Multiphoton microscopy for imaging infectious keratitis: demonstration of the pattern of microbial spread in an experimental model

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ABSTRACT

The purpose of this study is to assess the application of multiphoton fluorescence and second harmonic generation (SHG) microscopy for imaging and monitoring the disease progress of infectious keratitis in an experimental model, and to investigate the possible correlation of tissue architecture with spreading patterns of pathogens in an experimental model. Porcine eyes are to be obtained from slaughter house and processed and placed in organ culture system. Fungal infections by common pathogens of infectious keratitis are to be induced in porcine cornea buttons. Multiphoton fluorescence and SHG microscopy will be used for imaging and for monitoring the progression and extension of tissue destruction and possibly the pattern of pathogen spreading. We found that SHG imaging is useful in identifying alterations to collagen architecture while autofluorescence microscopy can be used to visualize the fungi and cells within the stroma. In summary, multiphoton fluorescence and second harmonic generation microscopy can non-invasively demonstrate and monitor tissue destruction associated with infectious keratitis. The pattern of pathogen spreading and its correlation with the tissue architecture can also be shown, which can be useful for future studies of the tissue-microbial interactions for infectious keratitis.

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1. Introduction

Corneal infection remains a major cause of vision impairment which required intensive medical attention. Although corneal infection is global eye disease, regional differences in the pathogens do exist. In the developed countries, contact lens usage is the major causative factor of corneal ulcer, while in the tropical countries, fungi may be an important pathogen[1,2]. The timing and accuracy of diagnosis and the application of proper antimicrobial may be crucial for determining visual outcome. Currently the clinical diagnosis relies mainly on the laboratory culture system. However, some recalcitrant infectious keratitis may be difficult to be differentiated[3-5]. Therefore, an imaging technique that can provide early in vivo diagnosis is of great value for dealing corneal infections. Confocal microscopy has been widely applied for imaging corneal pathologies[6,7], including corneal infections[8-13]. The pathogens with relatively large size such as fungi and Acanthamoeba can be identified with confocal microscopy in the reflected mode[9-12]. However, there are some limitations for this technique. The resolution may be hindered while the media is turbid, which is mostly in the case of corneal infections. And the destruction of extracellular matrix cannot be visualized with the reflected model.

Recently, multiphoton fluorescence microscopy has been widely applied in the biomedical imaging[14,15]. The non-linear exciation of fluorescence photons using ultrafast, near-infrared excitation source have provided advantages in its ability to acquire enhanced axial depth discrimination images, reduced photodamage, and increased imaging penetration depths. The reduced photodamage enables multiphoton fluorescence microscopy to be applied for observation of living cells without detectable damages[16,17]. In addition to structural information, characteristic autofluorescence from various cellular and extracellular components can help to investigate subjects of interest.[18,19] In addition to multiphoton fluorescence excitation, another non-linear polarization effect, second harmonic generation (SHG), also can provide morphological information from certain biological structures lacking inversion symmetry. A variety of biological materials such as collagen, muscle fibers and microtubules have been shown to be effective SHG generators[19-23]. Collagen is a particularly important structure for SHG imaging due to the fact that it is widely distributed in tissues, including cornea. Moreover, cornea may be a great candidate of the application of multiphoton fluorescence and SHG microscopic system due to its transparent nature. Combined multiphoton autofluorescence and second harmonic generation technique for biological imaging had been applied to various clinical medical categories, including dermatology and ophthalmology.[24-26] Previously, we had demonstrated the application in mapping structure of normal porcine cornea[27]. In this work, we extend our work of the applications of multiphoton fluorescence and SHG microscopy for imaging corneal infections using in vitro porcine corneal model. With direct imaging of the migrating and invading

pattern of pathogen during infectious model, as well as the host inflammatory responses, we hope that we can establish a model and the potential of our approach for investigating the pathogenesis of corneal infections.

2. Experimental Method

Specimen preparation

The porcine eyes used in this study were obtained directly from local market. The porcine eyeballs were sent for processing at the early possible time. The eyeballs with intact epithelium and clear media were selected for further processing. The whole eyeballs were disinfected with 10% proviodine in order to eradicate the normal surface flora. The corneal buttons were cut from the intact eyeballs with 8mm corneal trephine. The corneal buttons were then left to equilibrate for 48 hours in Eagle's minimum essential culture medium with containing antibiotics.

