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Excitation-emission matrices for detection of colorectal tumors – initial investigations

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Detection and evaluation of cancerous changes in gastrointestinal tract (GIT) are performed with standart endoscopy. Its limitations are significant challenge and initiate development of novel diagnostic modalities. Therefore many spectral and optical techniques are applied recently into the clinical practice for obtaining qualitatively and quantitatively new data from gastrointestinal neoplasia with different level of clinical applicability and diagnostic success. Investigations for new diagnostic techniques by applying spectral and optical methods in clinical practice show very promising direction for an improvement of the tumour detection. One of the most promising technologies in this area is the fluorescence spectroscopy. The technique is very topical with its practical application in intra-operative, image-guided resection of tumors, because it permits minimal surgical intervention and friendly therapeutic conditions. In our investigation we evaluate the fluorescence of in vitro samples of lesions and healthy tissue of lower gastrointestinal tract. Autofluorescence of biological tissues is based on endogenous fluorophores response to an excitation in 280 - 440 nm spectral range and is observed in spectral range 320-550nm. On the basis of the spectral data analysis we try to estimate the options for application of autofluorescence spectroscopy techniques in clinical practice for in vivo diagnostic of lower GIT tumors. Autofluorescence detection could make the entire diagnostics procedure more personal, patient friendly and effective and will help for further understanding of tumors nature and to improve patients' lives. These investigations are part of the concept to proof the feasibility of such a system for a real clinical application. Therefore, we plan to gain more significant data base for the main spectral characteristics of lower GI neoplasia. We foresee as well to develop appropriate algorithms for benign/malignant tissue differentiation, based on the spectral features, obtained for normal mucosa and colorectal pathologies. Initial results obtained are promising that the specific differences are observed between normal mucosa and tumor, as well as for dysplasia-tumor pairs.

Key words: autofluorescence spectroscopy, colorectal cancer, excitation-emission matrices

INTRODUCTION

Colorectal cancer is the third most commonly diagnosed cancer and a major cause of cancer-related deaths worldwide [1]. At its early stage colorectal cancer usually do not reveal significant structural changes, this characteristic makes its detection and evaluation with standard endoscopy rather difficult and strongly related to physician's experience. Autofluorescence spectroscopy is one of the optical techniques applied for improving diagnostic sensitivity of standard endoscopy [2,3]. Light-induced fluorescence imaging and spectroscopy are one of the most sensitive optical approaches for detection of neoplasia of gastrointestinal mucosa, especially fluorescence spectroscopy, because of its rapid and highly sensitive response to early biochemical and morphological changes in the tissues [3]. Autofluorescence signal of biological tissues depends on endogenous fluorophores presence and concentration. Metabolic and of the tissues reflect in concentration and appearance of endogenous fluorophores [2]. Therefore cancerous tissues and tumors exhibit different fluorescence in comparison with the healthy tissues. Endogenous fluorophores which have a major contribution in fluorescence signal from GIT mucosa depend on the applied excitation wavelength, but at general they are amino acids, lipids, enzymes and coenzymes and some structural proteins. Optimal excitation wavelengths and maxima of the fluorescence emissions of the primary endogenous fluorophores are presented in Table 1.

Fluorescence signal obtained from the biological tissue depends not only from the endogenous fluorophores but also from the native pigments – chromophores in the tissues. The chromophore, which mostly affects fluorescence spectra of GIT mucosa in the visible range, by absorbing in 400–700 nm, is hemoglobin [4]. For the needs of clinical diagnostic of GIT there are just a few existing endoscopic systems that combines white light and fluorescent mode. Some of them are digestive tract videoscopes, for example one such produced by Olympus Inc., is used

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Table 1. Maximum of excitation and fluorescence emission of the main endogenous fluorophores in GIT mucosa. NADH – nicotinamide adenine dinucleotide; FAD – flavin adenine dinucleotide

Endogenous fluorophores	Maximum of excitation, nm	Maximum of emission, nm
Amino acids		
Tryptophan	280	350
Tyrosine	275	300
Porphyrines	400-450	630, 690
Enzymes and coenzymes		
NADH	450	535
FAD	290, 351	440, 460
Structural proteins		
Collagen	325, 360	400, 405
Elastin	290, 325	340, 400

