Steady state and time-resolved autofluorescence studies of human colonic tissues

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Steady state and time-resolved autofluorescence spectroscopies are employed to study the autofluorescence characteristics of human colonic tissues *in vitro*. The excitation wavelength varies from 260 to 540 nm, and the corresponding fluorescence emission spectra are acquired from 280 to 800 nm. Significant difference in fluorescence intensity of excitation-emission matrices (EEMs) is observed between normal and tumor colonic tissues. Compared with normal colonic tissue, low nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) and flavin adenine dinucleotide (FAD), and high amino acids and protoporphyrin IX (PpIX) fluorescences characterize high-grade malignant tissue. Moreover, the autofluorescence lifetimes of normal and carcinomatous colonic tissues at 635 nm under 397-nm excitation are about 4.32 ± 0.12 and 18.45 ± 0.05 ns, respectively. The high accumulation of endogenous PpIX in colonic cancers is demonstrated in both steady state and time-resolved autofluorescence spectroscopies.

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Changes in the morphological and biochemical properties of cells and tissues, related to physiological state or induced by the occurrence of pathological processes, result in modifications of the concentration and distribution of endogenous fluorophores and the chemical-physical properties of their microenvironment. Therefore, analytical techniques based on laser-induced autofluorescence monitoring can be used to obtain information about morphological and physiological states of cells and tissues[1,2]. Moreover, optical biopsy with autofluorescence spectroscopy can be performed in real time without removing the tissue and without the need for fixing or staining of the specimens, providing improved diagnosis of pre-cancerous lesions. Autofluorescence alone has been applied to cancers for many organs of the human body, including the nasopharyngeal carcinoma, lung, oral cavity, esophagus larynx, stomach colon, cervix, bladder, breast, liver, skin, and brain[3−5]. Detection of colonic cancer is particularly of interest because it is one of the most frequently occurring cancers and also due to the failure to diagnose neoplastic lesions at an early stage[6−9]. In this letter, steady state and time-resolved autofluorescence spectra are simultaneously utilized to explore the autofluorescence characteristics of human normal and carcinomatous colonic tissues *in vitro*.

Adenomatous colonic tissue samples ($n = 7$) were obtained from six patients during surgery for malignant tumors of the colon, and normal tissue samples ($n = 5$, matched controls) were also collected from the same patient. Each tissue sample was cut into two specimens. One specimen was sent to the pathologist for standard histopathological evaluation, the other unstained and unfixed sample was placed on a non-fluorescence quartz substrate for laser-induced autofluorescence within 1—3 h of resection.

Laser-induced autofluorescence excitation-emission matrices (EEMs) were performed with a FLS920 spectrofluorimeter (Edinburgh Instruments, UK). In order to minimize the detection of the backscattered excitation light, the wavelength selected excitation light illuminated onto the sample with a 60° incident angle and the fluorescence emission was collected at 45° with respect to the normal quartz substrate. The slit sizes of excitation and emission monochromators were 1.5 and 0.5 mm, respectively. The wavelength dependence of the excitation power and the detector response was corrected and calibrated for all emission spectra. Fluorescence EEMs were recorded by measuring the fluorescence emission spectra over a range of excitation wavelengths. The excitation wavelengths varied from 260 to 540 nm in 20-nm increment. At each excitation wavelength, the fluorescence emission spectra were recorded from 280 to 800 nm at 5-nm interval. These wavelength ranges enabled characterization of endogenous fluorophores, such as amino acids, structural proteins, enzymes and co-enzymes, vitamins, lipids, and porphyrins present in tissues in the ultraviolet, visible, near infrared (UV/VIS/NIR) spectra range[1,2,10]. Fluorescence data from each single measurement were then assembled into EEM containing fluorescence intensity as a function of both excitation and emission wavelengths, which resulted in a total recording time of about 25 min for each EEM.

