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3-Dimensional Assessment of In Vivo Corneal Wound Healing using a Modified HRT-RCM Confocal Microscope

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INTRODUCTION

The Heidelberg Retinal Tomography Rostock Corneal Module (HRT-RCM) is a relatively new instrument that provides excellent resolution, contrast and optical sectioning for imaging the cornea *in vivo* (Fig. 1). However, challenges arise in changing the focal plane over large distances, since the thumbscrew objective housing must be rotated by hand. This limits the ability to perform quantitative 3-D imaging.

A method termed confocal microscopy through focusing (CMTF) was previously developed in our laboratory to collect 3-D information from the cornea using earlier confocal systems.[1,2] With this technique, corneal scans are acquired by scanning from the epithelium to the endothelium at constant lens speed. The images acquired in the z-series are stored into computer memory and are analyzed using custom developed software, which interactively displays the images along with their depth within the cornea. In addition, intensity vs. depth curves can be generated. By measuring the area under these curves, a relative estimate of corneal backscattering (haze) can also be obtained.[3] Unlike other 3-D imaging technologies such as high frequency ultrasound, quantitative 3-D confocal microscopy can provide a series of high resolution images which allow assessment of depth-dependent changes in cell morphology, density and reflectivity.[3-5]

This goal of this study was to test hardware and software modifications for the HRT-RCM which allow similar quantitative 3-D corneal imaging techniques to be applied, using a freeze injury model in the rabbit.

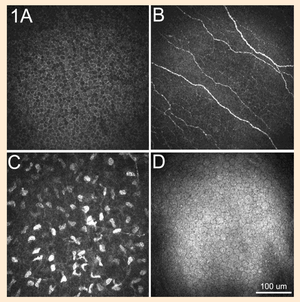

Figure 1. HRT-RCM images of a normal human cornea. Due to its high magnification objective lens and superior axial resolution, intra-epithelial sectioning can be achieved, and wing cells and basal cells (1A) can be easily distinguished. The sub-basal nerved plexus (1B), stromal cells (1C) and corneal endothelium (1D) are all imaged with higher contrast and better SNR than the TSCM.

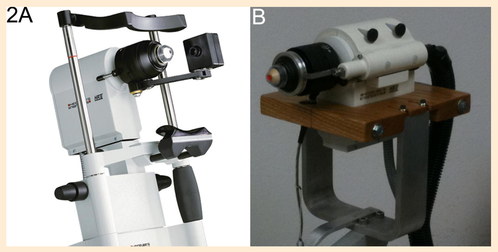

Figure 2. (A) Heidelberg Engineering HRT-RCM confocal microscope. (B) Modified HRT-RCM with rotational motor drive to allow automated through focusing, and a modified support structure (slit lamp stand) to facilitate positioning.

METHODS

System Modifications:

A Newport LTA precision motor drive was enclosed in a housing that attached to the HRT scan head, and a spring loaded drive shaft was used to couple the actuator to the front section of the HRT-RCM. This assembly ensured proper alignment of the motor drive shaft with the z-axis of the HRT-RCM (Fig. 2). The CMTF program was modified to control the position of the Newport motor via a serial interface. The HRT–RCM was mounted on a slit lamp stand to facilitate alignment. Finally, a thin silicone washer was placed on the Tomocap to eliminate reflections that can interfere with superficial epithelial imaging.

The standard HRT software only collects 100 images during a sequential acquire, which results in a large step size (> 5 µm) between images in a CMTF stack of the full thickness cornea. We recently obtained beta software from Heidelberg Engineering that allows real-time "streaming" of images to the hard drive during an examination. With this software, much larger sequences can be obtained (maximum 14,525 images). All images in a sequence are combined into a single ".vol" file, in which each image contains a 384 byte header followed by the 384x384 pixel data. The CMTF software was modified so that the HRT ".vol" files could be directly loaded. The header information from each image was also decoded to determine its exact time of acquisition, and its relative z-position was then calculated based on the known scan speed.

Experimental Procedures:

To test the new system, nine New Zealand White rabbits were anesthetized and studied. For CMTF, the left and right eyes were scanned from the endothelium to the epithelium at a constant lens speed (60 µm/sec), while collecting images using the HRT streaming software function at a rate of 30 frames/second. Following the pre-operative scans, a transcorneal freeze injury (FI) was performed on the left eye; the right eye was used as an experimental control. The rabbits were scanned post-operatively at 7, 10, and 28 days after surgery.