to observe blood vessels in mucous membranes under infrared light in the regions 790–820 nm and 905–970 nm. Another endoscopic system - Xillix-LIFE-GI, jointly developed by Xillix and Olympus, is applied for autofluorescence detection of stomach neoplasia. In this system blue-light excitation is used in the range of 400–450 nm, with additional simultaneously excitation with red-near-infrared light in the newer version of the system. Fluorescence signal is evaluated by two CCD cameras with maximum sensitivity in the green (480–560 nm) and in the red (630–750 nm) spectral ranges. Fluorescence signal is processed and displayed as false-color image in real-time. On those images normal mucosa appears colored in green and neoplastic mucosa appears colored in red [5]. Fluorescence technique is very topical with its practical application in visualization and discrimination of neoplastic tissues during open surgical interventions for tumor excision in lower GIT. This kind of intra-operative, image-guided resection of tumors permits precise tumor excision along with minimal surgery intervention and friendly therapeutic conditions [6,7]. Although several systems have been developed, the existing clinical limitations are one significant technical challenge and initiate a development of new diagnostic modalities based on different spectral and optical techniques. Also the reported specificity is not satisfactory as the rate of false-positive results in differentiating dysplastic from inflamed tissues is one of the main drawbacks. On the way of solving those problems is to find excitation wavelengths that provide fluorescence signal consisting unique spectral characteristic features, correlated with the tissue pathologies and establishing of a robust algorithm that can extract and compare those features of the autofluorescence signal. Therefore different research groups work in the direction of optimization of fluorescence detection that could improve implementation and contribution of the fluorescence techniques in to the clinical diagnostic practice for lower GIT tumour detection.

MATERIALS AND METHODS

In our study, we investigated fluorescence of 8 samples of neoplastic tissue and healthy tissues from 5 patients. Normal and tumour's GIT mucosa samples are received after surgical excision during procedures for a removal of GIT neoplasia lesions. Normal tissue investigated is a part of the safety area around the tumor excised during its removal. Neoplastic tissue in samples has been histological identified in 5 samples as carcinoma, in 2 samples as polyp and in 1 sample as metastatic lymph node. All patients received and signed written informed consent and this research is approved by the ethical committee of University Hospital "Queen Giovanna", Sofia. After surgical removal biological samples are transported in isothermal conditions and safe-keeping solution from the hospital to the spectral laboratory, where their fluorescence is investigated with point-by-point spectroscopy.

For fluorescence measurements we used spectrofluorimeter FluoroLog 3 (HORIBA Jobin Yvon, France). This system allows performance of steady-state and time-resolved fluorescence measurements with light source xenon lamp with power 300 W and performance range 200 - 650 nm and PMT detector with performance range of 220–850 nm for the fluorescence detection. Our tissue samples cannot be put into a standard cuvette, since they vary in shape and dimensions. Therefore we use additional fiber-optic module of Fluorolog 3 – F-3000, which allows investigation of samples outside of the sample chamber. The measurements of the fluorescence signals are obtained in a regime of so called excitation-emission matrices (EEMs) for all tissues investigated. This three-dimensional fluorescence spectral maps provides information about the fluorescence spectra of biological tissue samples, which is needed for determining excitation wavelengths that gave emission fluorescence spectra containing the most significant diagnostic information for a further clinical diagnostic analysis.

Excitation applied to the samples was in 280–440 nm spectral region. Fluorescence emission was measured in the range of 300 to 800 nm. After the spectroscopic measurement procedure the samples were kept in a formalin solution.

RESULTS AND DISCUSSION

Fluorescence spectroscopy of gastrointestinal tissues ex vivo demonstrated that autofluorescence spectra depend on the applied excitation wavelength and tissue type. Different spectral characteristics of autofluorescence spectra of tumours in comparison with autofluorescence spectra of normal GIT mucosa give information about intrinsic sources of fluorescence, that correlate with the biochemical and morphological changes in the neoplastic tissue.

Autofluorescence of the safe-keeping solution, where the tissue samples are kept during transportation and autofluorescence of the surface where the tissue sample is placed during the measurements are evaluated and signals have been found negligible low in comparison with the fluorescence intensities detected from the tissue samples themselves for all excitation wavelengths applied in our investigations.

In Figs. 1.a and 1.b are presented excitation – emission matrices of normal mucosa and of the tumour, in Figs. 1.c and 1.d are presented the autofluorescence spectra respectively for the normal and carcinoma tissues from a colorectal part of GIT. Several major autofluorescence sources could be addressed in the samples investigated. Those are amino acids – tryptophan and tyrosine, structural proteins – elastin and collagen, and their cross-links and coenzymes NADH and FAD. Their maxima of excitation and emission are presented in Table 2.

