Scanning all-fiber-optic endomicroscopy system for 3D nonlinear optical imaging of biological tissues

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Abstract

An extremely compact all-fiber-optic scanning endomicroscopy system was developed for two-photon fluorescence (TPF) and second harmonic generation (SHG) imaging of biological samples. A conventional double-clad fiber (DCF) was employed in the endomicroscope for single-mode femtosecond pulse delivery, multimode nonlinear optical signals collection and fast two-dimensional scanning. A single photonic bandgap fiber (PBF) with negative group velocity dispersion at two-photon excitation wavelength (i.e. \(\sim 810\) nm) was used for pulse prechirping in replacement of a bulky grating/lens-based pulse stretcher. The combined use of DCF and PBF in the endomicroscopy system made the endomicroscope basically a plug-and-play unit. The excellent imaging ability of the extremely compact all-fiber-optic nonlinear optical endomicroscopy system was demonstrated by SHG imaging of rat tail tendon and depth-resolved TPF imaging of epithelial tissues stained with acridine orange. The preliminary results suggested the promising potential of this extremely compact all-fiber-optic endomicroscopy system for real-time assessment of both epithelial and stromal structures in luminal organs.

1. Introduction

Two-photon fluorescence (TPF) and second harmonic generation (SHG) microscopy has become a powerful tool for three-dimensional (3D) deep tissue imaging \([1,2]\). The quadratic dependence of the incident light intensity in TPF/SHG, enables optical sectioning for depth-resolved microscopic imaging without the use of a confocal pinhole. The out-of-focus photodamage and image blurring are significantly reduced with the nonlinear optical process. The use of near-infrared radiation in TPF/SHG microscopy, combined with wide-field detection, enhances the sampling depth in highly scattering biological tissues. Despite these attractive features, \textit{in vivo} TPF and SHG imaging of biological tissues has been mainly limited to skin due to the lack of proper miniature probes to access internal organs. In order to translate these powerful imaging technologies to clinical and general \textit{in vivo} applications, a flexible endomicroscopy system becomes essential. An easy-to-use and cost-effective miniature endomicroscopy system would also promote the diffusion of these nonlinear optical imaging technologies into more basic research laboratories. Major challenges, however, exist for developing such a fiber-optic endomicroscopy technology, namely, how to achieve efficient excitation light delivery, nonlinear optical signals collection, beam scanning, and probe miniaturization etc. \([4-7]\).

In this paper, an all-fiber-optic endomicroscopy system was developed for TPF and SHG imaging of biological tissues. A conventional double-clad fiber (DCF) was used for single-mode femtosecond excitation laser pulse delivery, multimode TPF/SHG collection and fast
two-dimensional beam scanning. A single photonic bandgap fiber (PBF) was introduced to compensate the positive dispersion of femtosecond pulses in the double-clad fiber, which dramatically reduced the size of the overall endomicroscopy system and made the entire endomicroscopy system basically a plug-and-play unit. The temporal pulse widths at different powers delivered through the endomicroscope were characterized. Utilizing the compact, fully integrated endomicroscopy system, we performed real-time intrinsic ex vivo SHG imaging of rat tail tendon. We also conducted 3D TPF imaging of pig corneal tissue and rat oral tissue ex vivo (stained with acridine orange) with the rapid 2D en face scanning achieved by a miniature fiber-optic scanner built in the endomicroscope and the slow axial scan achieved by moving the probe with respect to the sample with a precision translation stage. The preliminary results strongly suggested the potential of the all-fiber-optic scanning endomicroscopy system for real-time and in vivo imaging of internal organs.

