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ANATOMICAL PATHOLOGY

Non-linear optical imaging and quantitative analysis of the pathological changes in normal and carcinomatous human colorectal muscularis

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Summary

Non-linear optical (NLO) imaging based on two-photon excitation (2PE) and second harmonic generation (SHG) has been widely used to image microstructures of biomedical specimens over the last two decades. We employed NLO imaging technology to investigate the histology of normal and carcinomatous human colorectal muscularis in transverse and longitudinal views. Results show there are different patterns of pathological changes of muscularis in tissue structure and cell morphology from both views. The NLO imaging provides identical histological information as the H&E images but requires neither stain nor tissue processing. Our study indicates that NLO imaging technology shows more detailed microstructure, which is a critical complementary tool in pathological diagnosis of colorectal tumours. It suggests that NLO imaging could be a very important diagnostic tool to help pathologists realise the real time early detection of human colorectal tumours in the foreseeable future.

Key words: Non-linear optical imaging; NLO imaging; two-photon excitation; 2PE; second harmonic generation; SHG; colorectal tissue; smooth muscle.

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INTRODUCTION

Colorectal cancer is the malignant tumour developed from human colon or rectum. Colorectal cancer has a 5-year survival rate of about 65%, however, this depends on how advanced the cancer is, whether or not all the cancer can be removed with surgery, and the patient's overall health. Globally, colorectal cancer is the third most common type of cancer making up about 10% of all cases. In 2012, there were 1.4 million new cases and 694,000 deaths from this disease. It is more common in developed countries, where more than 65% of cases are found. It is less common in women than men.[1]

The smooth muscle is a type of connective tissue in many organs of the human body, usually adjacent to submucosa. When tumour cells infiltrate into or beyond the muscularis propria, it is called advanced carcinoma (staging at T2) bearing a much worse prognosis, with a 5-year survival rate of 60% or less[2] Surgery is the primary treatment for colorectal cancer, and the procedures including total colectomy, subtotal colectomy, partial colectomy, and endoscopic mucosal or submucosal dissection depend on staging[3] Although the accurate pre-operative staging information is generally obtained using endoscopic ultrasonography and computer assisted tomography, oncologists still rely more on histopathology which provides structure information at cellular level. Visible suspicious regions identified with colonoscopy are targeted for biopsy or to be removed endoscopically. There are some unavoidable disadvantages caused by multiple biopsies or endoscopic interventions, such as bleeding and infection, time-consuming tissue processing, and non-representative biopsies lead to an underestimation of the diagnosis[4] Therefore, the capability to perceive cellular and subcellular details with real-time histological endoscopy is one of the major goals in diagnosis and treatment of colorectal cancer.

Non-linear optical (NLO) imaging technology, including two-photon excitation (2PE) and second harmonic generation (SHG), is an emerging and promising new optical imaging method for the early detection and diagnosis of human tumours in real time[5–7] NLO imaging technology is based on processes such as 2PE and SHG to acquire high resolution images of biomedical specimens at cellular and subcellular levels[8,9] It generates cellular images of human tissue with intrinsic fluorophores, which include reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotides (FAD). The elastic fibre has strong 2PE emissions, while collagen usually generates intense SHG signals[10,11] NLO imaging has some advantages over other imaging modalities, including label-free, deeper penetration, lower photo-toxicity, inherent three-dimensional resolution, and the capability of providing quantitative information[12,13] Previous studies have paid most attention to imaging the colorectal mucosa and submucosa[14] while rarely focused on the colorectal muscularis.

In this study, NLO imaging was employed to examine the pathological changes in microstructure of normal and carcinomatous human colorectal muscularis transversely and longitudinally to evaluate the capability of NLO imaging to discriminate the histological difference between normal and carcinomatous human colorectal smooth muscle tissue.

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Table 1 Patient demographics and tumour characteristics (colorectal carcinoma, $n = 30$)

Patient variables	
Age, years, median (range)	62 (49–72)
Gender, male / female	17 / 13
Body mass index, kg/m2, median (range)	26 (21–30)
Tumour size, cm, median (range)	5 (1–9)
Tumour location, right colon / left colon / rectum	5 / 12 / 13
Tumour differentiation, well / moderate / poor	7 / 13 / 10
AJCC/UICC staging (I / II / III / IV)	3 / 11 / 15 / 1

MATERIALS AND METHODS

Sample preparation

The normal and carcinomatous specimens with muscularis of human colorectal tissue were provided by Beijing 301 Hospital, China. Written consents were obtained from patients prior to participation. The specimens included 30 pairs of normal and carcinomatous *ex vivo* human colorectal tissue originating from 30 patients with carcinomas invading into the muscularis which underwent radical resection. Patient demographics and cancer characteristics are summarised in Table 1. The normal tissue was collected 6 cm away from the cancer margin. The size of tissue specimens varied from 3 to 10 mm. After being removed surgically, the specimens were placed in a standard pathological transport container covered with ice and then sent to the histology laboratory. The colorectal specimens were cut with a cryotome into two portions, one perpendicular and another parallel to the axial direction of the smooth muscle layer, so they comprised complete transverse and longitudinal cross-sections of the colorectal muscularis. To better compare the NLO images to the correlated haematoxylin and eosin (H&E) images, two out of three consecutive sections (10 μm in thickness) were prepared for NLO imaging while the other section (5 μm in thickness) was processed for H&E histology. To prevent dehydration while imaging, the specimens were sprinkled with PBS solution (pH 7.4) to keep certain moisture. The fresh tissue sections were placed directly on microscope slides for imaging.