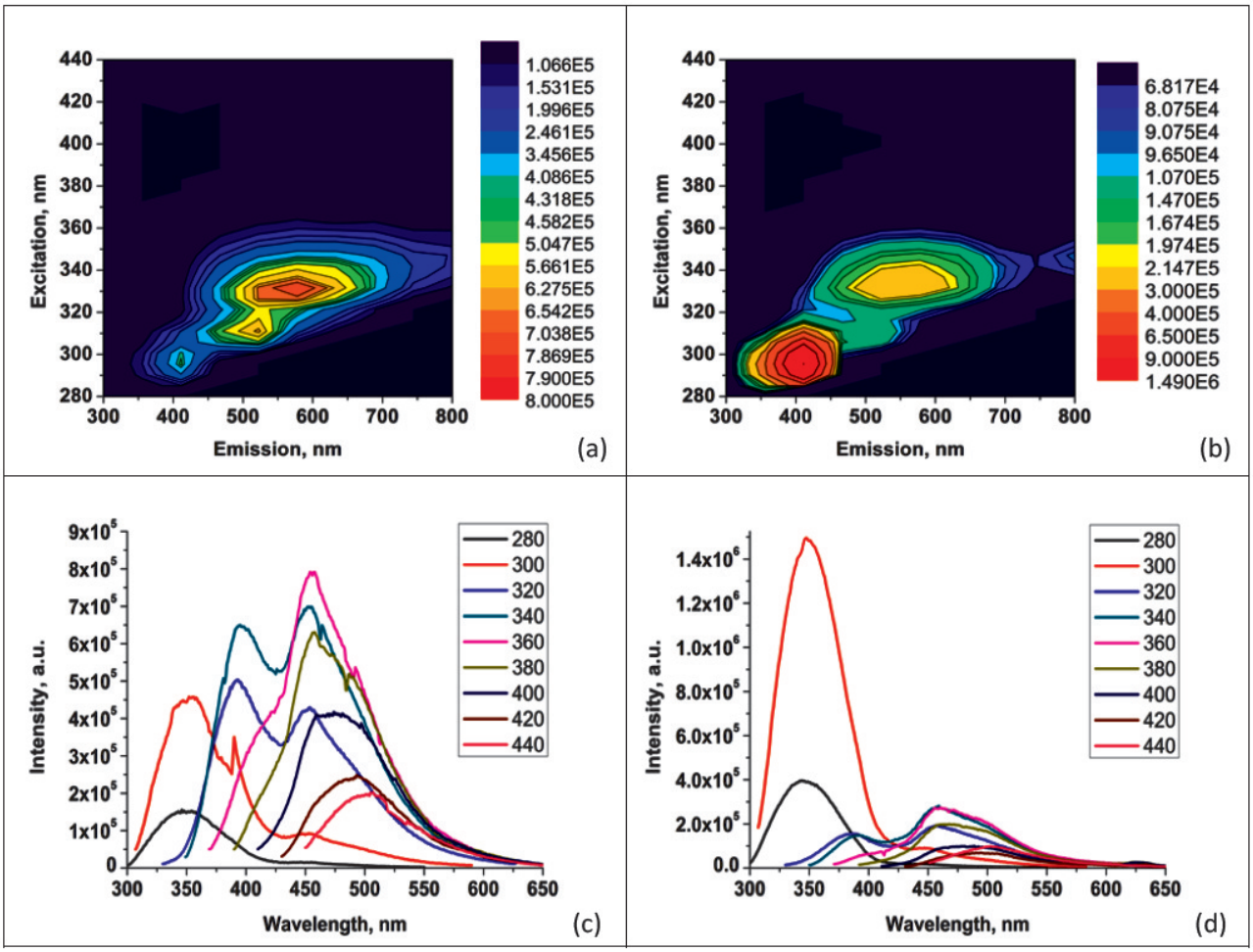

Fig. 1. (a) EEM of normal mucosa; (b) EEM of carcinoma; (c) fluorescence spectra of normal mucosa; (d) fluorescence spectra of carcinoma from the colorectal area of GIT.

Table 2. Endogenous fluorophores identified as main autofluorescence sources in the lower GIT tissues investigated, with their maxima of excitation and emission

Endogenous fluorophores	Maximum of excitation, nm	Maximum of emission, nm
Tryptophan and Tyrosine	280-300	320-360
NADH	340-380	440-480
FAD	340-400	500-530
Collagen and Elastin	320-360	400, 460-500
Protein cross-links	290, 325	340, 400

Coenzymes NADH and FAD are degrading fast in the tissue samples after their surgical excision, therefore the fluorescence arising from those endogenous fluorophores could be detected only in the freshly excised samples. Main difference between autofluorescence of the normal and tumour tissue is in about two times lower intensity of the fluorescence signal from tumour tissue in the spectral range 400 - 600 nm, where the primary source of fluorescence are structural proteins and their cross-links. This could be addressed to decrease of the signal detected in unit volume of the tissue from collagen fibers and collagen cross-links. In the case of tumour lesion, the intercellular matrix is relatively loosened, due to increased tumor cells size and general reduction of collagen and elastin concentration on volume unit. Similar observations of fluorescence signal reduction are reported from different research groups and are proposed to be used as an indication of tumor lesion presence for diagnostic analyses [8,9].

