Endogenous and exogenous fluorescence diagnosis of tumors in the lower part of the gastrointestinal tract

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Abstract. Endogenous fluorescence measurements using UV-VIS excitation wavelengths revealed a variety of natural fluorophores, including the amino acids tyrosine and tryptophan, coenzymes – NADH and flavin, collagen and elastin. Deep minima in the tumor fluorescence signals were observed in the region 540 – 575 nm related to re-absorption of hemoglobin. Such high haemoglobin content was also found as an indication of the tumor’s vascularization and it was clearly pronounced in all dysplastic and tumor sites investigated \textit{ex vivo}. A photosensitizer from the family of porphyrins was applied as an exogenous fluorescent marker, namely, delta-aminolevulinic acid/protoporphyrin IX (5-ALA/PpIX). The 5-ALA was administered \textit{per os} six hours before endoscopic observation and spectral measurements at a dose of 20 mg/kg. A high-power light-emitting diode at 405 nm was used as an excitation source (LED-405, 25 mW, CW, Polironik Ltd., Russia). A fiber was introduced through an endoscopic instrumental channel in order to retrieve information about the fluorescence to a USB4000 micro-spectrometer (OceanOptics Inc., Dunedin, USA). The fluorescence detected from \textit{in vivo} tumor sites has very complex spectral origins. It consists of autofluorescence, fluorescence from exogenous fluorophores and re-absorption from the chromophores accumulated in the investigated tissue. However, the fluorescence of 5-ALA/PpIX was clearly pronounced in the 630 – 710 nm region, having a significant contrast with the surrounding normal mucosa pale fluorescence in the blue-green spectral region. The precancerous mucosa also revealed a red light signal, but with lower intensity levels than the carcinoma lesions observed \textit{in situ}. False-positive signals were recognized in case of inflammations in the colon and rectum areas, where 5-ALA/PpIX was accumulated as well.

1. Introduction

In the last few decades, endoscopic techniques have been significantly improved; however, despite the technical advances, the conventional endoscopic screening usually detects lesions in patients who already have symptoms of obstruction, pain, or bleeding caused by the tumor. Conventional white light endoscopy is not optimal for detecting dysplasia, which leads to missed cases of diagnosing initial
lesions. Another disadvantage of the conventional technique is the difficult detection of dysplasia in areas of chronic inflammation of the mucosa. These limitations pose a significant challenge to clinicians and has initiated the development of new photodiagnostic techniques to be applied in addition to the diagnostic capabilities of the standard endoscopic techniques. These new technologies are based on the relative differences in the interaction of light with normal and pathologically altered tissues, which, as a result of disease transformations, acquire new optical properties. While conventional endoscopy is limited to detecting pathological formations based on their significant morphological changes, the new optical methods offer a new strategy for endoscopic detection – they can detect early mucosal changes at microstructural, biochemical and molecular level in real time [1, 2].

Fluorescence spectroscopy is one of the most frequently explored optical modalities, because of its rapid and highly sensitive response to early biochemical and morphological changes in bio-tissues [3-5]. It could be based on endogenous signals obtained by internal fluorophores in the tissues, such as coenzymes, structural proteins and amino acids [5], or on exogenous signals, from applied specific fluorescent markers [2, 4, 6].

The first study that demonstrated the potential of fluorescence spectroscopy in the localization and diagnosis of neoplasms was conducted in 1961 by Lipson and his team [6]. They studied the accumulation of fluorescent compounds – porphyrins, in malignant neoplasms. During endoscopic examinations, this research group used excitation at 400 nm and observed red fluorescence from the neoplasms studied [6].

Through the years, fluorescence spectroscopy has evolved increasingly, improving both the technical means – new and more sensitive detectors, and accumulating a database of intrinsic tissue fluorophores, the spectral properties of biological tissues and their pathologies. Algorithms for differentiation have been created, allowing the development of clinical methods for diagnostics with high sensitivity and specificity based on endogenous and exogenous fluorescent substances used as tumor markers [7, 8].