2. Endomicroscopy system and experimental methods

2.1 Miniature high-speed fiber-optic scanning head

Figures 1(a) and 1(b) show the schematic and a photo of the endomicroscope probe, respectively. The probe basically consisted only of a small tubular piezoelectric actuator, a double-clad fiber and a focusing unit. DCF has been shown to be very effective for delivery of the excitation femtosecond laser pulses and collection of the nonlinear optical signals [7, 8]. Here, a commercial DCF (Fibercore Ltd.) was used for single-mode laser delivery by the core (3.5 μm diameter and 0.19 NA) and wide-field collection of TPF/SHG signals by the inner cladding layer (103 μm and 0.24 NA) along with the core. A GRIN lens with a 0.22 pitch and a 1.8-mm diameter (NSG America, Inc.) was implemented as the focusing unit with a magnification of 0.5 from the DCF tip to the sample. The distal end of the probe, including the piezoelectric actuator/scanner and the GRIN lens, was housed in a thin-wall hypodermic tube with an overall diameter of 2.4 mm, as shown in Fig. 1(b).

In the endomicroscope, two-dimensional lateral beam scanning was realized by resonantly scanning the DCF in a spiral pattern with the tubular piezoelectric actuator. The scanning mechanism was detailed in our previous reports [7, 9]. Briefly, DCF was passed through the center of the piezoelectric tube and fixed to one end of the tube. The DCF extending outside the tube functioned as a freely standing cantilever. The outer surface of the piezoelectric tube was divided symmetrically into four quadrants, forming two orthogonal pairs of drive electrodes. Each parallel quadrant pair could be driven independently and a circular scanning by the DCF tip could be achieved when the two pairs of electrodes were driven respectively with a sine and cosine waveform at the mechanical resonant frequency of the fiber cantilever. Furthermore, by triangularly modulating the drive voltages, an open-close spiral scanning pattern would be produced. Our current endomicroscope had a resonant scanning frequency of ~1,690 Hz, which was mainly determined by the length of the fiber cantilever (i.e. the resonant scanning frequency is inversely proportional to the length of the cantilever). At this resonant frequency, 1,690 scanning circles per second would be generated, resulting in a frame rate of 3.3 frames/second with each frame consisting of 512 circular scans. An ~320-μm scanning diameter traced by the DCF tip could be achieved with a relatively low peak-to-peak drive voltage of ~±30 V. This corresponded to a beam scanning area of ~160 μm over the sample when using the GRIN lens with a magnification of 0.5.

2.2 Pulse dispersion management

To compensate the temporal pulse broadening caused by the positive dispersion of femtosecond pulses in the single-mode core of DCF, a grating/lens-based pulse stretcher can be used for negative prechirping before the pulse is launched into the DCF [10]. However, the grating/lens pulse stretcher consists of bulky optics with a double-pass configuration which is generally
very sensitive to alignment and has suboptimal throughput. In our compact system, a single photonic bandgap fiber (PBF, Crystal Fibers Ltd., HC-800-02) was used for dispersion management. The PBF guides light in a hollow core through Bragg reflection [11], and offers a negative group velocity dispersion (GVD) (e.g. -71675 fs$^2$/m at 840 nm) over a more than 60-nm wide spectral range. For the wavelength ranges studied in our experiments (i.e. 805±4.5 nm and 810±18 nm), the dispersion of the PBF and DCF was measured using an intensity autocorrelator. Table 1 summarizes the measured GVD parameter ($\beta_2$) and dispersion parameter (D). The reference values of a conventional silica core single-mode fiber (SMF) at 810 nm are listed [12]. As can be seen, the measured GVDs of the DCF are ~45,127 fs$^2$/m at the 805±4.5 nm band and ~43,065 fs$^2$/m at the 810±18 nm band, whereas the PBF offers a negative GVD of ~ -14,622 fs$^2$/m (at 805±4.5 nm) and ~ -35,246 fs$^2$/m (at 810±18 nm). As a result, the positive dispersion of a DCF can be compensated by a PBF when the length ratio of the PBF to DCF is ~3.1 and ~1.1 at 805±4.5 nm and 810±18 nm, respectively.