Measurements

The ".vol" files were opened in in the modified CMTF program, and plots of intensity vs. depth were automatically generated. In the normal rabbit cornea, the three major peaks on the curve correspond to the epithelium, basal lamina, and endothelium (Fig. 3). By right clicking on the peaks, it was possible to make depth measurements while navigating through the stack, thereby observing the exact frame that corresponds to each point on the curve. A relative measure of stromal backscatter (haze) was determined by calculating the area under the intensity curve. This was measured by right clicking points at the top and bottom of the stroma using the *area* function within the CMTF software (Fig. 5).

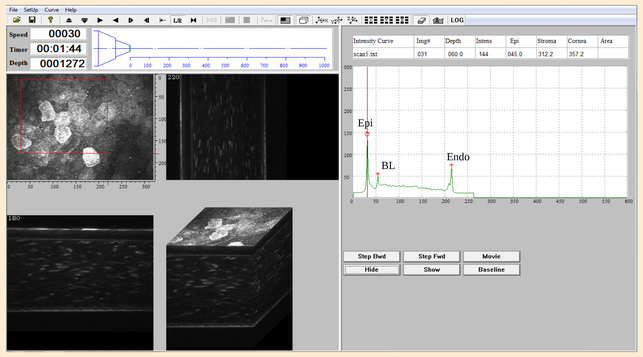

Figure 3. Screen shot of CMTF program (Confo). Right side shows corneal intensity curve with intensity peaks at the superficial epithelium (Epi), basal lamina (BL), and endothelium (Endo). Images on the left are reconstructions of the image stack collected by CMTF imaging shown at different projection angles. Scan shown was collected at a speed of 60 µm/second.

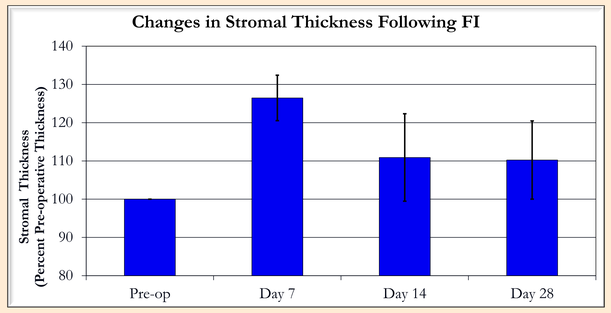

Figure 4. Graph showing changes in stromal thickness as a percent of pre-operative thickness of nine rabbits following FI. Of the four time points, edema is highest at day 7 and begins to level off beginning at day 14. Bars show mean ± standard deviation.

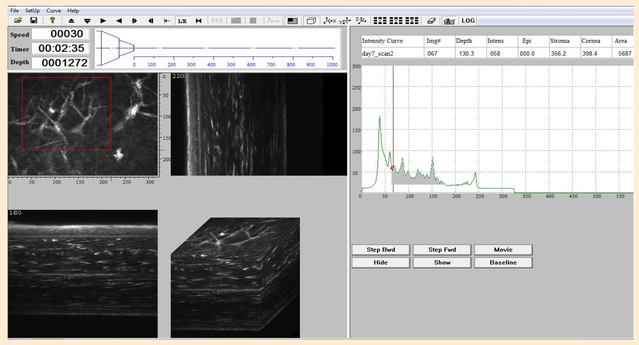

Figure 5. Screen shot demonstrating the use of the *area* function on the Confo program to measure stromal haze on a corneal scan taken 7 days following FI.

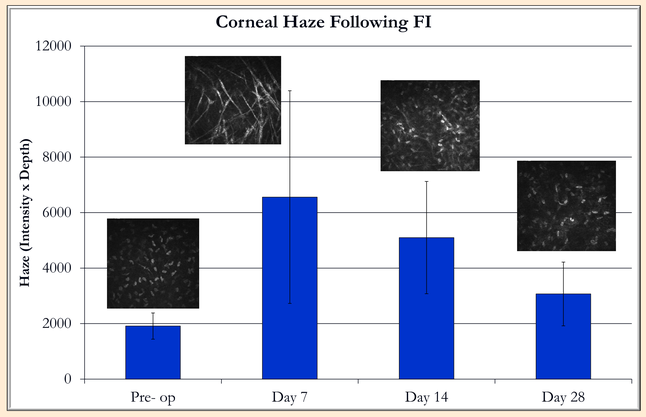

Figure 6. The graph displays corneal haze measurements acquired by taking the area under the intensity vs. depth curves. Of the four time points, haze is greatest at day 7 and begins to decrease at day 14. The insets show typical cell morphologies in the anterior stroma associated with each time point. Bars show mean ± standard deviation.