No distinct difference was observed in the autofluorescence spectra of normal and cancerous tissues. However, remarkable differences in fluorescence peaks and intensities were detected between normal and adenomatous tissues from the same patient. The typical EEMs for the normal colonic tissue and adenomatous colonic tissue are shown in Figs. 1 and 2, respectively. The gray scales represent different fluorescence intensities. The plots are shown on a log contour scale, where each contour connects points of equal fluorescence intensity.

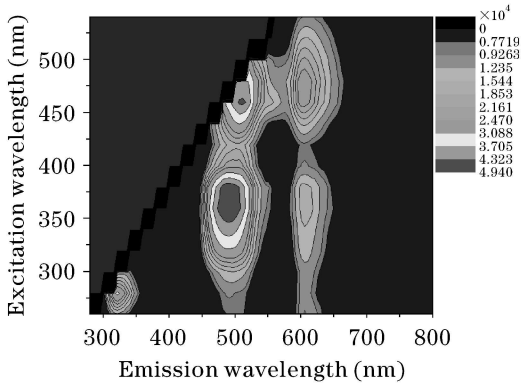

Fig. 1. Autofluorescence EEM of normal colonic tissue.

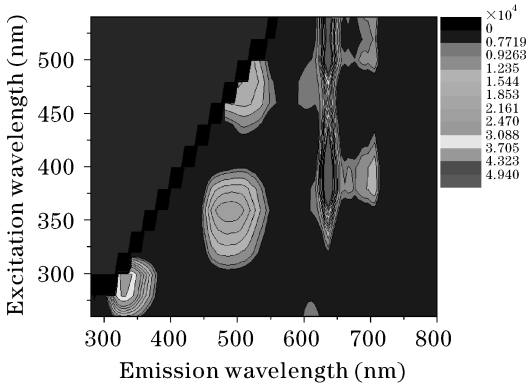

Fig. 2. Autofluorescence EEM of cancerous colonic tissue.

Although the fluorescence intensities are shown in arbitrary units, the same experimental setups were maintained throughout our measurements for comparison.

The primary fluorescence peaks of normal colonic tissue occurred at the excitation-emission wavelength pairs of 280-330, 350-480, 350/460-605, 460-520 nm. According to the previous studies, these fluorescence peaks were ascribed to the amino acids tryptophan and tyrosine, reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH), ceroid and flavin adenine dinucleotide (FAD), respectively. However, adenomatous tissues exhibited red emission peaks at 635 and 710 nm that were not observed in the emission spectra of normal colonic tissues. These remarkable peaks were the contribution of naturally occurring high endogenous phophyrins of protoporphyrin IX (PpIX) in cancerous tissues[11,12], and the fluorescence spectra closely resembled the spectra of the PpIX standard. Our results indicate that the fluorescence intensity of the amino acids tryptophan and tyrosine is dominant in both normal and cancerous colonic tissues at 280-nm excitation, which demonstrates the fact that amino acids are the basic structural units of protein and play an important role in biological tissues. These results are found to be in good agreement with those of Pradhan *et al.*[13], who suggested that there is an increase in tryptophan as cells progress from normal to cancerous state. Secondly, we observed that the intensity in the blue region of the autofluorescence spectra significantly reduced in adenomatous colonic tissues compared with normal tissues, which is in substantial agreement with the early work showing that the concentrations of NADH and NADPH in adenomatous tissues are lower than those in normal tissues[7,11]. Moreover, Anidjar *et al.* have demonstrated a decrease of FAD in cancerous tissues[14], which is proved in this study. As a result, low NAD(P)H and FAD, but high amino acids and PpIX accumulated in the human colonic malignancy. It should be considered that because of the absence of emission specific peaks from collagen and elastin in our tissue EEMs except for two cases, our future studies should reduce the 20-nm interval for the excitation spectra to improve the spectral resolution. An evaluation of the average fluorescence spectrum at 260-nm excitation recorded at the end of each EEM indicated that its peak fluorescence intensity was within 10% of that acquired at the beginning of each EEM. This means that minimal photobleaching occurred during the process of the 25-min EEM measurement.

