Ophthalmic Imaging Using Multiphoton Microscopy

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Abstract

This purpose of this study is to demonstrate the feasibility of using multiphoton microscopy in ophthalmologic imaging. Without the introduction of extrinsic fluorescence molecules, multiphoton induced autofluorescence and second harmonic generation signals can be used to obtain useful structural information of normal and diseased corneas. Our work can potentially lead to the in vivo application of multiphoton microscopy in investigating corneal physiology and pathologies.

Keywords: multiphoton microscopy, second-harmonic generation, fluorescence, cornea.

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1. Introduction

Recently, multiphoton microscopic system has been widely applied to different fields of life science, including neurobiology, oncology, transdermal delivery, hepatology, and deep-tissue imaging[1-14]. It provides numerous advantages over the conventional single photon fluorescence microscopic system. The multiphoton technique induces nonlinear, point-like, excitation of fluorescent molelcules using photons with roughly half the energy of the single photon used in single photon excitation. Since the near infrared excitation wavelengths used are absorbed and scattered less than the ultraviolet or visible radiation used in conventional microscopy, multiphoton imaging can achieve deeper penetration than conventional single photon techniques[1,2]. The combined capability of detection the autofluorescence of NAD(P)H from cytoplasm, and the second harmonic generation signal from collagen, the multiphoton microscopic system has also been applied for corneal imaging[15,16].

The potential of acquiring cornea structural information with no intervention and destruction as real-time disease diagnosis and monitoring may be invaluable. Traditionally, the diagnosis of corneal diseases relied heavily on histological procedures in probing the morphological changes. However the tissue processing, fixation, and staining may lead to morphological artifacts. The alternative approach of confocal microscopy has been successfully applied in the field of ophthalmology[17]. However, stroma collagen may not be easily imaged using this technique. In this study, we will examine the feasibility of multiphoton microscopic system for imaging full-layered normal corneal structure and several pathological ones. Our work can lead to the eventual application of multiphoton microscopy for in vivo application.

2. Methodology

2.1 Multiphoton microscopic system

The experimental set-up of our multiphoton microscopy instrument used in this study has been described[9]. In this experiment, the 760 nm, 80MHz output of a diode (Millennia X, Spectra Physics, Mountain View, CA) pumped, titanium-sapphire laser (Tsunami, Spectra Physics) was used to induce autofluorescence excitation and SHG from eye specimens. The linearly polarized laser source is circularized by the combination of a polarizer and half-wave plate. Circularly polarized laser is guided towards a pair of x-y scanning system (Model 6220, Cambridge Technology, Cambridge, MA) which steers the laser source two-dimensionally. At the entrance of the upright microscope, the laser is beam expanded and then reflected by a dichroic mirror (700DCSPRUV, Chroma Technology, Brattleboro, VT) into the back aperture of a water-immersion objective (Fluor 40x, NA 0.8; Nikon). The average power at the sample was 12-16 mW. The autofluorescence and SHG signals generated are collected by the same objective, and passed through the dichroic prior to reaching the photodetectors. Prior to reaching the detectors, the autofluorescence and SHG signals were separated by a secondary dichroic

mirror (435DCSX, Chroma Technology). The SHG signal centered at 380 nm was reflected and further filtered using a bandpass filter (HQ380/40, Chroma Technology) while the longer wavelength autofluorescence passes through the secondary dichroic mirror and an additional bandpass filter (HQ490/40, Chroma Technology) before being detected. In this fashion, the angular deviation of the x-y scanning system is translated to linear scanning of the focal spot across the cornea specimens. Both the autofluorescence and SHG signals were detected using single-photon counting photomultiplier tubes (R7400P, Hamamatsu, Japan). For large area scan of the eye specimens, a two-dimensional stage scanning system (H101, Prior Scientific, UK) was used for specimen translation.

2.2 Tissue preparation

The porcine eyes were obtained from slaughter house within two hours after the sacrifice. The extraocular soft tissue was removed and immersed into PBS buffer for imaging.

For multiphoton imaging of diseased human corneas, diseased corneal specimens were obtained with the permit consent from patient. The specimens were immersed into balanced salt solution after being removed from surgery before being imaged. After the imaging was taken, the corneal buttons were sent for standard hematoxylin and eosin histological examination.

One case of diseased cornea is demonstrated and described below.

Case Report - Corneal Scar

The second damaged cornea specimen was from a 51 year-old male who experienced an episode of trauma induced eyeball rupture about 20 years ago. In this case, primary corneal repair with lens extraction was done immediately after injury. Secondary intraocular lens implantation was done months later, and his best spectacle-corrected visual acuity was 20/30 till 6 months before. At that point, bullous keratopathy was impressed. Therefore, he has received penetrating keratoplasty due to endothelial decompensation.