Inoculation of corneal buttons and organ culture

Two days later, pathogen suspensions were injected into corneal stroma. A syringe with a 25-gauge needle was used. The inoculation was undertaken in sterile conditions. Corneal buttons were inoculated with *Aspergillus flavimatus*. The corneal buttons were then placed culture plates containing optimal culture medium and maintained at 37^{o}C in sterile conditions. The medium was changed every 24 hours and the buttons examined with multiphoton microscopy.

Multiphoton autofluorescence and SHG microscopy

The multiphoton autofluorescence (AF) and SHG microscopic system used in this study is similar to the experimental set up that was previously used for corneal studies[27]. It is a home-built system based on a commercial upright microscope (E800, Nikon, Japan). A titanium-sapphire (ti-sa) laser (Tsunami, Spectra Physics, Mountain View, CA) pumped by a diode-pumped solid state laser (Millennia X, Spectra Physics, Mountain View, CA) was used as the laser excitation source. The 760 nm output of the ti-sa laser is used for inducing autofluorescence and SHG from cornea. The multiphoton images were acquired using a water-immersion objective (Fluor WI 40x, NA 0.8, Nikon). To ensure optimal focusing, the laser was beam expanded to ensure overfilling of the objective's back aperture and a short-pass dichroic mirror (700DCSPRUV, Chroma Technology, Brattleboro, VT) was used to reflect the incident excitation laser source into the back aperture of the focusing objective. The multiphoton fluorescence and SHG signals are collected by the same focusing objective, and passed through the dichroic and additional filters before the signals reach the photo-detectors. Prior to reaching the detectors, the AF and SHG signals are separated by a secondary dichroic mirror (435DCSX, Chroma Technology). The SHG signal centered at 380nm was reflected by the secondary dichroic and filtered using a band-pass filter (HQ380/20, Chroma Technology), while the longer wavelength AF signal passes through the dichroic mirror and an additional broad-band filter (E435LP, Chroma Technology) before being detected. Both AF and SHG signals were detected using single-photon counting photomultiplier tubes (R7400P, Hamamatsu, Japan).

3. Results

Multiphoton fluorescence and SHG imaging can provide the structural information in corneal infectious model. In the fungal infected model, we can demonstrate the alterations of stoma collagen and also, identification of the pathogen due to its autofluorescence. In fig.1, we can demonstrate the fungal hyphae within the basal epithelial layer with its autofluorescence. The differentiation between autofluorscence fungal hyphae and epithelial cells is evident due to the morphological characteristics. Corneal stromal collagen fibers can be identified with high resolution and contrast with SHG signals. And the diminished SHG with corneal stroma may represent the degree of destruction of the corneal stroma during infection processes. Fig. 2 is the multiphoton imaging within corneal stroma. In addition to the demonstration of pathogens and the destruction of corneal stroma, we can also identify the presence of autofluroescent, stellate-shaped activated keratocytes, which may implicit the ongoing inflammatory host response taking place.

