CMTF curve (intensity $\times$ distance). Area was measured from the beginning of the basal lamina peak to the end of the endothelial peak (three images past the endothelium). A baseline intensity of 17 was used for the area calculations, which was above the background intensity obtained from the anterior chamber (12–14), and below the average image intensity of baseline stromal images.

In Situ Imaging

At 3 and 7 days postoperatively, both eyes from six rabbits were collected for in situ imaging. Corneas were fixed via anterior chamber perfusion with 1% paraformaldehyde solution consisting of 1% dimethyl sulfoxide, 1% Triton X-100, and 5% Dextran for 10 minutes immediately after animals were killed, as previously described.⁵¹ Corneas were removed and fixed in the same solution for an additional 10 minutes. Corneal blocks were washed in PBS for 20 minutes (10 minutes per wash), placed in Alexa Fluor 488 phalloidin (Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA) in PBS (1:20) for 3 hours at 37°C, then washed in PBS three times (30 minutes per wash). In some samples, nuclei were labeled by adding 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes) at a 1:500 concentration in PBS for 1 hour at room temperature.

Samples were mounted epithelial side down on Mattek glass bottom dishes in glycerol:PBS (1:1), and imaged using laser scanning confocal microscopy (Leica SP8, Heidelberg, Germany). An Argon (488-nm) laser was used for imaging *f*-actin, and a UV laser (405-nm) was used to image DAPI. Stacks of optical sections (z -series) were acquired using a $\times 25$ water immersion objective (0.95NA, 2.4-mm free working distance). Sequential scanning was used to image double-labeled samples to prevent cross-talk between fluorophores. To expand the effective field of view, montages of overlapping images were created using the programmable motorized stage and image tiling feature within the Leica software. Multiphoton fluorescence and SHG imaging were also performed on some samples, using a Zeiss LSM 510 confocal microscope (Carl Zeiss Microscopy, Thornwood, NY, USA) with a Chameleon multiphoton laser (Coherent, Santa Clara, CA, USA) set at 900 nm. A z -series of the forward and backscattered SHG signal from the collagen lamellae was acquired simultaneously along with *f*-actin images, using a $\times 40$ water immersion objective (0.8 NA, 3.6-mm free working distance).

Alignment of cells and collagen was quantified from *f*-actin and SHG images using the “directionality” plug in within Image J

(<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA), which uses a Fourier Transform algorithm to determine the percentage of image content aligned at each radial angle within the image. Plots showing both cell and matrix directionality were generated to allow direct comparison of the angle distributions.

Statistics

All statistical analysis was carried out using the analysis tool box in SigmaPlot (version 12.5; Systat Software, Inc., San Jose, CA, USA). Analysis of variance was used to compare group means. Post hoc multiple comparisons between groups were performed using the Holm-Sidak method. Linear regression analysis was used to assess the correlation between cell and ECM directionality.

RESULTS

The CMTF scans were successfully obtained using the HRT-RCM at all of the time points studied. Raw images are shown in Figure 1; images with contrast adjusted for better visualization are shown in Supplementary Figure S1. As detailed in the Methods section, scans were collected using gains of both “6” and “10.” The signal from wound-healing fibroblasts was often saturated when using a gain of 10, so scans with a gain of 6 were used for all reconstructions and analyses. Consistent with previous studies, in preoperative scans the primary signal in the stroma was derived from the keratocyte nuclei (Fig. 1A, a–c; Supplementary Movie S1). Corneal stromal nerves were also visualized in some regions (not shown). Endothelial images looked similar to those obtained using specular microscopy (Fig. 1A, d). Note that the superficial epithelium is obscured in these image stacks by the bright reflection from the Tomocap.

