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MULTIPHOTON MICROSCOPIC IMAGING OF MOUSE INTESTINAL MUCOSA BASED ON TWO-PHOTON EXCITED FLUORESCENCE AND SECOND HARMONIC GENERATION

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Multiphoton microscopy (MPM), based on two-photon excited fluorescence and second harmonic generation, enables direct noninvasive visualization of tissue architecture and cell morphology in live tissues without the administration of exogenous contrast agents. In this paper, we used MPM to image the microstructures of the mucosa in fresh, unfixed, and unstained intestinal tissue of mouse. The morphology and distribution of the main components in mucosa layer such as columnar cells, goblet cells, intestinal glands, and a little collagen fibers were clearly observed in MPM images, and then compared with standard H&E images from paired specimens. Our results indicate that MPM combined with endoscopy and miniaturization probes has the potential application in the clinical diagnosis and *in vivo* monitoring of early intestinal cancer.

Keywords: Multiphoton microscopy; second harmonic generation; two-photon excited fluorescence; mucosa; intestine; colonic; duodenal.

1. Introduction

Intestinal cancer is one of the leading worldwide cancers with high mortality, including colorectal cancer and small intestine cancer. Colorectal cancer is the third most commonly diagnosed cancer, with over 1.2 million new cancer cases and 608,700

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deaths estimated to have occurred in 2008.[1] Compared to colorectal cancer, small intestine cancer is relatively rare, but the survival rate is very low, especially for duodenal adenocarcinomas.[2,3] It has been reported that the overall median survival of patients with duodenal adenocarcinomas is only 18 months and the 5-year survival is 23%.[4] Early diagnosis of intestinal cancer is the key to increase cure rates and lower mortality. But the procedure of endoscopic biopsy, which remains the standard practice for the pathological diagnosis of intestinal cancer, results in bleeding and is time-consuming. And the available imaging modalities, such as ultrasonic endoscopy and magnetic resonance endoscopy, lack the sensitivity and specificity to the early diagnosis in the intestinal cancer.[5,6]

Recently, multiphoton microscopy (MPM) uses ultrafast, near-infrared laser as excitation sources and bases on nonlinear optical signals of intrinsic fluorophores in tissues including two photon-excited fluorescence (TPEF) and second harmonic generation (SHG). It can provide enhanced imaging penetration depth in scattering samples and reduced overall specimen photodamage, photobleaching and phototoxicity.[7] MPM has become a valuable optical technique to be used in biology and medicine area, showing high resolution and high sensitivity in cellular and subcellular imaging of tissue.[8−11] With the advent of the multiphoton-based endoscopy, the application of MPM *in vivo* digestive tract will attract attention.[12−14] TPEF is well suited for high-resolution imaging of intrinsic molecular signals in cells, such as nicotinamide adenine dinucleotide hydrogen (NADH) and flavins, and SHG enables direct imaging of anisotropic biological structures, such as collagen.[15] Owing to the ability to generate images based on the natural intrinsic fluorescence properties of tissue, MPM has been used in imaging of intestinal mucosa for potential diagnosis of intestinal cancer.[14,16] In this paper, we try to use MPM based on SHG and TPEF to characterize the microstructure of mucosa in the duodenal and colonic tissue and want to see whether this technology has the ability to show the main components in this layer.

2. Materials and Methods

2.1. *Sample preparation*

The intestines of experimental mouse were examined in this study. Six fresh specimens were excised from six living healthy experimental mice with eight months old, provided by the Animal Center of Fujian Medical University. The regions of interest are the normal duodenum and colon. We chose two close sections of the intestine. One was opened longitudinally and sandwiched between the microscope slide and a piece of cover glass with the topmost mucosa layer facing the objective. The other was set to make paraffin section and H&E staining. Moreover, to avoid dehydration or shrinkage during the SHG and TPEF imaging process, the specimen was sprinkled with PBS solution (pH = 7.4).