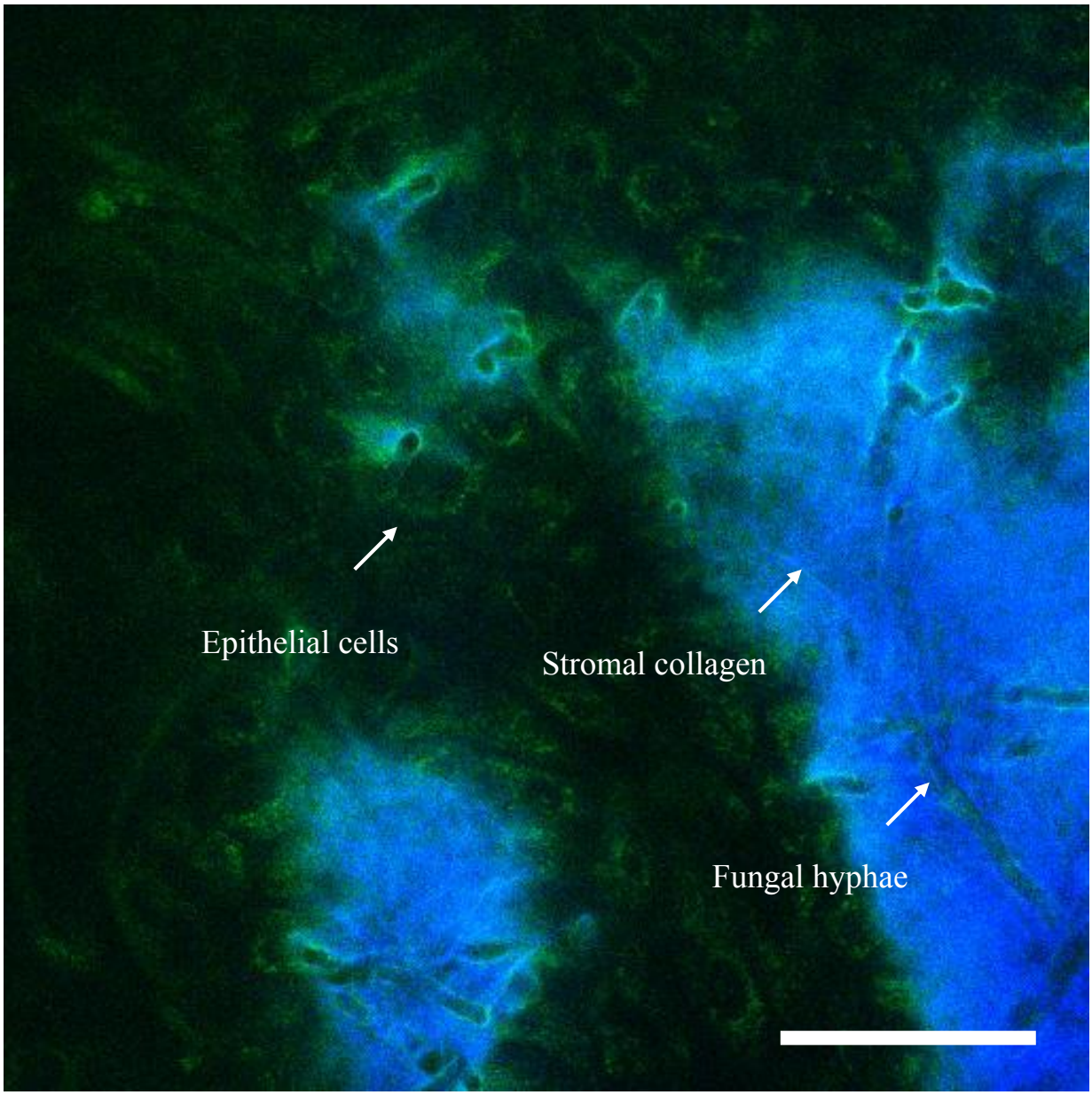

Fig.1 Multiphoton imaging of superficial cornea in fungal infection model. (scale bar: 20 μm)

