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Integrated multimodal endomicroscopy platform for simultaneous *en face* optical coherence and two-photon fluorescence imaging

Jiefeng Xi¹, Yongping Chen¹, Yuying Zhang¹, Kartikeya Murari¹, Ming-Jun Li^{1,2}, and Xingde Li^{1,*}

¹Department of Biomedical Engineering, Johns Hopkins University, Baltimore, Maryland 21210, USA

²Science and Technology, Corning Incorporated, Corning, New York 14831, USA

Abstract

We report an all-fiber-optic scanning, multimodal endomicroscope capable of simultaneous optical coherence tomography (OCT) and two-photon fluorescence (TPF) imaging. Both imaging modalities share the same miniature fiber-optic scanning endomicroscope, which consists of a double-clad fiber with a core operating in single mode at both the OCT (1310 nm) and two-photon excitation (1550 nm) wavelengths, a piezoelectric two-dimensional fiber-optic beam scanner, and a miniature aspherical compound lens suitable for simultaneous acquisition of *en face* OCT and TPF images. A fiber-optic wavelength division multiplexer was employed in the integrated platform to combine the low coherence OCT light source and the femtosecond two-photon excitation laser into the same optical path. Preliminary imaging results of cell cultures and mouse tissue *ex vivo* demonstrate the feasibility of simultaneous real-time OCT and TPF imaging in a scanning endomicroscopy setting for the first time.

Optical coherence tomography (OCT) and two-photon fluorescence (TPF) microscopy are recently developed high-resolution optical imaging modalities that hold strong promise for performing noninvasive “optical biop-sies” of biological tissues at a resolution approaching that of standard histology without the need for tissue removal. OCT is capable of visualizing micrometer-scale tissue structural morphologies with the imaging contrast predominantly sensitive to the intrinsic tissue scattering [1]. In contrast, TPF provides depth-resolved submicrometer-scale images with the imaging contrast coming from endogenous or exogenous fluorophores, thus providing molecular or biochemical information about biological tissues that cannot be obtained by OCT [2]. The two complementary imaging modalities provide important yet different optical information based on unique contrast mechanisms. There is, hence, a strong motivation for developing an integrated platform for performing both OCT and TPF imaging. Previous works have demonstrated the possibility of combining the two imaging techniques using a benchtop scanning microscope platform [3-5], which involves free-space optics and is generally bulky. *In vivo* applications and potential clinical translation of the OCT-TPF dual-modality imaging technology, particularly for imaging internal organs, requires a flexible and compact platform. This Letter reports the development of such a compact dual-modality imaging platform, consisting of a miniature endomicroscope and small footprint fiber laser sources.

Previously, we successfully developed a forward-viewing piezoelectrically actuated scanning OCT and TPF endoscopes at various wavelengths (e.g., 800, 1310, and 1550 nm) [6-11]. In this Letter we present an all-fiber-optic, multimodal endomicroscope that integrates both 1310 nm *en face* OCT and 1550 nm TPF imaging with the same miniature fiber-optic probe (see Fig. 1). The advantages of choosing these two wavelengths for this integrated multimodal imaging platform include: (1) compact fiber-optic light sources and components are widely available at both wavelengths, (2) both wavelengths can be delivered in the same fibers [i.e., SMF-28e or a customized double-clad fiber (DCF)], and (3) the single-mode fiber SMF-28e and the customized DCF show similar propagation and dispersion characteristics at both wavelengths.

The multimodal system consists of two modules: 1550 nm TPF and 1310 nm *en face* OCT endomicroscopy systems. In the TPF module, a 1550 nm passive mode-locked amplified fiber laser generates ultrashort laser pulses (i.e., ~300 fs with a repetition rate of 42.5 MHz) with a maximum average power of ~155 mW in soliton mode, as previously described in [11], so that the laser pulse remains relatively unchanged inside either the single-mode fiber (SMF-28e) or the customized DCF. Therefore, the TPF system does not require any further dispersion compensation [11]. Furthermore, reduced scattering at the near-infrared (NIR) two-photon excitation and emission wavelengths potentially improves the imaging penetration depth. In the OCT module, the light generated by a fiber-coupled superluminescent diode serves as a compact light source, with a 13 mW output power and a central wavelength of 1300 nm with a 3 dB bandwidth of 80 nm, and is delivered into a Michelson interferometer of the OCT system. A high-isolation wavelength division multiplexer (WDM) made of SMF-28e is employed to combine the 1310 nm OCT light source with the 1550 nm TPF excitation laser, which significantly simplifies the procedure of integrating the two imaging modalities.