Three days after transcorneal FI, an acellular region was observed in the central cornea, as was significant stromal edema. Closer to the edge of the wound, the acellular region was limited to the anterior stroma (Fig. 1B, a). Beneath this area, highly reflective structures were observed (Fig. 1B, b, c). The morphology of these structures was consistent with that of polarized migratory corneal fibroblasts.^{7,52} Interestingly, these structures were generally arranged in long, parallel lines. Distinct groups of these aligned structures were consistently observed, particularly in the posterior stroma (Supplementary Fig. S1; Supplementary Movie S2). Elongated, reflective cells were observed at the level of the corneal endothelium (Fig. 1B, d). The f-actin labeling confirmed that these were fibroblastic endothelial cells, as indicated by a loss of cortical f-actin and expression of intracellular stress fibers (Supplementary Fig. S2). This is consistent with previous studies demonstrating that migrating endothelial cells undergo fibroblastic transformation following FI in the rat, rabbit, and cat.^{49,53–55} To better visualize the 3-D relationship between structures, images from the posterior 100 μ m of the stroma were automatically aligned using the *linear stack alignment* plug-in in ImageJ (Fiji version), in order to compensate for movements that occur during in vivo imaging. As shown in Supplementary Movie S3, distinct “layers” of parallel linear structures were more clearly revealed in these reconstructions. Interestingly, the alignment of these structures shifted from one layer to the next.

Seven days postoperatively, highly reflective, interconnected cells were observed in the anterior stroma (Fig. 1C, a). In the mid and posterior stroma, parallel groups of presumptive corneal fibroblasts were consistently observed (Fig. 1C; Supplementary Movie S4). Reflective areas were often observed at the level of the endothelium in the center of the wound (Fig. 1C, d). These areas were generally thicker than the adjacent normal endothelium, consistent with previous studies demonstrating that a retrocorneal fibrous membrane (RCFM) forms after FI in the rabbit and cat.^{49,50,53} This RCFM results

from transformation of the endothelium to a fibroblast and/or myofibroblast phenotype.^{56–58} Similar to 3 days after FI, aligned stacks from the posterior stroma showed distinct “layers” of parallel linear structures, with the alignment changing from one layer to the next (Supplementary Movie S5).

Beginning at 14 days after injury, the amount of cell activation began to decrease (not shown), and by 28 days the corneal stroma images looked similar to those obtained preoperatively (Fig. 1D, a–c; Supplementary Movie S6). The endothelium appeared to have a more cobblestone appearance than it did preoperatively (Fig. 1D, d), suggesting that the cell-cell junctions had not completely reformed. These cells covered the posterior surface of the RCFM, which persisted in some areas, consistent with previous studies.^{49,53}

Quantitative analysis was also carried out using the modified CMTF software. As shown in Figure 2, CMTF curves had a strong peak derived from the Tomocap, which obscured the peak from the superficial epithelium normally observed when using the TSCM or Confoscan 4 system. However, the basal lamina and endothelium were easily identified at all time points, which allowed measurements of corneal stromal thickness to be obtained. A significant increase in stromal thickness was observed 7 and 14 days after injury, as compared with preoperative values (Fig. 2C). Furthermore, a striking increase in stromal backscattering was measured from the CMTF curves at days 7 and 14 (Fig. 2D, also compare Figs. 2A, 2B). At 28 days, stromal backscattering was reduced to near baseline levels, and no haze was noted by gross examination (Fig. 2D), although a small amount of stromal edema persisted.

To verify that the aligned structures identified in the in vivo images were corneal fibroblasts, and to further investigate their organization, corneas were fixed and labeled in situ with phalloidin (for f-actin) and DAPI (for nuclei). As shown in Figure 3A, in control (unoperated) corneas, keratocytes had a stellate morphology and were interconnected by dendritic cell processes.⁵¹ Three days after FI, an abrupt transition was found in the anterior stroma between dendritic keratocytes adjacent to the wound (Fig. 3B, left side) and activated corneal fibroblasts migrating into the wounded stroma (Fig. 3B, right side). Fibroblasts were characterized by an elongated, polarized morphology and more intense f-actin labeling. Fibroblasts formed an interconnected network that extended from the wound edge to the leading edge of the migratory front, and trains of cells moving along the same path were often observed (see Supplementary Movie S7). In the posterior stroma, long, interconnected lines of cells were observed (Fig. 3C, wound is on bottom right of image). Parallel groups of these cell chains were consistently detected (Supplementary Movie S8), with the orientation shifting from one layer to the next. At 7 days, the central cornea was completely repopulated with corneal fibroblasts. Fibroblasts continued to be arranged in parallel streams in the mid and posterior cornea (Figs. 3D, 3E). These fibroblasts were thin and elongated, and did not have the broad morphology and prominent stress fibers that are characteristic of myofibroblast transformation.