2.3 Overall endomicroscopy system

Figures 2(a) and 2(b) show the schematic and a photo of the entire endomicroscopy system with a home-built Ti:Sapphire laser, respectively. The laser operated at a center wavelength of 810 nm with a bandwidth of 36 nm and a measured temporal output pulse width of 60 fs. The laser pulse was coupled into a 0.86-meter long PBF by a fiber-launching lens with an effective NA of ~0.2 and then directed into the endomicroscope (i.e. to the core of the 0.76-meter long DCF). Note the influence of the short GRIN lens (3.27-mm long) on the pulse dispersion was insignificant and could be neglected [13]. Thus the length ratio of the PBF to DCF was kept at ~1.1 to achieve optimal dispersion compensation for this excitation band (810±18 nm). Figure 3 illustrates the intensity correlation curves of the pulses exiting from the endomicroscope at different powers. For the power of 10-20 mW delivered through the DCF core, the measured pulse width at the imaging target (e.g. after the PBF, DCF and the GRIN lens) was about 130 fs. The pulse width slightly increased with the increase of the delivered power which might be caused by the self-phase modulation and/or other nonlinear effects [12]. Nonetheless, a pulse width of less than 200 fs could be still achieved even with a 70 mW power delivered through the DCF core. Overall, the PBF worked well for dispersion compensation. In addition to its compactness, a single PBF also greatly simplified the system alignment (compared to the use of a pulse stretcher) and improved the system stability. Furthermore, the PBF helped to reshape the laser beam from the laser [14], thus improving the coupling efficiency from the PBF to the DCF core by almost a factor of 2 compared to the use of a pulse stretcher (i.e. from 20-30% with a pulse stretcher to ~50% with a PBF). The use of a PBF for dispersion compensation works conveniently when the excitation wavelength is fixed to avoid the need for changing the length of the PBF, which is ideal when an extremely compact fs fiber laser is used for excitation. This simple dispersion compensation approach made the entire nonlinear endomicroscopy system compact, reliable and essentially plug-and-play. As shown in Fig. 2(b), the entire endoscope system, including all the fiber-launchers, dispersion unit and the photon detector (except the excitation light source), can be put together within a box of a small footprint (21 × 15 × 7 inch), leaving the flexible scanning endomicroscope probe extending outside the box and freely accessible. The compact size and the plug-and-play feature greatly simplify the operation of the endomicroscope system and facilitate their applications for both laboratory research and future in vivo studies.

For imaging experiments, the laser power delivered to the samples was controlled at no more than 55 mW for intrinsic SHG imaging of tissues and 15 mW for TPF imaging of stained tissues. The SHG and TPF signals were collected back through the same DCF (core and inner clad), separated from the excitation light at the proximal end of the endomicroscope by a dichroic mirror and then directed to a photomultiplier tube (PMT). The residual excitation light was further blocked by a short-pass filter mounted in front of the PMT. Appropriate band-pass
filters were also employed for the detection of either the SHG or TPF signal. The photo current from the PMT was amplified, converted to voltage and digitized. Fluorescent beads were used to test the system performance. Using a phantom made of 0.1-μm fluorescent beads, the lateral and axial resolution (FWHM) was measured by scanning through the center of a single bead along the lateral and axial direction, respectively. The Gaussian fits to the fluorescence intensities shows that the endomicroscopy system has ~1.6 μm lateral resolution and ~11.4 μm axial resolution (See Figs. 4(a) and 4(b), respectively). According to the mode-filed diameter of the DCF (D = 4.1 μm) and the magnification of the endomicroscope (M = 0.5), the theoretical lateral and axial resolution (FWHM) should be 0.85 μm (as predicted by $D \times M \times \sqrt{\ln 2/2}$) and 5.75 μm (as predicted by $D^2 \times M^2 \times \sqrt{2\pi}/4\lambda$ where $\lambda$ is the excitation wavelength), respectively. This discrepancy is probably mainly attributed to the chromatic aberration (and possibly the geometrical aberration) of the GRIN lens, which can be improved with customized achromatic objective lenses.