3. Results

3.1 Normal porcine cornea

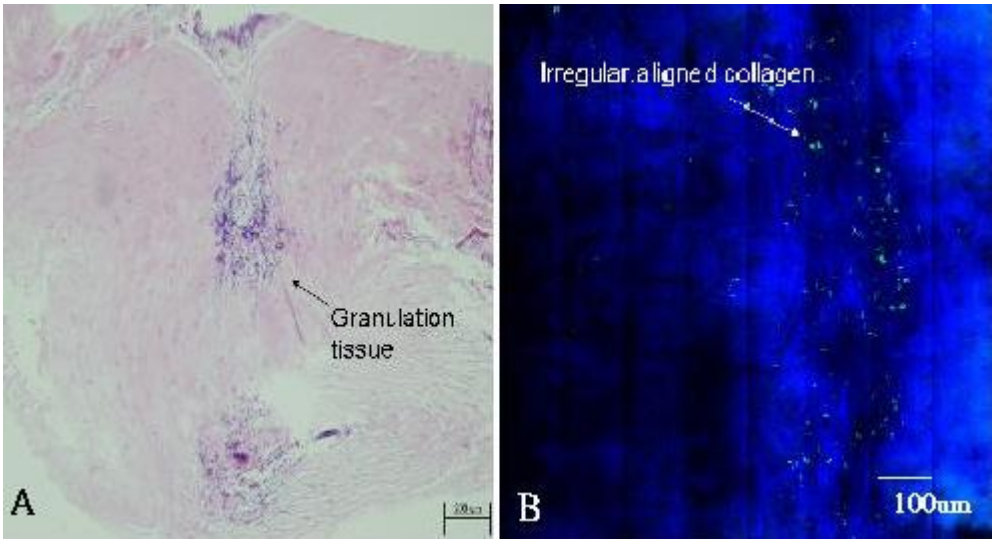
The multiphoton images of the whole-layer porcine cornea can be demonstrated. The luminescence at the superficial layer originates from intracellular autofluorescence (green). Each individual epithelial cell is clearly visible as the nuclei lacking autofluorescence. As the imaging depth increases, the source of sample luminescence becomes that of the SHG signal of collagen fiber (blue), indicative of the collagen fiber orientations. The collagen fiber orientation changes as the depth increase. At the innermost layer, the endothelium is also visible for the intracytoplasmic autofluorescence. Therefore we demonstrate imaging throughout the corneal structure with the combined usage of autofluorescence

and SHG.

3.2 Diseased Cornea

The histological and multiphoton images of this example are shown in Fig 1. The multiphoton images of the superficial cornea layer show significant disruption of Bowman's layer with the irregular growth of collagen fiber into epithelium layer, which is consistent of the pathological finding following corneal penetrating injury. As the imaging depth increased (Fig. 1b), the SHG signal diminished inside the groove at the injured stromal site. This observation seems to support the fact that there was little regeneration of collagen fiber occurred with over decades, consistent with the histological images showing the formation of the granulation tissue within the injured stroma. In addition, the orientation of regenerated collagen fibers along the incised wound was clearly visible. At the anterior portion, the regenerated collagen oriented irregularly. However, the collagen fibers in the posterior domain aligned parallel to the incised wound margin.

Fig. 1. Corneal scar: A. The histological image of the full-thickness incised wound. B. The large scale multiphton images at the depth of 200 μ m.



4. Conclusion

In this study, we applied multiphoton microscopy for imaging whole layer normal and diseased corneas. We demonstrated that autofluorescence can be used to identify epithelial and endothelial cells. And the SHG is useful for imaging stromal collagen. The orientation and packing of collagen fiber can be imaged through the whole layer. With additional developments, this approach may be applied for the in vivo investigation of corneal physiology. As for the diseased corneas, the combined usage of

autofluorescence and SHG was useful in revealing the severity and extension of destructed stroma. The long term regeneration pattern of collagen fiber following penetrating injury can also be demonstrated with SHG signal, which may provide useful information of the disease and the wound healing processes. In conclusion, our study demonstrates that the minimally-invasive imaging modality of multiphoton autofluorescence and SHG microscopy may offer valuable morphological and spectroscopic information in corneal physiology and pathology. With additional developments, this approach, this technique may be applied in vivo for disease diagnosis and tissue biology in ophthalmology.

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