2.2. *Imaging system*

The multiphoton microscopic imaging system used in this study has been described previously.[17] The system is an Axiovert 200 microscope (Zeiss LSM 510 META) equipped with a mode-locked femtosecond Ti: sapphire laser (110 fs, 76 MHz), operated at 810 nm (Coherent Mira 900-F). A Plan-Apochromat $40\times$ (N.A. = 0.75) objective (Zeiss) was employed to focus the excitation beam and collect the backward signals. The META detector has eight detecting channels. Two independent channels from the eight channels were used. One channel (398−419 nm) was used to detect SHG signal of collagen. The other channel (430−708 nm) was employed to detect TPEF signal of cells in the tissue of intestinal mucosa. Moreover, the highest power is approximately 25 mW, and no photobleaching and photodamage were observed in a series of multiphoton imaging for the mouse intestinal mucosa. An optional HRZ 200 fine focusing stage (Carl Zeiss) was used to move the motorized x-y scanning stage for obtaining a large-area image and change the focus position for recording various optical sections.

3. Results and Discussion

3.1. *Multiphoton images of duodenal mucosa*

Combining TPEF and SHG, high contrast and high resolution large-area MPM images ($691.02\times 691.02\,\mu$m) from the duodenal mucosa are obtained, as shown in Fig. 1. Figure 1(a) shows the collagen with mesh morphology in mucosa layer, imaged alone by one channel (red color-coded). Figure 1(b) shows the cells in the duodenal mucosa, clearly visualized via the other channel (green color-coded).