3. Results and discussions

SHG imaging of rat tail tendons *ex vivo* was performed using the all-fiber-optic endomicroscopy system. Microscopically, the main constituent of tendon is collagen which comprises 80% dry weight of tendon [15]. The tendon collagen fibers are mostly type I collagen, which is the most efficient SHG source in tissue due to the highly noncentrosymmetric structure [16,17]. In this study, tendons were extracted from 5 fresh rat tails and kept in phosphate buffered saline (PBS) during imaging to prevent potential tissue dehydration. All imaging was conducted within two hours after the dissection of the rats. Figures 5(a) and 5(b) show representative real-time (at a frame rate of 3.3-frames/sec) and 10 frames averaged backward SHG images, respectively. The SHG signals were detected by a band-pass filter with a pass-band of 400-420 nm. As displayed by the SHG images, fine tendon structures (i.e. collagen fiber bundles) are clearly identifiable. The collagen fiber bundles are closely packed with a diameter of ~2.8 μm which is consistent with the fiber bundle size (~0.4 - 4.8 μm) reported in literature [18]. The 3D animation in Figure 5(c) shows the layered intrinsic SHG images of rat tail tendon with an axial step of 5 μm controlled by a precision translation stage. Each section represented the average of 10 frames. As shown in the animation, different collagen bundles can be observed at different depths. Moreover, all the collagen fiber bundles at different depths are parallel. Overall, these preliminary *ex vivo* imaging results are consistent with the general structures of tendon. It has been shown that modification of the collagen matrix in biological tissues is associated with various physiologic/pathological processes, such as wound healing, diabetes and cancer [19,20]. Therefore, this compact all-fiber-optic SHG endomicroscopy system can be potentially used for real-time assessment of collagen fiber network morphology under various clinically relevant conditions for diagnosis and treatment monitoring.

Two-photon fluorescence (TPF) imaging of epithelial tissues was also performed using the scanning fiber-optic endomicroscopy system. Figures 6(a) and 7(b) show the typical 10 frames averaged images of a formalin fixed pig corneal limbus (from the tissue surface) and corneal stroma (100 μm below the cornea surface), respectively. The tissue was stained after fixation with 1% acridine orange (AO) in PBS, the dye commonly used in single and two-photon fluorescence imaging for enhancing the nuclei contrast [21, 22]. The single-photon excitation/emission maximum of AO when bound to DNA are at 487/520 nm and the two-photon fluorescence spectrum with 780-900 nm excitation is similar to the single-photon fluorescence spectrum [23]. The densely packed epithelial cells in the limbus (near tissue surface) and sparsely distributed keratocytes in the stroma (100 μm below the tissue surface) can be clearly identified from the images. In addition, the nuclei of epithelial cells and keratocytes exhibit different morphologies, which is consistent with the textbook histology [24]. These images
demonstrate that the system has sufficient resolution to resolve nuclei with a diameter of about 5 μm.

To further demonstrate the potential of the endomicroscopy system for depth-resolved imaging of internal organs, 3D TPF images of fresh rat oral tissues stained with AO were acquired by axially translating the 2D-scanning endoscope with a precision translation stage. Figures 7(a) and 7(b) show representative images of the oral tissue at depths of 10 and 50 μm below the tissue surface, respectively. The movie in Figure 7(c) illustrates a series of en face TPF images of the oral tissue as a function of depth. The depth separation between two adjacent images was 5 μm and the total imaging depth was 120 μm. The epithelial cell nuclei can be identified by the endomicroscopic TPF imaging. As seen from the depth-resolved images, the nucleus density increases from the superficial layer to the basal layer, which is consistent with the well-known histology of stratified squamous epithelium [24]. Considering neoplastic oral mucosa exhibit depth-dependent changes in nuclei packing density and pleomorphism [25], these ex vivo two-photon imaging results strongly suggest a potential role of this system for neoplasia detection by real-time assessment of epithelial structures with cellular and subcellular resolution. Higher resolution to resolve more detailed intracellular structures is also essential and may be achieved by equipping the endomicroscope with a customized lens assembly with a higher NA.