The fluorescence spectroscopy technique is applicable to detecting gastrointestinal tract (GIT) tumors as well. Despite of all new therapeutic modalities and instruments for diagnosis of GIT neoplasia, it is still one of the most severe types of pathologies with high mortality and morbidity rates for patients worldwide.

The number of studies on the autofluorescent properties of normal mucosa and of benign and malignant gastrointestinal tract lesions using fluorescence techniques is still limited. Some research groups have used single sources of excitation [9], several discrete excitation wavelengths [7] or suggested the use of exogenous fluorescent markers to allow good visualization and discrimination of GIT tissue types, including the use of photosensitizers [10], or multiplex molecular fluorescent markers [11].

In our work, we used both endogenous and exogenous fluorescence spectroscopy of gastrointestinal tumors with the purpose of evaluating the feasibility of both techniques, as well as the diagnostic accuracies that could be achieved if intrinsic or external contrast agents are used as a basis for tissue type discrimination.

2. Methods and materials
Fluorescence spectroscopy of malignancies in the lower part of the gastrointestinal tract was carried out on ex vivo samples obtained after surgical removal of tumors for endogenous fluorescent studies, as well during in vivo endoscopic observations for detection of malignancies after sensitization with exogenous delta-aminolevulinic acid (5-ALA), which is a precursor of the fluorescent protoporphyrin IX (PpIX).

Fluorescence spectra ex vivo were measured of pairs of benign or malignant tissues and healthy tissue from the safety zone of samples surgically excised from 18 patients (22 lesions). The procedure for obtaining the tested samples started with their excision during surgery to remove lesions of colorectal neoplasia. After surgical removal, the lesions were divided into two parts – for histological and for spectral analysis. For the spectroscopic measurements, the biological samples were transported under isothermal conditions and in a safe-keeping solution from the hospital to the spectral laboratory, where
their fluorescent properties were investigated. All patients signed a written informed consent and this study was approved by the Ethics Commission of the University Hospital.

A FluoroLog 3 Spectrofluorimeter (HORIBA Jobin Yvon, France) was used for the fluorescence measurements of the ex vivo tissue samples. Since our tissue samples varied by shape and dimensions, their fluorescence properties were investigated by using an additional F-3000 fiber-optical module outside of the sample chamber. The measurements of the fluorescence signals were conducted in the spectral range 300 – 700 nm, with the excitation applied in the 280 – 440 nm spectral range with a step of 10 nm. After the spectroscopic measurements, the samples were stored in formalin solution.

Fluorescence spectra in vivo were measured during endoscopic observations procedures in the University Hospital on three patients with suspicion for development of malignant lesion. As excitation source was used a light emitting diode (LED) illuminator – AFS-405 (Polironik Ltd., Moscow, Russia) at 405 nm with 25 mW output power at the end of the fiber tip. A quartz-polymer fiber probe was applied through a standard endoscopic instrumental channel to feed the excitation light and return the fluorescence signal back to a USB4000 micro-spectrometer (spectral range 350 – 1000 nm, FWHM ~ 2 nm, OceanOptics Inc., Dunedin, USA). A computer was used to control the spectrometric system and to store and display the data measured using the SpectraSuite specialized software (OceanOptics Inc., Dunedin, USA).

In the exogenous fluorescence studies, 5-ALA/PpIX (“ALASENS”, NIOPIK JSCo, Russia) was used as an additional fluorescent marker for dysplasia and tumor detection in the colorectal region of the GIT. The 5-ALA was administered per os six hours before measurements at a dose of 20 mg/kg weight.

3. Results and discussion
Overcoming the standard endoscopy limitations in detecting dysplastic changes in mucosa is a significant challenge and has initiated the development of new photo-diagnostics techniques, additional to standard endoscopic equipment. Fluorescence detection of early alterations in the G|I|T mucosa would be very useful for clinical diagnostic applications during standard endoscopic examinations of patients with predispositions/factors for GIT cancer development (genetic, eating behavior, smoking, etc.).

