

The Combination of multiphoton and reflected confocal microscopy for cornea imaging

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ABSTRACT

The purpose of this work is to demonstrate the combination of reflective confocal microscopy and multiphoton microscopy and its application in imaging cornea. The difficulty of optically imaging the highly translucent cornea has prevented the development of an effective non-invasive system for the clinical monitoring of the physiological or pathological states of corneas. In this work, we combine reflective confocal microscopy with multiphoton microscopy to demonstrate the potential of our methodology in the minimally invasive imaging of the cornea. The visible reflection signals from cornea can provide structural information of interfaces of different refractive indices while the multiphoton signals generated from the use of near infrared excitation allows deep tissue penetration and reduced photo-damage. In multiphoton imaging, the second harmonic generation (SHG) signal is used to detect collagen in the stroma of the cornea, and the reflective confocal imaging allows detection of the cellular components located in the epithelium. The combination of reflective and multiphoton imaging can be used to reveal complementary structural information of the corneal architecture.. The system is first tested on porcine eye cornea. Assessment of the result on the porcine eye will be used to evaluate the potential of the system as a technique for in vivo clinical applications.

Keywords: multiphoton microscopy, second-harmonic generation, reflected confocal microscopy, cornea.

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1. Introduction

Real-time acquiring cornea structural information with no intervention and destruction may be crucial for investigating and diagnosing all corneal pathologies. Traditionally, the investigation of corneal pathologies relies heavily on histological procedures for probing the morphological alterations. However, the processing and fixating procedures can inevitably lead to morphological artifacts. The alternative non-invasive approach of confocal microscopy has been widely applied in corneal pathological imaging¹⁻⁹, including infections, dystrophies, and various wound healing processes following surgical procedures. Cellular components, nerve, and certain pathogens may be visualized in vivo with reflected confocal microscopy. However, stromal collagen may not be easily imaged using reflected mode. Multiphoton microscopy is another new modality with minimal invasion that has been applied in ophthalmology^{10,11}. Multiphoton microscopy using the nonlinear excitation from ultrafast, near-infrared light source provides several advantages for the application in biomedical sciences. The key features of multiphoton microscopy include the enhancement of axial depth discrimination, reduced photodamage, and increasing imaging depth^{12,13}. In addition to multiphoton fluorescence excitation, the nonlinear optical effect of second harmonic generation (SHG) has also been provided for imaging biological structures that lack of an inversion symmetry. Several biological structures such as collagen, muscle fiber, and microtubules have been proven to be effective second harmonic generators^{14,15}. We had previously demonstrate multiphoton autofluorescence and SHG imaging of normal porcine cornea¹¹. The intracorneal cellular components can be visualized with multiphoton autofluorescence, and collagenous structures can be identified with SHG signals. In this work, we extend our investigation into the combination of reflected confocal microscopy with multiphoton microscopy for minimally invasive imaging of cornea. The use of near-infrared excitation source allows deeper tissue penetration and a lower potential for photodamage. Collagenous components within cornea can be identified with SHG signals, and the reflected confocal imaging allows detection of the cellular components. We provides this minimally invasive imaging technique for imaging porcine cornea ex vivo and our work demonstrates the feasibility of this optical technique as a clinical diagnostic and monitoring tool for imaging corneal pathologies.

2. Experimental method

Specimen preparation

The porcine eyes used in this study were obtained directly from local market at the earliest possible time. The eyeballs with intact epithelium and optically clear media were selected for further processing. The corneal buttons were created with 8mm corneal trephine and placed in phosphate buffered solution.

Multiphoton microscopy and reflected confocal imaging

The imaging system used in this study is a commercial laser scanning microscope (Meta 510, Zeiss, Germany). For

reflective microscopy, the 543 nm line from a visible source provided with the Meta 510 system was used. The multiphoton imaging of the porcine cornea specimen was achieved using the near-infrared excitation source from a titanium-sapphire laser (Tsunami, Spectra Physics, Mountain View, CA) pumped by a diode-pumped, solid state (DPSS) laser system (Millennia, Spectra Physics). The multiphoton excitation wavelength used was 780 nm and the we simultaneously acquired multiphoton autofluorescence and SHG signals (centered at 390 nm) from the cornea specimen. In our study, the reflected confocal and multiphoton images were separately acquired. The instrument set up of the reflective confocal and multiphoton microscope is shown in Fig. 1.