4. Conclusion

In summary, we have developed an extremely compact, all-fiber-optic and plug-and-play scanning endomicroscopy system for nonlinear optical imaging of biological samples. A commercially available double-clad fiber was employed in the endomicroscope for femtosecond pulse delivery, nonlinear optical signals collection and fast beam scanning. A single photonic bandgap fiber was introduced in replacement of a bulky grating/lens-based pulse stretcher for pulse prechirping which significantly reduced the system size and improved the power delivery efficiency through the endomicroscope. Real-time ex vivo 2D and 3D TPF/SHG imaging of biological tissues were performed with the scanning endomicroscope at cellular and subcellular resolution. The preliminary results strongly suggest the promising potential of this all-fiber-optic endomicroscopy technology as a basic laboratory research tool and a clinical tool for real-time assessment of epithelium and collagen fiber network under various clinically relevant conditions. It will be straightforward to combine this promising endomicroscopy technology with targeting fluorescence contrast agents for molecular imaging (ex vivo and in vivo). It is expected that the performance of the all-fiber-optic scanning endomicroscopy system will be further improved to enable imaging of intrinsic tissue fluorophores such as NADH and FAD which are important metabolic indicators and natural biomarkers of epithelial cancer.

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Fig. 1.
Schematic (a) and photo (b) of the distal end of the fiber-optic scanning nonlinear optical endomicroscope probe. The piezoelectric transducer tube (PZT), fiber-optic scanner, and GRIN lens were encased in a hypodermic tube with an overall outer diameter of 2.4 mm.
Fig. 2.
(a) Schematic of the all-fiber-optic scanning nonlinear optical endomicroscope imaging system. The combination of a DCF and PBF made the system all-fiber-optic, in which the DCF was used for both nonlinear optical excitation and TPF/SHG collection, whereas the PBF was employed for dispersion management. FL: Fiber launcher; PBF: Photonic bandgap fiber; M: Mirror; DM: Dichroic mirror; DCF: Double-clad fiber; L: Focusing lens; F: Short-pass filter and/or band-pass filter; PMT: Photomultiplier tube; DAQ: Data acquisition. (b) Photo of the all-fiber-optic scanning nonlinear optical endomicroscope imaging system inside a box with the flexible endomicroscope probe extended outside the box and placed on the cover. The endomicroscope probe can be easily plugged into the system and becomes freely accessible.
Fig. 3.
Second-order intensity autocorrelation curves of laser pulses existing from the endomicroscope with different powers delivered through the core of the DCF. A pulse width of less than 200 fs could be achieved even with a power of 70 mW in the DCF core.
Fig. 4. Fluorescence intensity profiles (dots) across the center of a 0.1-μm fluorescent bead along (a) the lateral and (b) axial dimension. Black traces are Gaussian-fitted curves. The measured lateral and axial resolution given by the FWHM in (a) and (b) are ~1.6 μm and 11.4 μm, respectively.
Fig. 5.
Representative intrinsic SHG images of rat tail tendon: (a) real-time image (3.3 frames/second) and (b) 10 frames averaged image. (c) animation of layered intrinsic SHG images as a function of depth. The SHG signals are solely attributed to type I collagen.
Fig. 6.
Exogenous TPF images of pig cornea tissue stained with acridine orange. (a) corneal limbus and (b) corneal stroma. The densely packed epithelial cells in the limbus and sparsely distributed keratocytes in the stroma can be clearly identified from the images.
Fig. 7.
Typical depth-resolved TPF images of rat oral tissue stained with acridine orange: (a) at the depth of 10 μm; (b) at the depth of 50 μm; and (c) movie of stacked 3D images. The depth-resolved TPF images reveal that the nucleus density increases from the superficial layer to the basal layer.
Table 1
Measured GVD parameter ($\beta_2$) and dispersion parameter (D) of DCF and PBF.

|                | $\beta_2$ (fs$^2$/m) | D (ps/nm/km) |
|----------------|----------------------|--------------|
| DCF (805 ± 4.5 nm) | 45,127               | -131.2       |
| DCF (810 ± 18 nm)  | 43,065               | -123.7       |
| PBF (805 ± 4.5 nm) | -14,622              | 42.5         |
| PBF (810 ± 18 nm)  | -35,246              | 101.2        |
| SMF (810 nm) *     | 34,120               | -98.0        |

For reference, the $\beta_2$ and D values of an SMF are cited from Ref. [12].