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Multiphoton Fluorescence and Second-Harmonic-Generation Microscopy for Imaging Structural Alterations in Corneal Scar Tissue in Penetrating Full-Thickness Wound

The transparency of the cornea relies on the special spatial arrangement of stromal collagen.[1] Any pathological abnormality that leads to the wound healing responses and consequential disruption of collagen alignment may hinder corneal transparency.

Understanding wound healing may be crucial for the successful applications of clinical procedures such as refractive surgery.[2] Therefore, the development of a noninvasive in vivo imaging technique is valuable for investigating the physiological response associated with corneal wound healing. In this article, we demonstrate multiphoton ex vivo imaging of full-thickness corneal scar tissue 10 years following wounding. We intend to demonstrate the ability of multiphoton imaging to reveal corneal structural alterations from the wound healing process and the potential of this imaging modality in clinical diagnosis and monitoring of corneal pathological abnormalities.

Report of a Case. A 52-year-old man had a penetrating linear corneal wound in his right eye and, owing to corneal decompensation, received penetrating keratoplasty 10 years following trauma. The trephined corneal button was bathed in balanced salt solution and sent for multiphoton imaging immediately. After image acquisition, the specimen was prepared for histological examination.

The multiphoton imaging system that we used is similar to that in our previous article on corneal imaging.[3] The 760 nm of a titanium-sapphire laser was used for sample excitation, and the images were acquired using a water-immersion objective (Fluor water immersion, ×40, 0.8 numerical aperture; Nikon, Tokyo, Japan).

The multiphoton images of the corneal scar specimen are shown in **Figure 1**. Three-dimensional projections of large-area fluorescence (green) and second-harmonic-generation (SHG) (blue) images from the depths of 0 to 1200 µm are shown (Figure 1A-F). In addition, selected regions of interests are shown (Figure 1G-I). A number of physiological and pathological features can be identified. First, the image at 0 µm (Figure 1G) shows a disruption of the Bowman membrane with protruding SHG stromal collagen. At this depth, one can easily

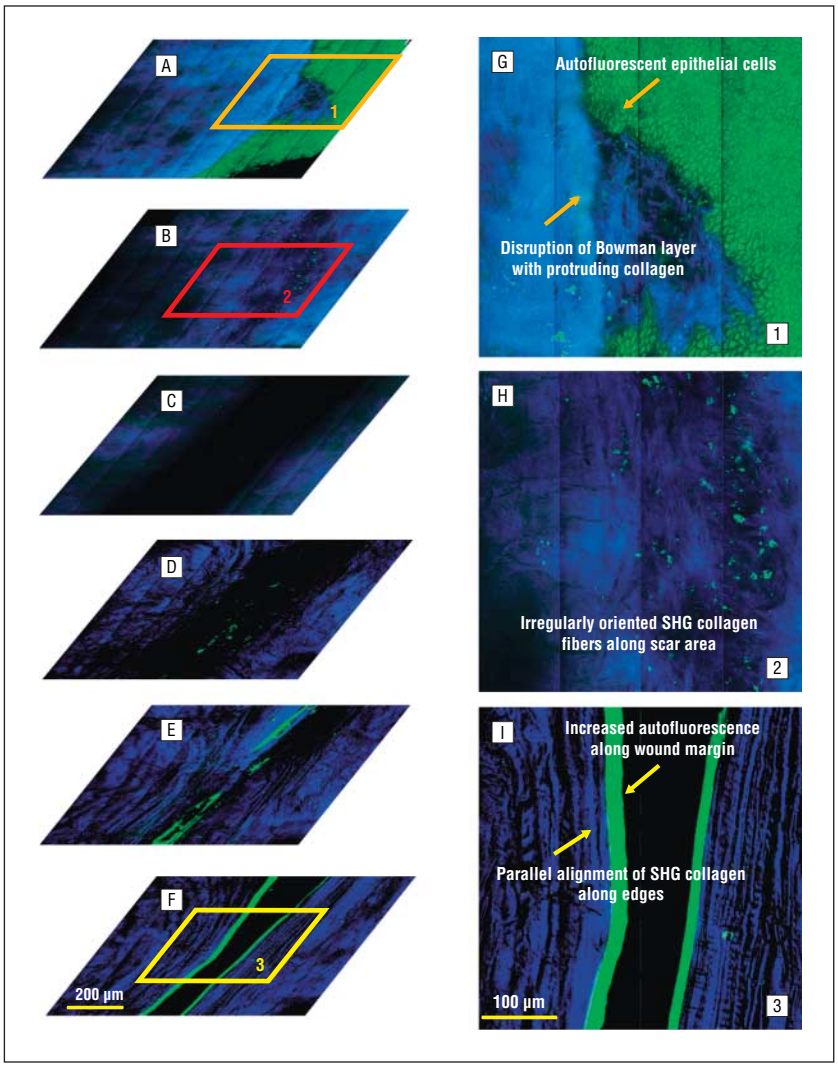

Figure 1. Ex vivo multiphoton images of the penetrating corneal scar tissue. Three-dimensional projected images at 0 µm (A), 200 µm (B), 400 µm (C), 800 µm (D), 1000 µm (E), and 1200 µm (F) from the surface. The boxes in A, B, and F correspond to magnified images from selected regions of interest in the epithelium (G), anterior stroma (H), and posterior stroma (I), respectively. At the superficial epithelial layer, aberrantly regenerated collagen fibers can be seen to have invaded the epithelial layer. As the depth increases, disorganized collagen fibers are visualized within the linear wounded area, while the surrounding stroma is composed of homogeneous second-harmonic-generating (SHG) collagen lamellae. In the posterior stroma, parallel aligned collagen fibers can be seen along the linear wounded area. Note that the thickness of the cornea is increased owing to a possible edematous condition during sample preparation. Green indicates fluorescence; blue, SHG signals.