Specific alteration in the metabolic activity in tumour cells causes oxidation of NADH, whose oxidized form NAD+ is non-fluorescent. That lead to significant loss of fluorescence signal in the 450–550 nm region. That is another essential difference between autofluorescence spectra of normal and tumor tissues, which could be applied for differentiation. As a summary the most significant fluorescence spectra differences are observed for the excitation wavelengths applied in the region of 300–360 nm, where NADH and structural proteins have maximum of excitation, because the alterations in their fluorescence spectra are the main difference between autofluorescence spectra of normal and tumour tissue. Excitation wavelengths from this range have been investigated by other research groups as well for the diagnostic meaning of the corresponding fluorescence spectra of normal and neoplastic GIT tissue [9,10]. The results obtained show high sensitivity and specificity for determining cancerous tissues on the basis of the obtained fluorescence spectra.

CONCLUSIONS

Implementing fluorescence techniques into the clinical practice has potential to improve determination of the site, borders and size of the lesions, not only for initial diagnostics but also in real-time monitoring of resection procedures and in the personalization of the patient healthcare. One of the main advantages of fluorescence techniques is that they are patient friendly, not using ionizing radiation and could help for further understanding of tumors nature, which will result in improving patients' lives. Our efforts are directed towards developing a robust algorithm for processing fluorescence spectra, which will result in reliable differentiation between normal and cancerous tissues and to be implemented in practical clinical diagnostics. This requires gaining more significant data base for the main spectral characteristics of lower GI neoplasia.

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МАТРИЦИ НА ВЪЗБУЖДАНЕ И ФЛУОРЕСЦЕНЦИЯ ЗА ДЕТЕКТИРАНЕ НА КОЛО-РЕКТАЛНИ ТУМОРИ – НАЧАЛНИ ИЗСЛЕДВАНИЯ

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(Резюме)

Диагностиката на неоплазии в областта на гастроинтестиналния тракт (ГИТ), се извършва със стандартни ендоскопски изследвания, чиито ограничения са предпоставка за търсенето и развитието на нови, по-точни и по-чувствителни диагностични методи. Търсенето на нови диагностични техники, чрез прилагане на спектралните и оптични методи в клиничната практика е обещаващо направление.

Флуоресцентната диагностика е един от най-разпространените оптични методи за диагностика, с добри перспективи за развитие. Методът се прилага за по-точно идентифициране на лезии и по време на операции, осигурявайки минимална намеса в състоянието на оперативното поле и цялостното състояние на пациента. Автофлуоресценцията на биологичните тъкани, дължаща се на излъчването на ендогенн флуорофори, се наблюдава в диапазона 320-550 nm и се получава при възбуждане в диапазона 280-440 nm.

В нашето изследване оценяваме флуоресценцията на in vitro образци от лезии и здрава тъкан на долен ГИТ, за да проучим възможностите за приложение на автофлуоресцентни техники за клинична диагностика на тумори на ГИТ in vivo.

Флуоресценцията на образците in vitro е изследвана чрез системата FluoroLog 3 (HORIBA Jobin Yvon, Франция), която използва като източник мощна ксенонова лампа (300 W., 200-850 nm), два двойни монохроматора, и детектор - ФЕУ с висока чувствителност в областта 220-850 nm. Автофлуоресцентните сигнали са детектирани чрез методиката за определяне на матрици на възбуждане и флуоресценция от образци на здрава тъкан, дисплазия и карцином на дебелото черво. Изследваните образци проявяват специфични спектрални характеристики в зависимост от състоянието на тъканта, като могат да се определят диагностично-значими особености, приложими в клинични алгоритми за диференциация на патологичните изменения.

Автофлуоресцентната техника може да направи диагностичната процедура по- ефективна, неинвазивна и съобразена с индивидуалните особености на пациента. Приложението ѝ може да допринесе за по-доброто разбирането на същността на неопластичните процеси и подобряване на качеството на живот на пациентите. Нашите усилия са насочени в посока на събиране и систематизиране на статистически данни за основните флуоресцентни характеристики на неоплазии на долния ГИТ, които да бъдат използвани за основа за развитие на подходящи алгоритми за разграничаване на здрава от туморна тъкан.

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