To investigate whether corneal fibroblast patterning correlated with the alignment of the corneal collagen lamellae, SHG imaging was performed. In control corneas, corneal keratocytes had a stellate morphology and the cell bodies were not polarized, thus no preferential alignment was detected (Fig. 4, top row). However, the dendritic processes connecting corneal keratocytes in the posterior cornea often appeared to be aligned parallel with the collagen lamellae. This is consistent with a recent study by Young et al.,⁵⁹ which demonstrated that dendritic keratocyte processes are closely associated with orthogonally arranged stromal collagen fibrils in the developing chick cornea.

At both 3 and 7 days after FI, a complex pattern of cell and matrix organization was found in the anterior stroma (Supplementary Movie S9). In en face image slices, no predominant

FIGURE 1. Three-dimensional stacks of images from CMTF scans collected using the HRT-RCM, before and after transcorneal freeze injury. (A) Preoperative scan. (B) Three days after FI. (C) Seven days after FI. (D) Twenty-eight days after FI. The *top row* shows 3-D reconstructions of the confocal z-stack. (a–d) Selected en face images from each stack.

alignment of either cells or collagen lamella could be identified (Fig. 4, middle row). In contrast, distinct, aligned layers of lamellae were observed in the posterior cornea (Fig. 4, bottom row). Corneal fibroblasts were consistently aligned parallel with the collagen lamella, resulting in high correlations between cell and ECM directionality (Supplementary Movie S10).

DISCUSSION

In vivo confocal microscopy is ideally suited for studying corneal wound healing in vivo. Confocal microscopy through-focusing has been used for quantitative assessment of sublayer thickness and depth-dependent cell and ECM backscatter following injury, toxic insult, contact lens wear, infection, and refractive surgery

using the TSCM system.^{2,20–33,42,43} The HRT-RCM produces corneal images with better resolution and contrast than the TSCM (which is no longer commercially available), and this has led to growing use of this instrument in recent years. We recently reported hardware and software modifications to the HRT-RCM that allow CMTF scans to be collected from the full-thickness rabbit cornea in vivo.⁴⁵ In the current study, we use this system to assess the stromal wound-healing response following transcorneal freeze injury in the rabbit.

Quantitative CMTF analysis demonstrated a significant increase in stromal thickness at 7 and 14 days after injury, as compared with preoperative values. Furthermore, a 4-fold increase in stromal backscattering was measured from the CMTF curves at days 7 and 14. Unlike other 3-D imaging

FIGURE 2. In vivo CMTF data collected from rabbit corneas using the modified HRT-RCM system preoperatively (**A**) and 7 days after transcorneal freeze injury (FI) (**B**). Corneal stromal thickness was measured by marking the location of the top of the stroma and the corneal endothelial peak. A relative estimate of stromal cell and ECM backscattering was obtained by measuring the area under the CMTF curve (*shaded areas under curves*, “area” on top right of each image). (**C**) Graph showing changes in stromal thickness over time (mean $\pm$ SD). A significant increase was found at 7 and 14 days after injury. (**D**) Graph showing changes in stromal backscatter over time (mean $\pm$ SD). Significant increases were identified 7 and 14 days after injury. ** $P < 0.01$; * $P < 0.05$.