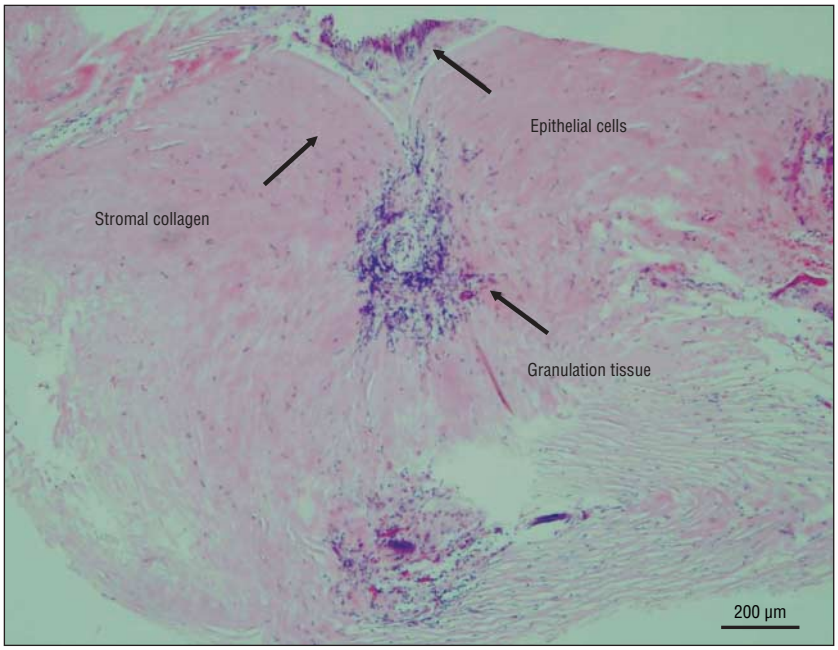

Figure 2. Histological analysis of full-thickness corneal scar tissue. Epithelium undulation was noted at the wounded area owing to contraction of scar tissue. Disorganized collagen fibers were found at the penetrating scarred area (hematoxylin-eosin).

visualize the fluorescent epithelial cells with less fluorescent nuclei. The corneal epithelial cellular autofluorescence was most likely due to nicotinamide adenine dinucleotide phosphate.[4,5] At 200 µm, the collagen pattern at the wound is irregularly arranged, which is in sharp contrast to the orthogonal packing of adjacent lamella found in normal stroma (Figure 1H). At imaging depths of 400 µm and beyond, regions lacking in SHG collagen were observed. In addition, the collagen fibers outside of the wound tended to align parallel to the wound edges. At the imaging depths of 1000 and 1200 µm, intense fluorescent lining (possibly from detached uveal tissue) along the wound edge was found (Figure 1I).

For comparison, the histological image is shown in **Figure 2**. Both the surface epithelial cells and the V-shaped corneal wound were visible. At greater depths, we also found granulation tissue (with cells). The existence of the corneal wound and granulation tissue may explain the lack of SHG collagen fibers within the wound.

Comment. Previously, it was shown that multiphoton microscopy can be used to image autofluorescent epithelial cells and SHG collagen fibers within the stroma of normal porcine cornea.[3] In this study, we extended this approach to the investigation of the structural alterations of a full-thickness linear corneal scar. The structural alteration of the cornea along the linear scar can be identified using the multiphoton technique without histological procedures. With additional refinement of scanning technology (increase in imaging speed) and a better characterization of possible tissue photodamage, multiphoton microscopy may be developed into a clinical diagnostic tool for in vivo monitoring and lead to a better understanding of corneal wound healing processes.

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Histopathologic Findings in Naturally Preserved Mummified Human Eyes

This study was undertaken to assess the suitability of 2 pre-Columbian, naturally preserved, mummified human globes for contemporary histopathologic analysis, to successfully rehydrate and process the unembalmed specimens, and to describe the gross and microscopic findings. The ability to analyze ancient human remains may expand our understanding of the prevalence of ophthalmic diseases and possible correlations involving diet, lifestyle, and heredity. Histopathologic analysis was performed on the 2 naturally preserved eyes from the Atacama Desert of northern Chile. One eye was from a 2-year-old boy and the other from a 23-year-old woman. Modifications to traditional tissue rehydration techniques were used. Mummified ocular tissues were successfully rehydrated. Processed tissues survived paraffin embedding and microtome sectioning. Tissue sections absorbed various conventional tissue stains. Light microscopy revealed uveal tissue, retinal pigmented epithelia, and intact sclera. Structures representing the inner lining of the embryonic optic cup were not recovered. Naturally preserved mummified tissues are amenable to contemporary histopathologic analysis. The