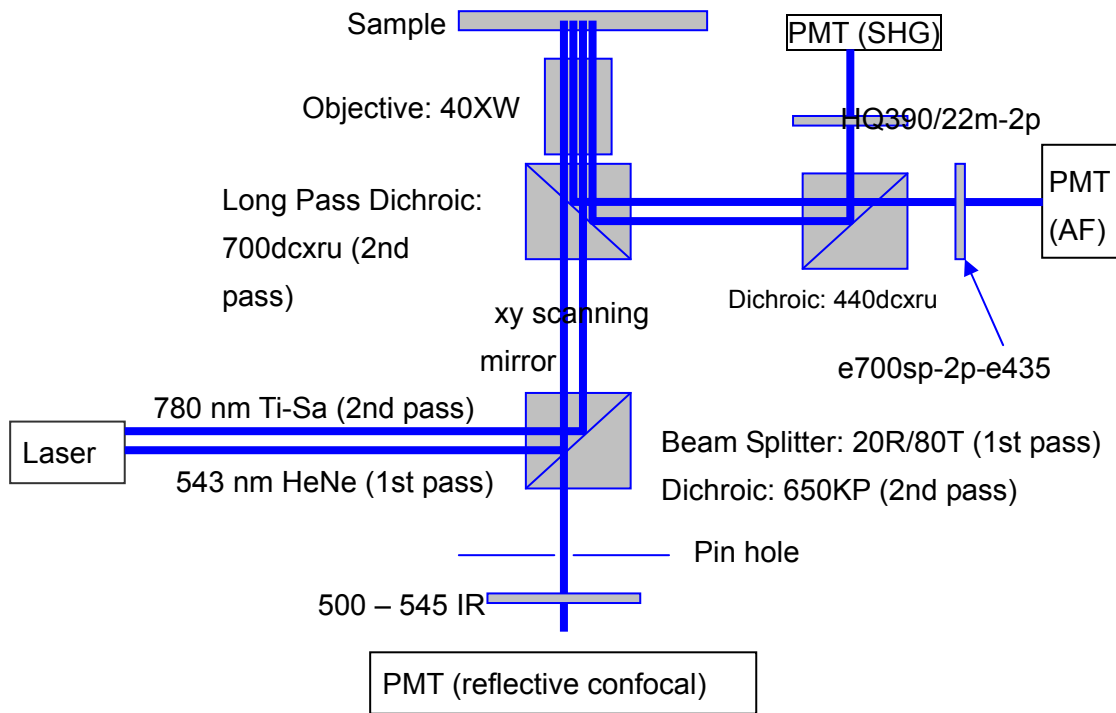


Fig. 1. The instrumentation setup of combined reflective confocal microscopy and multiphoton microscopy.

3. Results

The separated reflective and multiphoton images are shown in Fig. 2 and 3. The whole layered corneal structure can be imaged with multiphoton system. The corneal epithelium can be visualized with autofluorescence signals. In normal porcine corneal stroma, collagen fibers can be identified with SHG signals, while keratocytes cannot be easily detected with multiphoton autofluorescence excitation.

