Polarization sensitive excitation-emission matrices for detection of colorectal tumours – initial investigations

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Abstract. Excitation-emission matrices were used for detection of the autofluorescence properties of surgically excised normal and cancerous mucosa of lower gastrointestinal tract tissues (colon and rectum carcinoma). Linear polarization of the excitation and emission fluorescence light was additionally applied to evaluate the influence of anisotropic fluorophores presented in the tissues investigated. Excitation applied was in the region of 280-440 nm, using step of 10 nm for the scanning, fluorescence emission was detected in the region of 300-800 nm, with scanning step of 1 nm. Excitation and emission light were investigated in parallel and perpendicular linear polarization modes respectively. These investigations are part of the concept to proof the feasibility of autofluorescence system for a real clinical application. 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. In the current investigation major spectral features without and with linear polarization applied are addressed and lower GIT lesions’ emission properties are evaluated.

1. Introduction

The limitations of standard endoscopy for detection and evaluation of cancerous changes in gastrointestinal tract (GIT) 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 a different level of clinical applicability and diagnostic success. Fluorescence imaging has been one of the most promising technologies in this area. The technique is very topical with its practical application in intra-operative, image-guided resection of tumors, because it permits minimal surgery intervention and friendly observation conditions. It could be based on endogenous fluorophores response to an excitation in the end of UV-blue spectral range, or on exogenous fluorescence, when
fluorescent markers are applied to improve the detection sensitivity and the contrast ratio of the signals obtained from normal and abnormal tissue sites.

The first gastrointestinal laser-induced fluorescence spectroscopy (LIFS) of gastrointestinal tract was performed by Kapadia et al. in 1990 [1]. In that *ex vivo* study normal colonic tissue and adenomatous polyps were investigated, using an excitation at 325 nm and the emission was evaluated in the 350 - 600 nm range. Very promising results were reported due to the applied multivariate linear regression analysis of the emission spectra, which resulted 94% accuracy in differentiating hyperplasia and 100% accuracy in differentiating adenoma from normal colonic tissues.

Similar prospective results were observed during *in vivo* autofluorescence investigations of normal, hyperplastic and adenomatous polyps, [2] In that study excitation wavelength at 370 nm was chosen. Considering fluorescence characteristics of normal and cancerous tissue in the spectral range from 400 nm to 700 nm, the research group had developed probability-based algorithm for tissue classification with a sensitivity of 90% and a specificity of 82 %.

Due to the first promising results, many groups started to work in the next two decades in the field of autofluorescence detection of gastrointestinal neoplasia and dysplasia with different level of success, depending from the excitation wavelength applied and specificity of the autofluorescence spectra received.

About ten years later were proposed the endoscopic systems for clinical GIT autofluorescence detection. Several feasibility studies are reported based on several endoscope models produced by the specialized companies, working in the field of endoscopic equipment development.

Few autofluorescence endoscopic systems had been implemented for diagnostics of the GIT tumours, such as the LIFE-GI system and its second generation *LIFE II*; Xillix Technology Co., Canada, D-Light system of Karl Storz GmbH, DAFE system (Diagnostic AutoFluorescence Endoscope) of Richard Wolf GmbH, XCLV-260HP and XCF-Q240FAI; Olympus, Tokyo, Japan. The feasibility of those systems in diagnostic endoscopy of the esophagus, stomach and colon cancer diagnostic has been investigated by several research groups. [3-5]

However, to improve the diagnostic accuracy, multiple excitation wavelengths have to be applied, to cover the range of all endogenous fluorophores, which could add to the diagnostic value of the autofluorescence spectra detected from normal and cancerous GIT tissues.

For a first time, an excitation – emission mapping using several excitation wavelengths for gastrointestinal tissues autofluorescence detection was performed by the group of Richard-Kortum at al. [6], in the beginning of 1990s. The group performed investigation of the autofluorescence of colonic tissue *ex vivo* using excitation - emission matrices, for identifying excitation wavelengths, which would result an emission spectra with diagnostic meaning. They stated that excitation wavelengths at 330 nm, 370 nm and 430 nm were the most suitable for autofluorescence differentiation between normal and cancerous colonic tissues and 404, 480, and 680 nm were found to be the most useful emission wavelengths for diagnose.

Recent investigations in this field are related to the searching of some optimal excitation wavelengths and revealing of the fluorescence peculiarities, which could have diagnostic value for better differentiation between normal, dysplastic and malignant GIT mucosa.

Imaizumi et al., 2011. had investigated the fluorescence of NADH in human colonic tissue samples [7]. Diagnostic meaning of the fluorescence spectra in this study was evaluated by observing ratio images created by dividing the fluorescence spectra obtained with excitation wavelengths of 365 nm and 405 nm. The ratio images showed a higher signal intensity in the adenomas and hyperplastic polyps, respectively, than in the adjacent normal mucosa. This study showed that metabolic alteration of colonic adenomas could be detected by imaging mucosal autofluorescence in the wavelength range of 450 to 490 nm under excitation at 365 and 405 nm.

Another group applied excitation wavelength of 355, 361, 370, 380, 384, 390 and 410 nm to biopsied or surgically resected colorectal tissue samples from 50 subjects with different degrees of colorectal dysplasia [8]. Autofluorescence from the samples was observed in the spectral region of 400-570 nm. The most significant difference in the autofluorescence signal of normal, benign and
malignant tissue observed was the intensity at 460 nm of the emission, related to the NADH, which is lower for malignant and benign tissues. The obtained sensitivity, specificity and accuracy in differentiating between normal, benign and malignant tissue was found to be 100%, 90% and 96.7% respectively, which is an excellent perspective for further development of the autofluorescence excitation-emission mapping of the GIT mucosa for the needs of early tumor diagnosis.