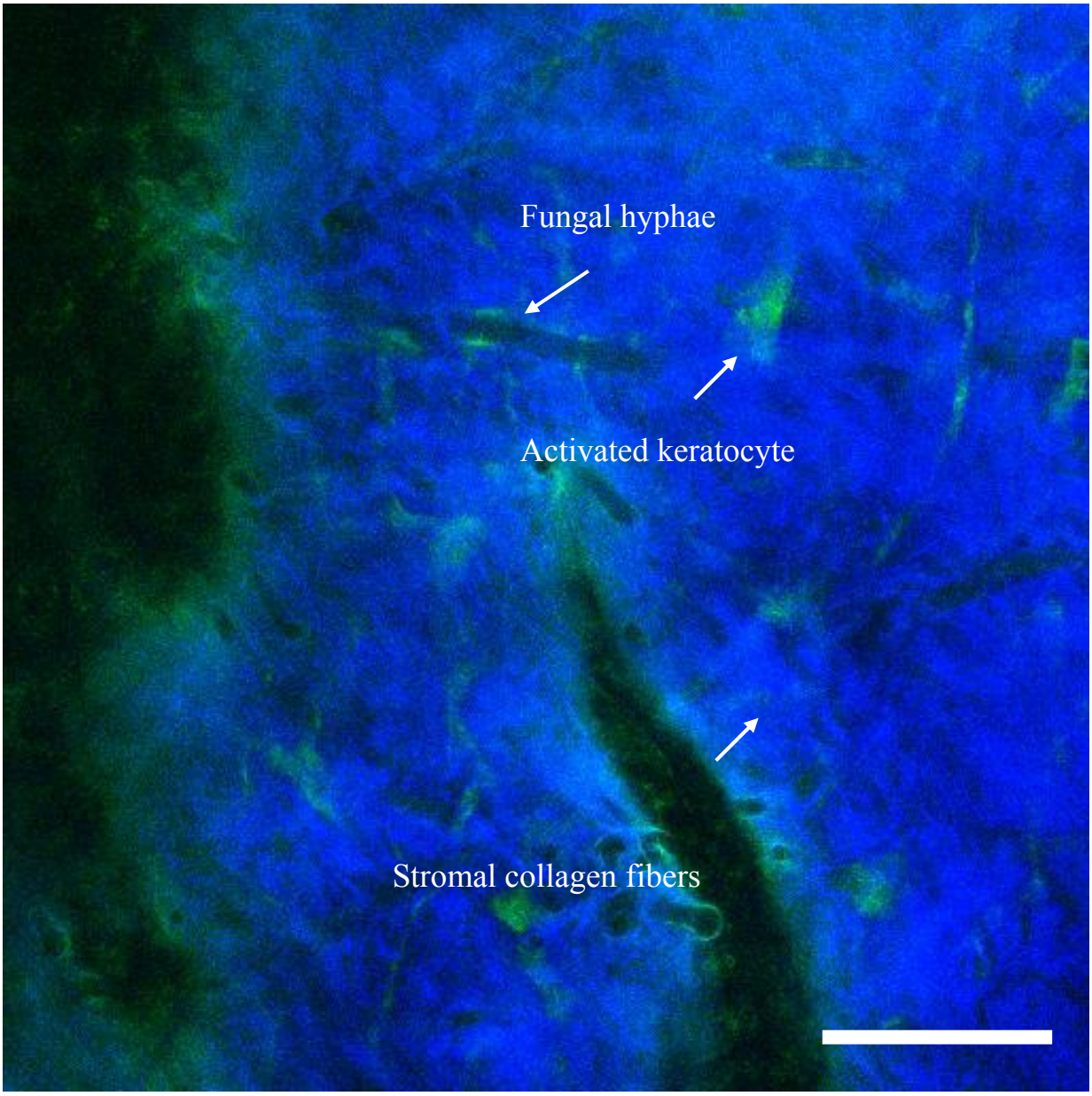

Fig. 2 Multiphoton imaging of corneal stroma in fungal infection model. (scale bar: 20 μm)

4. Conclusion

The corneal stroma composed of layers of collagen lamellae. The transparent properties may provides a unique opportunity for directly visualizing the infection processes in biological systems, including the spreading pattern for different pathogen, and the host immune response for pathogens. We had previously demonstrated the application multiphoton fluorescence and SHG microscopy for imaging normal cornea structure with subcellar resolution[27]. We herein further extend our work for demonstrating that multiphoton fluorescence and SHG microscopy can provide useful

structural information in corneal infections. Not only the cellular components within cornea can be visualized, the selected pathogens can also be visualized without additional processing due to the presence of autofluorescence, which may be of important value for clinical diagnosis. In addition, the decrease of SHG signals from stromal collagen fibers provides important structural information for the severity and extension of tissue destruction during infectious processes. And with corneal organ culture model, we will be able to monitoring the infection processes in vitro at different times. Not only the invasion and migrating pattern of individual pathogen can be identified and characterized, the host inflammatory responses can also be monitored. With additional development, we believe that the corneal infection model using multiphoton fluorescence and SHG imaging can provide a good model for investigating the pathogenesis of corneal infections.