FIGURE 3. Confocal images collected in situ from corneal blocks labeled with phalloidin (*green*) and DAPI (*blue*). (A) Control (uninjured) cornea. (B) Montage of tiled images from the anterior stroma, 3 days after FI. (C) Montage of tiled images from the posterior stroma, 3 days after FI. (D, E) Montages of images from the posterior cornea, 7 days after FI. “W” indicates the image area closest to the center of the wound.

FIGURE 4. Multiphoton confocal images collected in situ showing fluorescent signal from phalloidin (green) and forward-scattered SHG signal from stromal collagen lamellae (red). Graphs on right show the percentage of image content aligned at each radial angle within the image for both cells and collagen. *Top row:* Images from posterior of control (unoperated) cornea. *Middle row:* Images are from anterior stroma (10 μm below basal lamina), collected 7 days after FI. *Bottom row:* Images are from posterior stroma (20 μm above endothelium), collected 7 days after FI.

technologies, such as optical coherence tomography or high-frequency ultrasound, CMTF scans provides a series of high-resolution en face images that allow assessment of depth-dependent changes in cell morphology, density, and reflectivity.^{29,32,33} Previous studies have demonstrated that when keratocytes transform to a migratory fibroblast phenotype, they assume a polarized morphology, and have increased light scattering due in part to a downregulation of crystalline protein expression.^{60,61} Consistent with these studies, highly reflective linear structures were observed in the corneal stroma beginning 3 days after FI. Subsequent labeling of corneal tissue for f-actin and in situ confocal imaging verified the cellular origin of these structures. Specifically, corneal fibroblasts were characterized by an elongated, polarized morphology and more intense f-actin labeling. In situ imaging also verified the unique pattern of organization identified in the in vivo confocal images. In the anterior stroma, cells were randomly organized. However, in the mid and posterior cornea, long parallel trains of cells moving along the same path were often observed. Interestingly, the alignment of these structures shifted from one layer to the next.

Previous studies using SHG imaging in the rabbit have shown that in the anterior stroma, there is significant interweaving of the collagen lamellae, whereas in the mid and

posterior cornea, there is less interweaving and the lamellae form more distinct orthogonal layers.^{62,63} Based on the data from both in vivo and in situ imaging in the current study, we hypothesized that corneal fibroblasts migrate into the wounded, acellular stroma in a pattern that corresponds to the lamellar organization of the tissue. To test this hypothesis, cell and matrix organization were directly compared by using SHG imaging. During wound healing, a complex pattern of cell and matrix organization was found in the anterior stroma, and no predominant alignment of either cells or collagen lamella was found. In contrast, distinct, aligned layers of collagen were observed in the posterior cornea, and corneal fibroblasts were consistently aligned parallel with these lamellae.

Overall, our results suggest that the collagen lamellae provide contact guidance of intrastromal fibroblast migration during wound repopulation. This is consistent with in vitro studies demonstrating that topographical parameters (i.e., height, depth, width, and spacing) can have a significant impact on cell morphology, differentiation, and migration mechanisms.^{46,47,64–66} Individual Type I collagen fibers in the cornea are approximately 30 nm in diameter and have a center-to-center spacing of approximately 65 nm.⁶⁷ However, it is not clear if the pitch of individual fibers is responsible for the

lamellar guidance of cell migration observed in this study. Pot et al.⁶⁸ demonstrated that corneal fibroblasts spread and migrated parallel to collagen coated ridges and grooves with a pitch of greater than 1 μm , whereas spreading and migration were randomly oriented on planar substrates or substrates with smaller topographic features. Interestingly, cells also migrated faster when moving parallel to the aligned substrate features.⁶⁸ Aligned substrates also increase the alignment of cells and matrix within each layer of self-assembled sheets derived from corneal fibroblasts.^{48,69–73} Within 3-D matrices, migrating cells can establish tracks for spreading and migration by aligning fibrils via mechanical force generation.^{74–79} Similarly, strain-induced tension applied to 3-D matrices also can establish aligned tracks for cell migration.^{80,81}