In order to further identify the presence of endogenous PpIX in colonic cancers, time-resolved fluorescence was induced by a short pulsed diode laser emitting at 397 nm (LDH-C-400, PicoQuant GmbH, Germany) with a picosecond pulsed diode laser driver (PDL 800-B, PicoQuant GmbH, Germany). Subsequently, the average lifetimes of normal and carcinomatous colonic tissues at the emission wavelength of 635 nm were determined by least squares fitting from the time-resolved autofluorescence spectra. Fluorescence from biological tissue generally stems from several endogenous fluorophores. Therefore, for a multi-exponential decay of fluorescence after pulsed light excitation, the fluorescence intensity with time change is described as[15]

$$I(t) = A + B_1 \exp(-t/\tau_1) + B_2 \exp(-t/\tau_2) + B_3 \exp(-t/\tau_3) + \cdots + B_i \exp(-t/\tau_i). \quad (1)$$

As a result, the average lifetime could be computed from

$$\langle \tau \rangle = \frac{B_1\tau_1^2 + B_2\tau_2^2 + B_3\tau_3^2 + \cdots + B_i\tau_i^2}{B_1\tau_1 + B_2\tau_2 + B_3\tau_3 + \cdots + B_i\tau_i}, \quad (2)$$

where τ_1, τ_2, τ_3, $\cdots$, τ_i are the lifetimes of the different endogenous fluorophores and the pre-exponential factors, B coefficients, are a measure of the contribution of each endogenous fluorophores to the total autofluorescence decay.

The time-resolved autofluorescence decay profile of cancerous colonic tissue is shown in Fig. 3, and the decay curve for normal tissue is indicated in the inset. Autofluorescence lifetimes for the normal and carcinomatous colonic tissues at 635 nm under 397-nm excitation were 4.32 ± 0.12 and 18.45 ± 0.05 ns, respectively. The selective accumulation of PpIX with known long lifetime of 18 ns was exactly presented in colonic cancers, as we have demonstrated in above steady state measurements, which agrees well with the results reported by Moesta and Mayinger *et al.*[11,12]. However, the *in vivo* fluorescence decay of the photosensitizers 5-aminolevulinic acid hexylester hydrochloride-induced PpIX was previously measured in human bladder and found to be mono-exponential with a lifetime of 15.9 ± 1.2 ns[16]. This variation in fluorescence decay times of PpIX

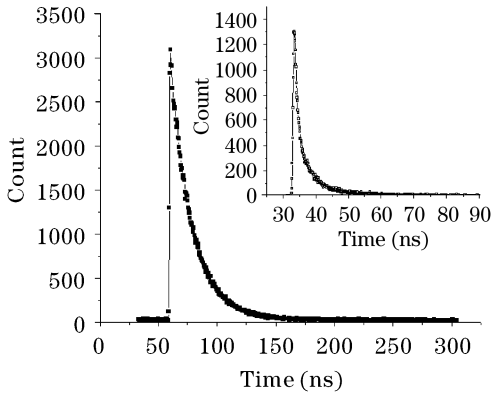

Fig. 3. Decay profiles of cancerous colonic tissues and normal colonic tissue (inset).

can be attributed to the different biochemical properties of human tissues during *in vitro* and *in vivo* measurements[17].

In conclusion, the transient decay of the autofluorescence intensity indicates the relative concentrations and the lifetimes of the endogenous fluorophores contributing to the emission. Laser-induced autofluorescence EEMs were used to exploit the mainly endogenous fluorophores responsible for the emission fluorescence of human colonic tissue. In particular, the high accumulation of endogenous PpIX in colonic cancers has been demonstrated in both steady state and time-resolved fluorescence spectroscopies, which could be treated as an important biomarker in diagnostic applications. Meanwhile, the combination of steady state and time-resolved autofluorescence spectroscopies can certainly provide more rich and precise spectroscopic information for the characterization of colonic tissues.

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