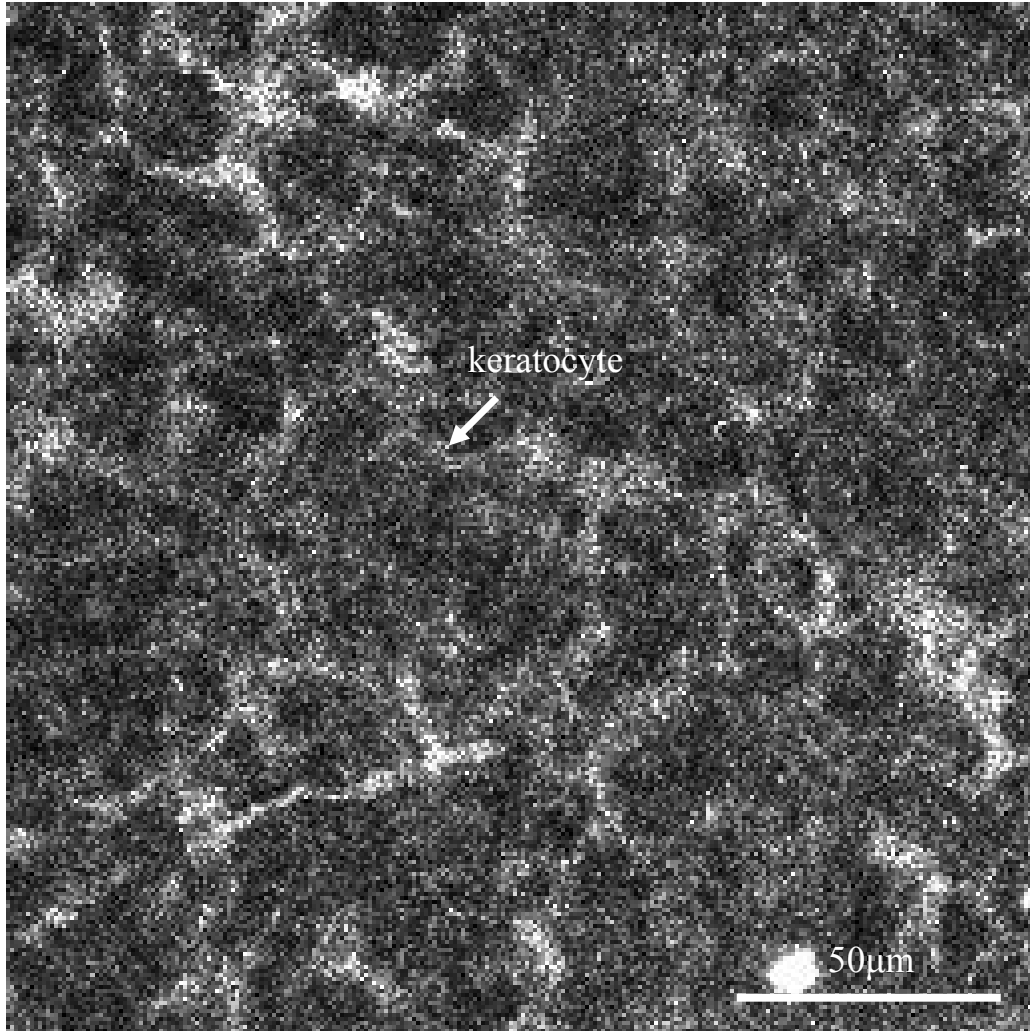


Fig. 2. Reflected confocal image of normal porcine cornea at the approximate imaging depth of 10 μm .

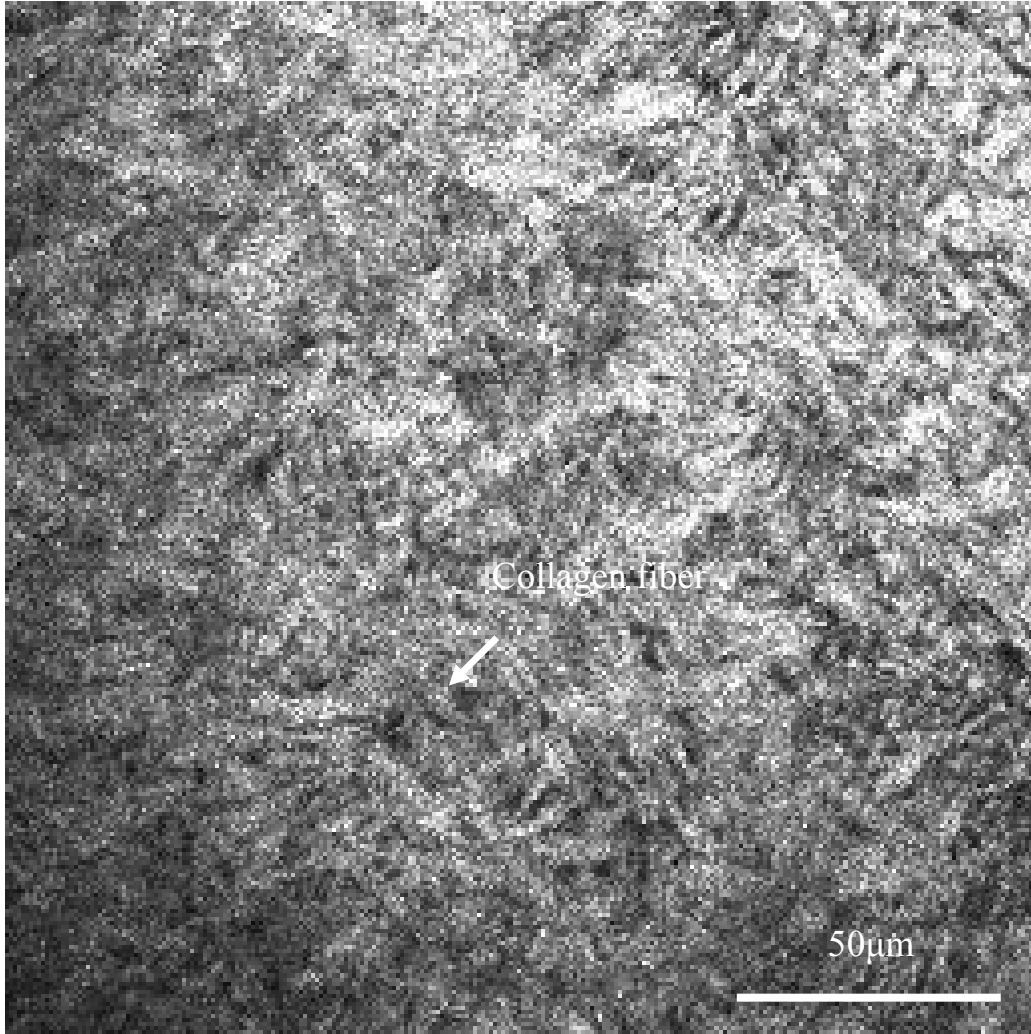


Fig. 3. The corresponding second harmonic generation image of figure 2 at the same position of normal porcine cornea.