Cell and collagen alignment has been assessed previously using SHG imaging following PRK in the rabbit.⁸² Two weeks after surgery, wound healing was characterized by myofibroblast transformation of corneal keratocytes and the development of fibrotic tissue on top of the photoablated stroma. Within this fibrotic tissue, stress fibers within corneal myofibroblasts and collagen fibers were shown to be coaligned, suggesting that cell-secreted collagen is organized via cell-matrix mechanical interactions. Both cells and matrix were randomly aligned within this fibrotic tissue layer (i.e., no specific pattern of organization was found). In contrast to this fibrotic layer that forms on top of the stroma, cells migrating within the stroma following FI in the current study were exposed to topographic cues from the native lamellae. These cues apparently led to cell alignment via contact guidance of migration.

In addition to developing a pattern of alignment that mirrored the lamellar structure of the corneal stroma, fibroblasts also appeared to form long streams of interconnected cells that extended from the wound edge to the leading edge of the migratory front. This pattern of collective cell migration is called “multicellular streaming,”⁸³ and is thought to be involved in directional guidance of neural crest cells during embryonic development. We recently identified a similar pattern of migration by corneal fibroblasts invading 3-D fibrin matrices, using a nested matrix model.^{84,85} In this model, fibroblasts initially extend their leading edge into the fibrin while remaining connected to cells behind them. These cells follow along the same paths, producing long streams of interconnected cells. Lateral protrusions between adjacent cells also become interconnected, resulting in the formation of a mesh-like structure. Fibrin fibrils are randomly organized in the nested matrix model. In contrast, the corneal stroma lamellae are made up of highly aligned collagen fibrils. In the current study, cells migrating within the mid and posterior corneal stroma were highly polarized, and did not appear to develop lateral processes. Thus interconnections between the streams of aligned cells were not observed. Interestingly, in fibrin, cells at the leading edge of the migratory front appear to create tracks through the matrix that are used by the cells behind them (Miron-Mendoza M, et al. *IOVS* 2015;56:ARVO E-Abstract 2043.)^{84,85} Future studies are needed to determine whether similar tracks are created during intrastromal migration in vivo.

Although 3-D CMTF scans were successfully obtained using the HRT-RCM, there are still some limitations to this system. One issue encountered when performing repeated CMTF scans using the HRT-RCM was that the layer of Gental between the objective and the back surface of the Tomocap progressively dissipated and shifted downward over time. If this Gental layer moved below the optical axis, the image intensity decreased. This generally could be avoided by checking the layer carefully before and after scanning each eye. In cases in which the Gental layer was found to shift, scans were discarded and repeated with a new cap. Another issue is that the flat applanating tip (Tomocap) can produce compression artifacts

that can distort cellular structures during clinical imaging. However, these artifacts are not generally observed when scanning the rabbit cornea. In addition, the reflection from the Tomocap generally obscures images of the superficial epithelial cells. Zhivov et al.⁴¹ reported that a thin polymethylmethacrylate washer can be placed on the Tomocap to eliminate these reflections. Using a similar device, we obtained full-thickness scans through the rabbit cornea that had clear images of the superficial epithelium, with no apparent change in the cellular backscattering detected (Supplementary Fig. S3).

In conclusion, this study demonstrated that the modified HRT-RCM allows in vivo measurements of corneal sublayer thickness; assessment of cell morphology, alignment, and connectivity; and estimation of stromal backscatter (haze) during wound healing. Interestingly, stromal cells repopulating the damaged tissue assumed an elongated and interconnected fibroblastic morphology, and parallel, interconnected streams of aligned corneal fibroblasts were often observed both in vivo and ex vivo during wound healing, particularly in the posterior cornea. This pattern of fibroblast alignment was highly correlated with the structural organization of the lamellae, suggesting contact guidance of intrastromal cell migration. To our knowledge, this is the first demonstration that ECM structure mediates the pattern of intrastromal corneal fibroblast migration during in vivo wound healing.

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