One of the key components in the system is a miniature endoscope that employed the customized DCF. The core diameter of the DCF is ~8 μm , similar to that of SMF-28e, ensuring that both 1550 nm TPF excitation light and 1310 nm OCT light can be delivered in single mode through the core. The DCF has a large inner cladding ($\phi 175 \mu\text{m}$) suitable for effective collection of the TPF signal. The NAs of the DCF core and inner cladding are 0.14/0.12 (at 1310 nm/1550 nm) and 0.267 (at 1550 nm), respectively. At the proximal (entry) end of the endoscope, the OCT source and TPF excitation light are coupled into the DCF core through a pair of aspherical lenses (i.e., the two CLs in Fig. 1). The distal end of the endoscope design is similar to what was published previously [6,8]. In essence, the DCF is attached to a tubular piezoelectric (PZT) actuator of a 2.0 mm diameter, with an ~10-mm-long fiber cantilever standing outside the PZT tube, forming a resonant fiber scanner once the PZT is actuated at the mechanical resonance frequency of the DCF cantilever (i.e., ~1.5 kHz in our case). The overall diameter of the endoscope is about 2.8 mm, including the protective metal tubing. The sweeping DCF tip is imaged to the sample by a miniature aspherical compound lens with a maximum NA of 0.8 and a magnification of ~0.22 (along the direction of fiber to sample) [11]. The microcompound lens offers a minimal chromatic focal shift from the OCT wavelength (1310 nm) to the TPF excitation wavelength (1550 nm), which is ~10 μm by ray tracing and ~11 μm by experiment. It is recognized that the TPF collection efficiency is suboptimal due to the estimated 670 μm focal shift between TPF excitation and fluorescence light.

On the collection (i.e., return) path, to separate the TPF and the OCT signal, a customized dichroic mirror (i.e., DM in Fig. 1), with transmission efficiency >90% for 1550 nm and >70% for 1310 nm and reflectivity >99.97% for 700–900 nm, is placed between the pair of coupling lenses. A photomultiplier tube (PMT) is used to collect the TPF signal reflected from the dichroic mirror. For OCT detection, although some of the backscattered OCT light

from the sample enters the inner cladding of the DCF in the endoscope, the SMF-28e in the sample arm of the OCT module (e.g., the WDM) can filter it out to avoid “ghost” OCT images. To perform optical heterodyne detection, an electro-optic modulator is inserted into the reference arm of the OCT module to introduce a Doppler frequency. A rapid scanning optical delay line (RSOD) is used to compensate the dispersion mismatch between the two OCT arms and to select or scan the imaging depth, as reported previously [12]. A balanced detection scheme is used in the OCT module to eliminate any DC components and increase the detection dynamic range.

It is noted that OCT and TPF imaging share the same scanning endoscope but have two independent detection paths; thus, the two imaging modalities can run simultaneously with the same field of view and imaging speed. Different from a typical benchtop microscope system, the dual-modality endomicroscope system is drawn upon fiber optics, except for a short-distance free space to place the dichroic mirror and separate the OCT and fluorescence signals. The overall system is, thus, very compact and easy to use, which is critical for future *in vivo* and clinical applications.

Simultaneous TPF and OCT imaging was performed on cell culture and biological tissue (*ex vivo*) to test the performance of the multimodal endomicroscopic imaging platform. The resolutions were $\sim 2.5 \times 10.0 \mu\text{m}$ (lateral $\times$ axial) in air for OCT and $\sim 1.2 \mu\text{m} \times 5.7 \mu\text{m}$ (lateral $\times$ axial) for TPF imaging. Imaging was performed through a cover glass placed on the sample and by a dry microobjective lens of a working distance of $200 \mu\text{m}$. The powers incident on the sample were ~ 30 to 50 mW (at 1550 nm for TPF) and $\sim 4.0 \text{ mW}$ (at 1310 nm for OCT). The sweeping range of the DCF fiber tip was either ~ 450 or $\sim 675 \mu\text{m}$ in this study, which resulted in an ~ 100 or $150 \mu\text{m}$ field of view on the sample after the microcompound lens. With a spiral scanning pattern, a frame rate of ~ 3.0 frames/s was achieved, with each frame consisting of 512 spirals. The fluorescence dye used for TPF imaging was Indocyanine Green (ICG), or ICG nanocapsules, which has an NIR emission peak around $\sim 810 \text{ nm}$. Details on cell culture and tissue preparation were described elsewhere [11,13].