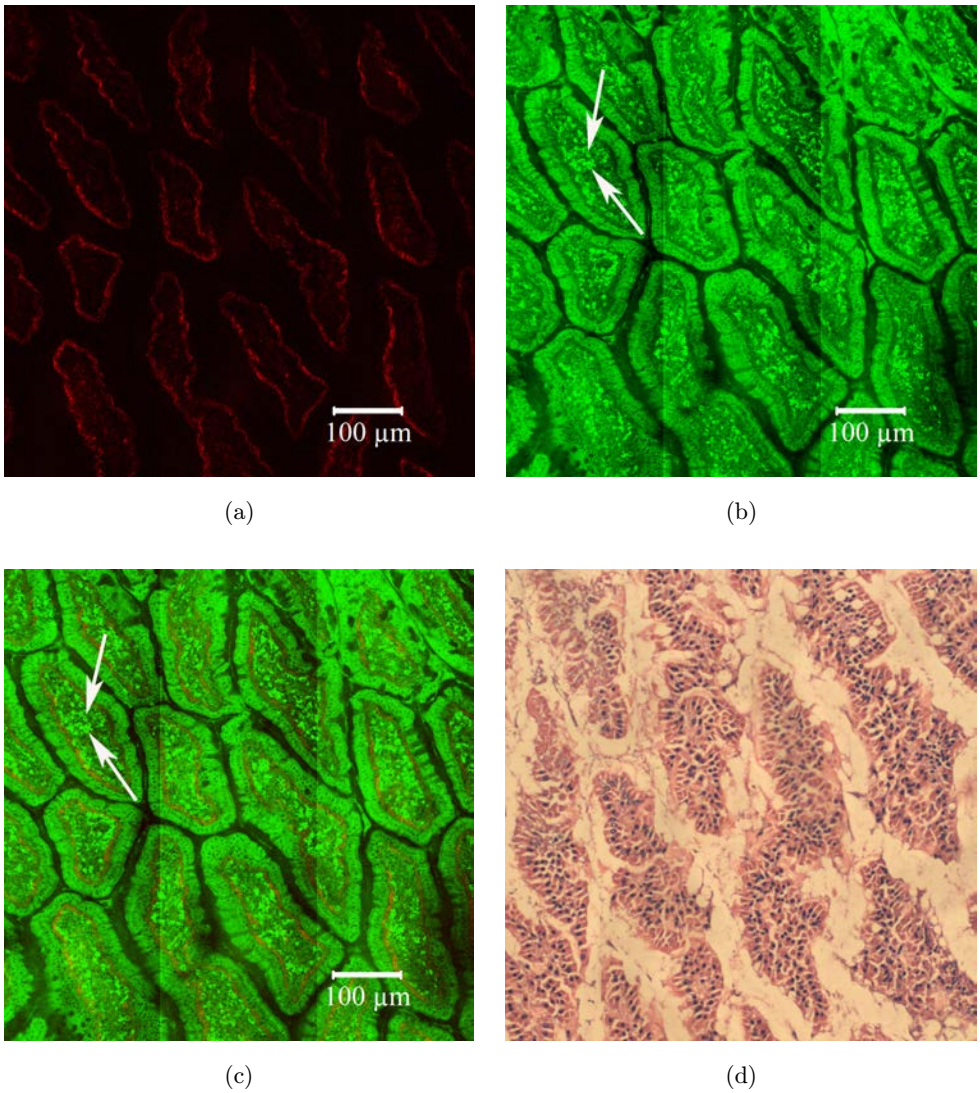
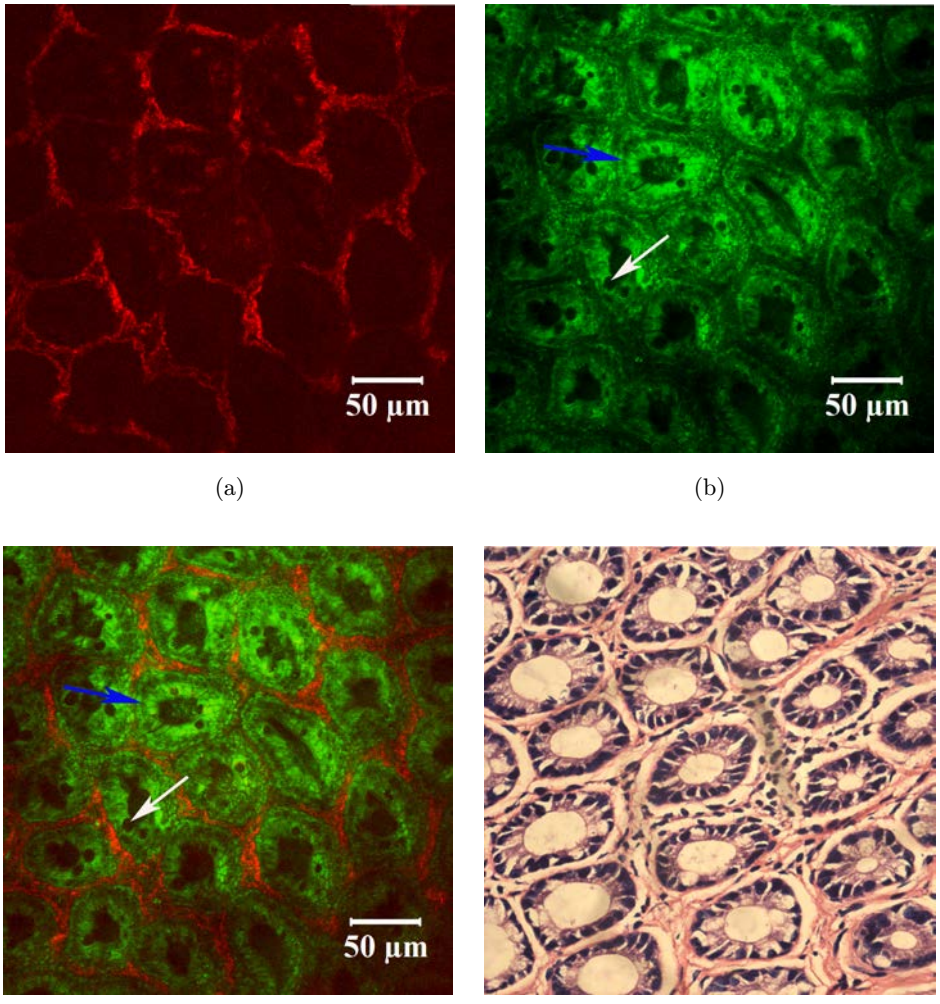


Fig. 1. Images of duodenal mucosa. (a) SHG image of collagen, (b) TPEF image of cells, (c) overlayed SHG/TPEF image, and (d) the corresponding H&E image (20×) showing a similar arrangement compared to the overlayed SHG/TPEF image. Scale bar is 100 μm. The white arrows indicate the lymphocytes or plasma cells.