Image acquisition

The NLO imaging system was based on an inverted optical microscope (A1 Observer; Carl Zeiss Microscopy, Germany) equipped with a mode-locked femtosecond Ti: Sapphire laser (110fs, 76 MHz, 1.0W) tunable from 700 nm to 980 nm (Mira 900F; Coherent, USA). An oil immersion objective lens (Plan-Apo 63×/1.40; Carl Zeiss Microscopy) was chosen to focus the excitation beam on the sample and to collect the 180° backscattered intrinsic emissions. The spectrally-resolved META detector with a high-quality, reflective grating and an optimised array of 32-channel PMTs was employed in our study. The 810 nm laser was used for excitation. The high-contrast 2PE/SHG images were collected simultaneously with two independent channels: one (387–419 nm) to collect SHG signals (colour-coded in green) from collagen (intramuscular septa), and another (430–716 nm) to collect 2PE signals (colour-coded in red) from elastic fibres. An IR beam block filter (KP650; Carl Zeiss Microscopy) was placed in front of the META detector to block the excitation wavelength. The acquisition time for a 256 × 256 image was 1.57 s with a pixel time of 2.56 μs and the acquired image was averaged 4 times to improve the signal-to-noise ratio. All images were acquired in 8-bit depth[15] The H&E histological images were acquired with a CCD camera on a bright field microscope (Eclipse E400; Nikon

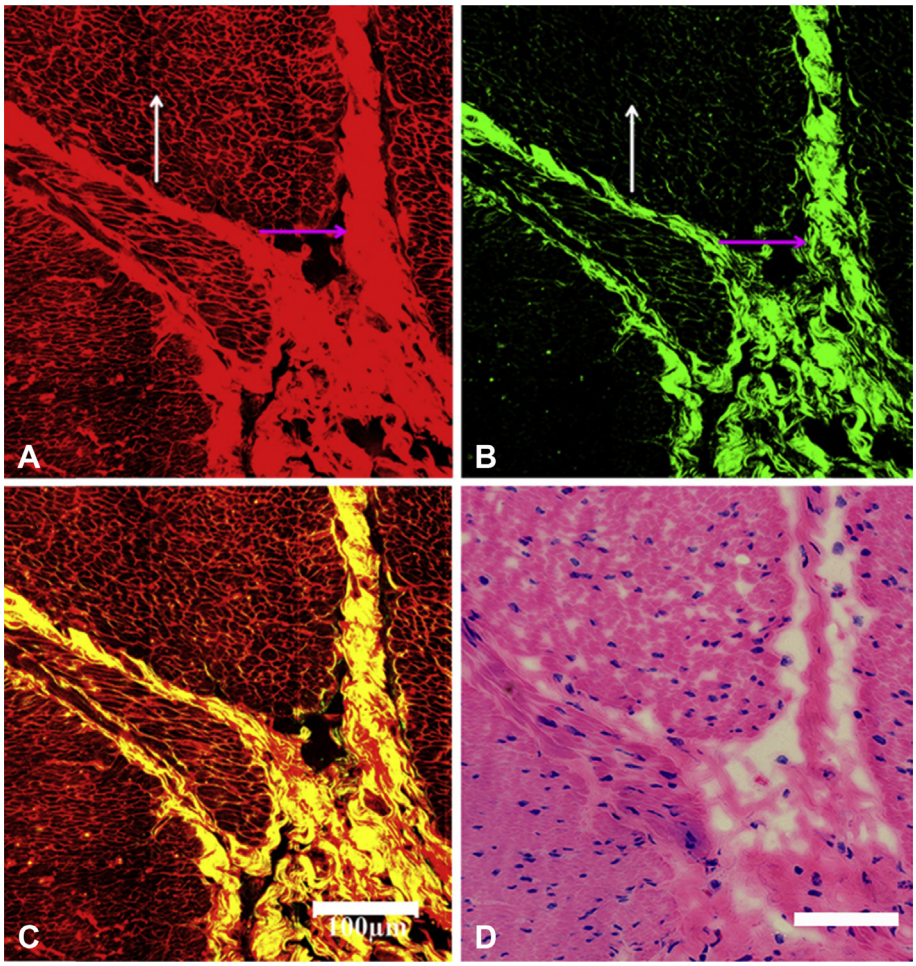

Fig. 1 NLO images and correlated H&E image of the transverse section of normal human colorectal smooth muscle. (A) 2PE (red), (B) SHG (green), (C) overlaid SHG/2PE, and (D) H&E. The pink and white arrows indicate collagen bundles and intramuscular septa, respectively.

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