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REFERRENCES

1. Dart J, Stapleton F, Minassian D. Contact lenses and other risk factors in microbial keratitis. Lancet **1991**; 338:650-3.
2. Houang E, Lam D, Fan D, Seal D. Microbial keratitis in Hong Kong: relationship to climate, environment and contact-lens disinfection. Trans R Soc Trop Med Hyg **2001**; 95:361-7.
3. Thomas PA. Fungal infections of the cornea. Eye **2003**; 17:852-62.
4. Bacon AS, Frazer DG, Dart JK, et al. A review of 72 consecutive cases of Acanthamoeba keratitis, 1984-1992. Eye **1993**; 7:719-25.
5. Radford CF, Minassian DC, Dart JK. Acanthamoeba keratitis in England and Wales: incidence, outcome, and risk factors. Br J Ophthalmol **2002**; 86:536-42.
6. Cavanagh HD, Petroll WM, Alizadeh AH. Clinical and diagnostic use of in vivo confocal microscopy in patients with corneal disease. Ophthalmology **1993**; 100:1444-54.
7. Jalbert I, Stapleton F, Papas E, Sweeney DF, Coroneo M. In vivo confocal microscopy microscopy of the human cornea. Br J Ophthalmol **2003**; 87:225-36.
8. Chew SJ, Beuerman RW, Assouline M, et al. Early diagnosis of infectious keratitis with in vivo real time confocal microscopy. CLAO J **1992**; 18:197-201.
9. Winchester K, Mathers WD, Sutphin JE. Diagnosis of Aspergillus keratitis in vivo with confocal microscopy. Cornea **1997**; 16:27-31.
10. Florakis GJ, Moazami G, Schubert H, et al. Scanning slit confocal microscopy of fungal keratitis. Arch Ophthalmol **1997**; 115:1461-63.
11. Avunduk AM, Beuerman RW, Varnell ED, and Kaufman HE. Confocal microscopy of Aspergillus fumigatus

keratitis. Br J Ophthalmol **2003**; 87:409-10.

12. Pfister DR, Cameron JD, Krachmer JH, Holland EJ. Confocal microscopy findings of Acanthamoeba keratitis. Am J Ophthalmol **1996**; 121:119-28.
13. Kaufman SC, Laird JA, Cooper R, Beuerman RW. Diagnosis of bacterial contact lens related keratitis with the white-light confocal microscope. CLAO J **1996**; 22:274-7.
14. Denk W, Strickler JH, Webb WW. Two-photon laser scanning fluorescence microscopy. Science **1990**; 248:73-6.
15. So PTC, Dong CY, Masters BR, and Berland KM. Two-Photon Excitation Fluorescence Microscopy. Annual Review of Biomedical Engineering **2000**; 2: 399-429.
16. Squirrell JM, Wokosin DL, White JG, Bavister BD. Long-term two-photon fluorescence imaging of mammalian embryos without compromising viability. Nat Biotechnol **1999**; 17:763-7.
17. Miller MJ, Wei SH, Parker I, Cahalan MD. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. Science **2002**; 296:1869-73.
18. Zipfel, WR. Williams RM, Webb WW. Nonlinear magic: multiphoton microscopy in the biosciences. Nat Biotechnol **2003**; 21:1369-77.
19. Zipfel, WR. Williams RM, Christie R, Nikitin AY, Hyman BT, Webb WW. Live tissue intrinsic emission microscopy using multiphoton-excited native fluorescence and second harmonic generation. P Natl Acad Sci USA **2003**; 100: 7075-80.
20. Zoumi A, Yeh A, Tromberg BJ. Imaging cells and extracellular matrix in vivo by using second-harmonic generation and two-photon excited fluorescence. P Natl Acad Sci USA **2002**; 99:11014-9.
21. Zoumi A, Lu X, Kassab GS, Tromberg BJ. Imaging coronary artery microstructure using second-harmonic and two-photon fluorescence microscopy. Biophys J **2004**; 7:2778-86.
22. Campagnola PJ, Loew LM. Second-harmonic imaging microscopy for visualizing biomolecular arrays in cells, tissues and organisms. Nat Biotechnol **2003**; 21:1356-60.
23. Brown E, McKee T, diTomaso E, et al. Dynamic imaging of collagen and its modulation in tumors in vivo using second-harmonic generation. Nat Med **2003**; 9:796-800.
24. Masters BR, So PT, Gratton E. Multiphoton excitation fluorescence microscopy and spectroscopy of in vivo human skin. Biophys J **1997**; 72:2405-12.
25. Sun Y, Su JW, Lo W, Lin SJ, Jee SH, Dong CY. Multiphoton polarization imaging of the stratum corneum and the dermis in ex-vivo human skin. Optics Express **2003**; 11:3377-84.
26. Yeh AT, Nassif N, Zoumi A, Tromberg BJ. Selective corneal imaging using combined second-harmonic generation and two-photon excited fluorescence. Optics Letters **2002**; 27:2082-4.
27. Teng SW, Tan HY, Peng JL, et al. Multiphoton autofluorescence and second-harmonic generation (SHG) imaging of ex-vivo porcine eye. Invest Ophthalmol Vis Sci **2006**. In press.