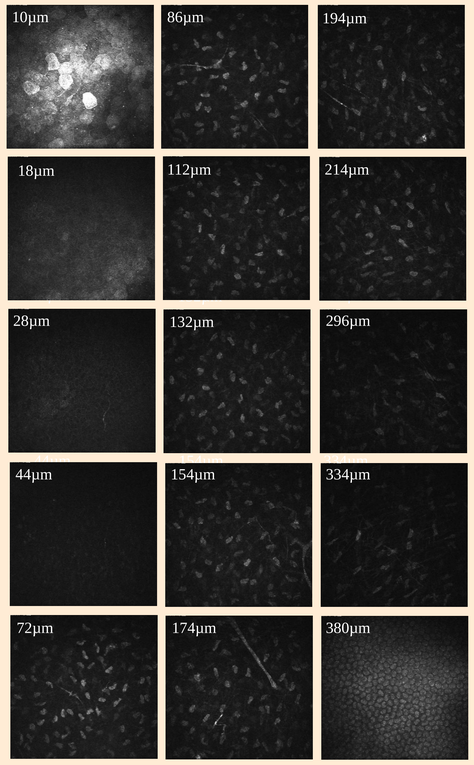

Figure 7. A sampling of images from a CMTF scan taken from a normal rabbit cornea *in vivo*. The position displayed in the upper left of each image is the depth relative to the front surface of the Tomocap. A speed of 60 µm/second was used for the CMTF scan.

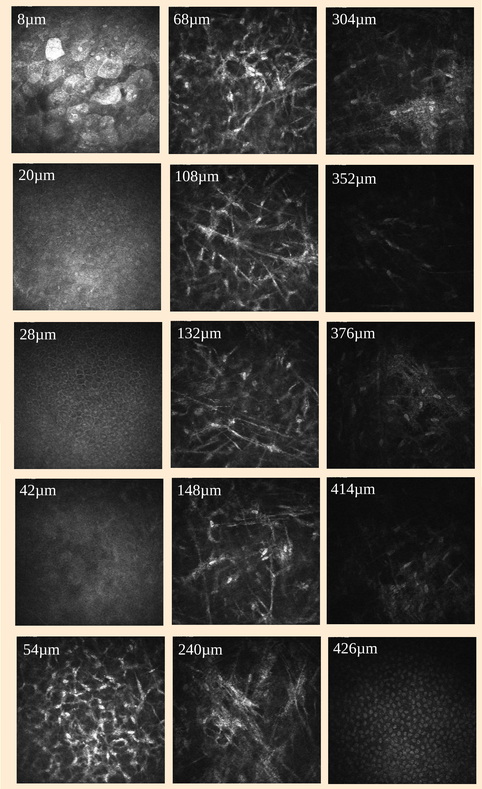

Figure 8. A sampling of images from a CMTF scan taken from a rabbit cornea *in vivo* 7 days post-operation. The position displayed in the upper left of each image is the depth relative to the front surface of the Tomocap. A speed of 60 µm/second was used for the CMTF scan.

RESULTS

Using the modified HRT-RCM, full thickness CMTF scans with clear images of all cell layers were consistently obtained (Fig. 7). Calculating the average pixel intensity and plotting versus z-depth produced curves with clear peaks corresponding to the superficial epithelium, basal lamina, and endothelium (Fig. 3).

FI induced damage to the epithelial and endothelial barrier, which led to edema and increased corneal thickness at 7 days (Fig. 4). Edema partially subsided at 14 and 28 days as these cell layers were re-established.

When keratocytes were in their quiescent state, only the cell nuclei were visible by confocal microscopy (Fig. 7). Following FI, the cells became elongated and more reflective (Figs. 5 and 8), characteristics which are consistent with transformation to a migratory fibroblastic phenotype.

Corneal haze increased following FI due to the transformation of cells in the stroma, and was maximum at day 7 (Fig. 6). It then gradually decreased as wound healing progressed.

CONCLUSIONS

The hardware and software modifications to the HRT-RCM allow high resolution 3-D image stacks to be collected from the entire cornea *in vivo*.

Using the modified CMTF program, these datasets can be used for interactive visualization of corneal cell layers, as well as quantitative assessment of sub-layer thickness.

The system also allows quantitative estimates of corneal backscattering, which has been shown to correlate with clinical "haze" that can reduce visual acuity in some patients.

Overall, the modifications significantly expand the capabilities of the HRT-RCM for quantitative corneal imaging.

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