Overlaying the two channels, a high-contrast TPEF/SHG image of duodenal mucosa was obtained as shown in Fig. 1(c). It can be readily observed from the overlayed image that the basement membrane containing collagen surrounded by the columnar epithelial cells. And the columnar epithelium is arranged to form a series of villi punctuated by occasional goblet cells characterized by absent fluorescence of cellular nuclei and mucus. The signals encompassed by the basement membrane represent the lymphocytes or plasma cells [white arrowheads in Figs. 1(b) and 1(c)] whose nonfluorescent nuclei live on the side of the cells. It is well consistent with the outcome of the corresponding H&E image of the duodenal mucosa in Fig. 1(d), showing the intestinal villous lined by a single layer of enterocytes with basally located nuclei and interspersed goblet cells with apically located clear mucous. The results show that MPM could demonstrate microstructures of this layer, which were comparable to the H&E stained image. As the carcinoma *in situ* penetrates through the basement membrane, it may become invasive carcinoma, which is the most common form of a cancer.[18] It will result in the microstructural alteration of basement membrane. Hence, the microscopic imaging of the mucosa layer in the duodenal tissue may further develop a new method for histological evaluation.

3.2. *Multiphoton images of colonic mucosa*

Figure 2 displays the large-area MPM images ($325.75 \times 325.75\,\mu$m) compared to H&E image of colonic mucosa. As can be seen from Fig. 2(a), a little collagen with mesh morphology in mucosa layer could be imaged alone by one channel (red color-coded). In Fig. 2(b), the mucous cells, columnar cells and goblet cells in a large number of intestinal glands could also be isolated from collagen using another channel (green color-coded). Overlaying the two channels yields a high-contrast TPEF/SHG image of mouse colon in Fig. 2(c), where the structural details of the sample can be distinguished. The overlayed image reveals regular tissue architecture and cell morphology, including a typical foveolar pattern with central, round crypt openings, and glands lined by columnar cells [blue arrowheads in Figs. 2(b) and 2(c)], and sporadic mucous cells [white arrowheads in Figs. 2(b) and 2(c)]. In addition, the intercellular space between individual cells was readily discerned, and the morphology of individual nuclei could be observed as well. Moreover, the glands are clearly surrounded with the connective tissues and the collagen fibers circles are quite distinctly observed. These features are well consistent with the H&E stained image in Fig. 2(d). Modifications of the collagen matrix structure, the circular crypts framework, and the cellular junctions are associated with various intestinal diseases, such as precancer and cancer.[16,19–21] Hence, the MPM



(a) (b)

(c) (d)

Fig. 2. Images of colomic mucosa. (a) SHG image of collagen, (b) TPEF image of cells, (c) overlayed SHG/TPEF image, and (d) the corresponding H&E image (40×) showing a similar arrangement compared to the overlayed SHG/TPEF image. Scale bar is $50\,\mu$m. The blue and white arrows indicate the columnar and mucous cells, respectively.

imaging of the mucosa layer in the intestine may further develop a new method for histological evaluation. In the next work, we will do further research in the different disease of the intestine base on SHG and TPEF imaging.

4. Conclusion

In summary, we demonstrated that MPM can clearly display the microstructure of the mouse intestinal mucosa. Our results show the potential of MPM as a powerful noninvasive technique to characterize intestine physiology and pathology. With the advent of the multiphoton endoscopy, MPM may be used for the *in vivo* diagnosis and monitoring of the status of intestinal cancer in the future.

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