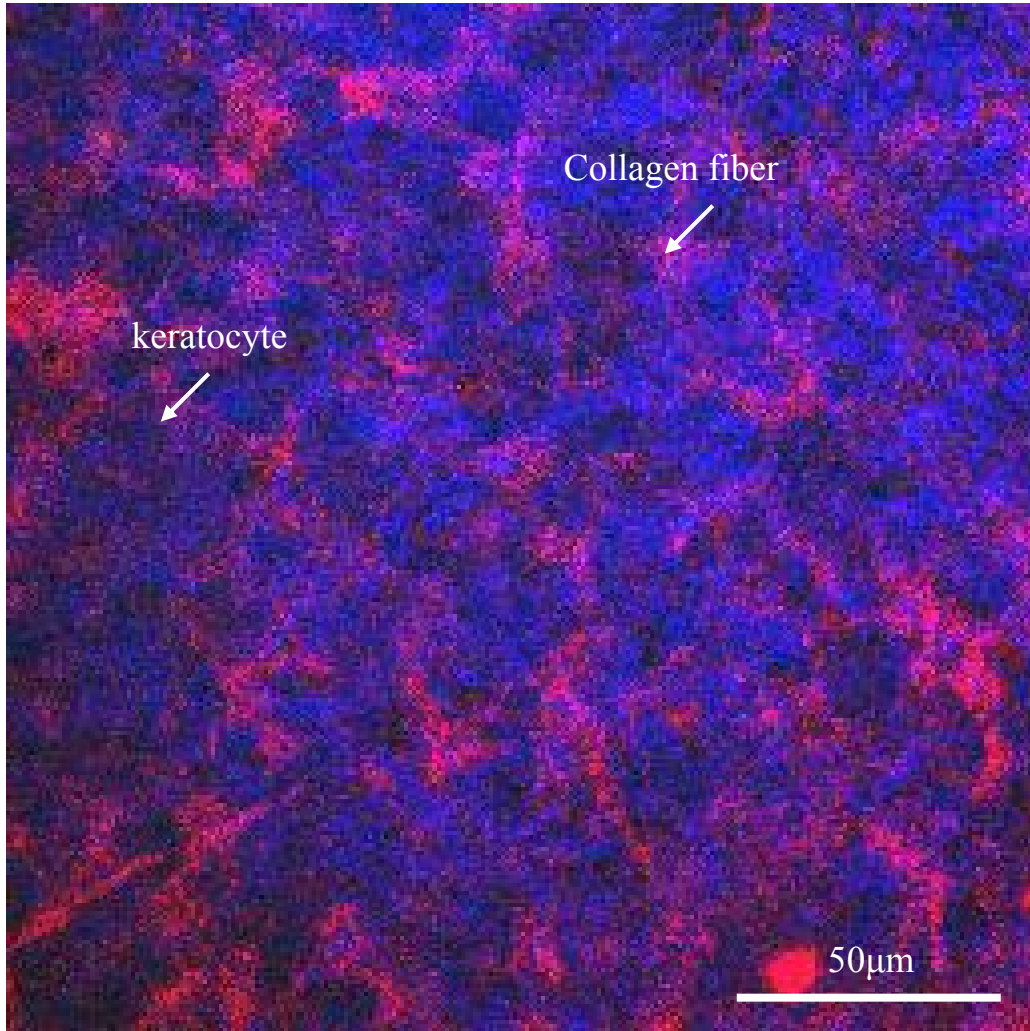


Fig. 4. The combined reflective and multiphoton imaging of normal porcine cornea at depth of 10µm below epithelium. Red color represents reflected signals while the blue color for SHG signals. Stromal collagen fibers are effective SHG generators, and keratocytes can be identified with reflective imaging. Notice that autofluorescence signals cannot be detectable here.