A431 cancer cells were incubated on a coverslip with anti-epidermal-growth-factor-receptor (EGFR) conjugated ICG micelles so that the cell membranes were immunostained with the ICG micelles [13,14]. Figures 2(a) and (b) show one set of representative OCT and TPF images of the same cell culture sample acquired simultaneously with the multimodal imaging platform. The superimposed OCT and TPF image is shown in Fig. 2(c), where the whole cell topology can be easily identified with OCT while the cell membrane is enhanced under TPF imaging. For thin, flat, and relatively transparent cell samples cultured on a coverslip, artifacts such as the ring pattern in Fig. 2(a) became visible, which could be manifested from any phase perturbation in the system, e.g., caused by the periodic instability in the galvanometer mirror in the reference arm.

For tissue imaging, different samples from a nude mouse were harvested 15–x20 min after local administration of $50 \mu\text{l}$ of $10 \mu\text{M}$ ICG solution. Figures 3(a) and (b) show representative OCT and TPF images taken simultaneously from adipose tissue. The adipocytes were clearly visualized under OCT with low reflectance from the large lipid droplets within the adipocytes (indicated by red arrows). In comparison, the locally administrated ICG was found mainly diffused among the adipocytes, as shown on the TPF image. The merged image from the two modalities is shown in Fig. 3(c), and the OCT and TPF images overlap well, particularly around the cell membranes. In addition to cell culture imaging, simultaneous OCT and TPF imaging was also performed on tissue samples from the small intestine, and the representative OCT and TPF images are shown in Figs. 3(d) and (e), respectively. The circular structures on the OCT image may represent the intestinal villi

(indicated by blue right-downward arrows) with the lacteals (indicated by red left-downward arrows) shown as the areas of lower backscattering on the OCT image. Similar structures were visualized on the TPF image, as well. The brighter fluorescent spots (indicated by lighter yellow arrows) on the villi may suggest that either the enterocytes or the lymphocytes actively absorbed the ICG molecules. The superposed image is shown in Fig. 3(f) and the nice overlapping features suggest that the endoscopic multimodal imaging platform is able to produce well-correlated images even with highly scattering tissues.

In summary, we demonstrated an all-fiber-optic scanning endomicroscopy system that enabled simultaneous *en face* OCT and TPF imaging on a compact endoscopic setting. To the best of our knowledge, this is the first endoscopy platform that fully integrates OCT (1310 nm) and TPF (1550 nm) imaging with a small footprint. *in vitro* cell culture and *ex vivo* tissue imaging experiments demonstrated the feasibility of the multimodal endoscopy system for simultaneously acquiring tissue morphological (by OCT) and molecular information (by TPF) using the integrated fiber-optic imaging platform. The small footprint, complementary contrast, and real-time imaging capability of the multimodal imaging platform hold potential for translating to *in vivo* and clinical applications.

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Fig. 1.

(Color online) Schematic of TPF/OCT multimodal endomicroscope system. BD, balanced detector; C, circulator; CL, coupling lens; DCF, double-clad fiber; DM, dichroic mirror; ENDO, miniature endoscope; EOM, electro-optic modulator; FC, fiber coupler; LP, long pass filter; M, mirror; PMT, photo-multiplier tube; RSOD, rapid scanning optical delay line; WDM, wavelength division multiplexer.

Fig. 2.
(Color online) (a) OCT and (b) TPF images of A431 cancer cells immunostained with anti-EGFR conjugated ICG micelles. (c) Superposition of the OCT and TPF images.

Fig. 3.

(Color online) (a) OCT, (b) TPF, and (c) superposed images of mouse adipose tissue with local ICG administration. Each red arrow in (a) and (b) indicates one of the adipocytes visualized under both imaging modalities. (d) OCT, (e) TPF, and (f) superposed images of mouse small intestine tissue with local ICG administration. Blue arrows (right downward) shown in (d) and (e) indicate villus structures and red arrows (left downward) indicate lacteals. The stronger fluorescence dots indicated by yellow arrows at center shown in (e) may be either enterocytes or lymphocytes. Both sets of images show great correlation between two imaging modalities.