Algorithms for differentiation could be created, allowing the development of diagnostic methods with high accuracy based on the fluorescent properties of the respective biological tissues and of additionally introduced fluorescent substances used as tumor markers. The endogenous fluorescence is based on the signals obtained from amino acids, structural proteins and co-enzymes present in the GIT mucosal tissues and covering the range from 300 – 650 nm. In the long-wavelength region (630 – 720 nm), the most pronounced signal was detected from endogenous porphyrins (650 – 700 nm) – a typical signal for an advanced stage of the tumor growth. Figures 1a and 1b present autofluorescence emission at different excitation wavelengths applied in normal colorectal mucosa and in carcinoma lesions.
Figure 1. (a) Autofluorescence spectra of normal colon mucosa using different excitation wavelengths in the region from 280 nm to 440 nm (b) Autofluorescence spectra of colorectal carcinoma lesion using different excitation wavelengths in the region from 280 nm to 440 nm

The endogenous fluorophores are characterized by the maximum fluorescent emission for the exact excitation wavelength applied. These pairs of excitation-emission wavelengths are unique for each of the intrinsic fluorophores and were identified in the spectra received, see table 1.

Table 1. Endogenous fluorophores responsible for the tissue autofluorescence in the lower GIT part for normal mucosa and cancerous lesions.

| Fluorophore          | Type              | Excitation [nm] | Emission [nm] |
|----------------------|-------------------|-----------------|---------------|
| Tryptophan, Tyrosine | Amino acids       | 280-300         | 300-400       |
| Collagen, Elastin    | Structural proteins| 310-350         | 350-420       |
| NADH, FAD            | Co-enzymes        | 370-440         | 400-600       |
| Endogenous PpIX      | Porphyrin         | 400-430         | 600-650       |

Figure 2. Fluorescence spectra of normal colon and mucosal tumor in vivo at 405-nm excitation

When analyzing the received signal (see figure 2) three areas were distinguished:
- range 450 – 630 nm, where an autofluorescent signal from the tissue is observed;
- range 630 – 710 nm, with pronounced maxima at 630 nm and 705 nm, typical of protoporphyrin IX;
- range 530 – 580 nm, with pronounced minima in the autofluorescent signal associated with its absorption by oxy-hemoglobin, which has absorption maxima at 545 nm and 575 nm.

One of the main differences between the fluorescence spectra observed for \textit{ex vivo} and \textit{in vivo} tissues arises from the absorption of oxy-hemoglobin. The effects of oxy-hemoglobin absorption could not be observed in the fluorescence of \textit{ex vivo} tissue samples, since after excision the blood could not be retained in the tissue without processing and additional alteration of its optical properties. Some attempts have been made to reduce the effect of blood absorption on the \textit{in vivo} tissue fluorescence by using polarized light and detecting fluorescence with a specific polarization \cite{12}.

The diagnostic accuracy, i.e., the endogenous fluorescence signals of normal \textit{vs} cancerous GIT mucosa, reached 91.67\% \cite{13}. In the cases when exogenous 5-ALA/PpIX was applied as an additional marker, specific PpIX fluorescence signal was observed for all tumor lesions investigated, but due to the small number of patients, the diagnostic accuracy is still under question.

4. Conclusions
The following characteristics stood out in the spectra obtained from normal and abnormal colorectal mucosa:
- Strong re-absorption of the autofluorescence signal by oxy-hemoglobin in tumor tissue;
- Intense fluorescence of protoporphyrin IX with maxima at 635 nm and 704 nm in the tumor formation;
- Well-expressed autofluorescence signal in the blue-green spectral region in normal mucosa with a maximum of about 530 – 560 nm;
- Absence of fluorescence in the red spectral range for healthy tissue, which is an indicator of the selective accumulation of 5-ALA/PpIX in tumor cells only;
- Absence of autofluorescent and exogenous fluorescent signal from necrotized tissue.

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