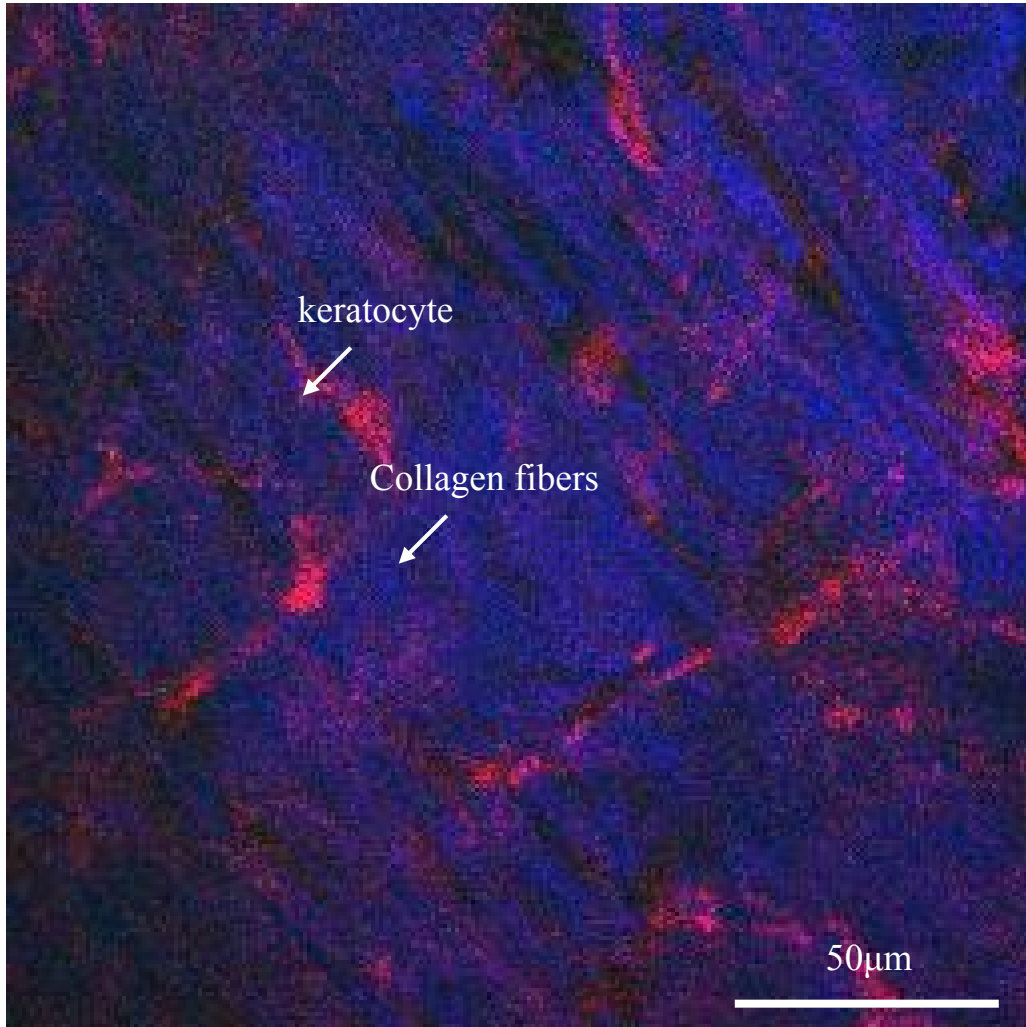


Fig. 5. The combined reflective and multiphoton imaging of normal porcine cornea at depth of 190µm below epithelium. Red color represents reflected signals while the blue color for SHG signals. Stromal collagen fibers are effective SHG generators, and keratocytes can be identified with reflective imaging. Notice that autofluorescence signals cannot be detectable here.

With the combined technique of multiphoton microscopy and reflective confocal microscopy, it can provide additional structural information. The corneal epithelium can be imaged with both the multiphoton autofluorescence excitation and reflective mode simultaneously. In the corneal stroma, collagenous structure can be identified with SHG signals (Fig. 4 and 5). As for the quiescent keratocytes, they can be identified in the reflected mode, but can be ineffective for generating autofluorescence. Therefore with the combination of SHG microscopy and reflective confocal microscopy as a minimally invasive imaging technique, both the cellular and extracellular information can be visualized.

4. Conclusion

While we have demonstrated the ability of multiphoton imaging in resolving cornea structure, we anticipate the addition of reflected confocal imaging will widen the capability of cellular level corneal imaging. If only morphological information is needed, one can select the second harmonic generating wavelength such as not to excite cellular autofluorescence. In such a case, structural details of both the corneal epithelium and stroma can be obtained without minimal photodamage associated with autofluorescence excitation. This approach has potential application for in vivo imaging of cornea pathologies.

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