We plan to gain more significant data base for the main spectral characteristics of lower GI neoplasia using excitation in the range of 280-440 nm, with a step of 10 nm. Additionally applied linear polarization for excitation and emission in two positions – parallel and perpendicular polarization for the light beams from the excitation source and from the fluorescent tissue sample will allow us to make some conclusions about the influence of structural proteins and other polarization-sensitive endogenous fluorophores in the GIT mucosa – normal and tumour. 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 with and without polarizing of the excitation/emission light pairs. Initial results obtained are promising that the specific differences are observed between normal/tumour, as well as for dysplasia/tumour pairs.

2. Methods and materials

Our investigations here are based on ex vivo measurements of excitation-emission matrices (EEM) for lower gastrointestinal (GIT) neoplasia to evaluate the applicability of autofluorescence technique for clinical observations of lower GIT tumours in vivo. Fluorescence signal from the surgically excised samples was obtained in three modalities – EEM – excitation –emission matrix without polarization optics, and with linear polarizers applied on excitation and emission pathway of the light in two positions – parallel and perpendicular for the excitation and emission light beams.

The EEMs are graphically represented with excitation wavelength on one axis, emission wavelength on the second, and fluorescence intensity forms the third axis. This method for three-dimensional fluorescence spectroscopy provides enough information about the fluorescence spectra of biological tissue samples for determining excitation wavelengths that gave emission fluorescence spectra containing the most diagnostic meaning for clinical diagnostic analysis. EEMs were performed over pairs of cancerous tissue and healthy tissue from the lower GIT from 9 different patients. The procedure of obtaining the investigated samples includes their excision during surgery for removal of GIT neoplasia lesions. After the 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. All patients received and signed written informed consent and this research is approved by Ethics committee of University Hospital “Tsaritsa Yoanna”, Sofia.

Spectrofluorimeter FluoroLog 3 (HORIBA Jobin Yvon, France) was used for the measurements. This system’s light source is Xenon lamp with power 300 W, performance range of 200-650 nm and PMT detector with performance range of 220-800 nm for fluorescence detection. Since our samples vary in shape and dimensions, their fluorescence was investigated with additional module F - 3000 of Fluorolog 3, which allows investigation of samples outside of the sample chamber.

Measurements of the fluorescence signals of the different tissue samples obtained in EEMs were performed with applied excitation in 280-440 nm spectral region with step of 10 nm and emission observed between 300 nm and 800 nm, with step of scanning - 1 nm. Polarization measurements were performed in the same spectral ranges for excitation and emission light, but on the way of the light beams were applied linear polarizers in two positions - parallel and perpendicular to each other and polarized EEMs were detected. After the performing of both types of spectroscopic measurements for healthy and cancerous tissue, the samples were stored in formalin solution. Histological analysis of the tissues detected by the autofluorescence EEM approach was used as a “gold” standard for the clinical diagnosis of the pathology investigated.
3. Results and Discussion

Main differences observed between the fluorescence spectra of healthy and cancerous tissue are in the intensity of the fluorescence originating from the amino acids – tyrosine and tryptophan, the enzymes and coenzymes NADH and FAD, and from the structural proteins elastin and collagen.

On Figure 1, are presented the results in a form of spectral curves (1a and 1b) and contour maps for EEMs (1c and 1d) of a healthy mucosa and a colon carcinoma. No polarization elements are applied.

The differences observed arise from the different metabolic rate and structural characteristic of cancerous cells to healthy cells. The higher metabolic rate of the cancerous cells results in intensive production of the amino acids tyrosine and tryptophan, hence we observe higher intensity of their fluorescence in comparison with other fluorophores in these tissues in the UV spectral region – 320-350 nm peaks. Cancer cells undergo aerobic glycolysis which results in elevated NADH:NAD+ ratios, where NAD+ is the non-fluorescent oxidized form of NADH. This may be one of the reasons for the observed lower intensity of the fluorescence maxima of NADH in cancerous cells (max at 440-460 nm region).

Figure 1. Comparison between (a) autofluorescence spectra of normal colon mucosa and (b) autofluorescence spectra of colon carcinoma using excitation wavelengths in the region of 280-440 nm; (c) EEM of normal colon mucosa (d) EEM of colon carcinoma.
Abnormal oversized growths of cancerous cells result in lack of structural proteins in volume unite of the cancerous tissues. This reduction of the quantity of the structural proteins affects the fluorescence spectra of cancerous tissue by lowering the intensity of fluorescence maxima of structural proteins, in comparison with the same maxima in the spectra of healthy tissue. The maximum of collagen type I fluorescence at 400-410 nm is significantly higher for normal mucosa in comparison with tumour site. The partial demolition of the extracellular matrix due to tumour growth also affected fluorescence signal resulted from protein cross-links in the region of 480-500 nm. This signal is about two times lower for the tumour vs. normal GIT mucosa.

Excitation-emission matrices were used for defining most suitable excitation wavelength for fluorescence diagnostic of colonic tissue ex vivo, also by Li et al. [9]. This group performed the measurement for excitation in the range of 260-540 nm with increment of 20 nm and emission observed in a range starting 20 nm above the excitation wavelength and extending to 800 nm at 5-nm intervals. They observe four maxima in the EEMs of the investigated normal and cancerous colonic tissue. The fluorophores, those maxima are arising from, are addressed as tryptophan and tyrosine for excitation of 280 nm and emission of 330 nm, NADH with excitation maxima of 350 nm and emission at 480 nm and 460-605 nm and FAD with excitation maxima of 460 nm and emission at 520 nm. These authors observed also additional peaks in the fluorescence spectra of adenomatous colonic tissue, at 635 nm and 710 nm, addressed to the endogenous porphyrins in cancerous tissues. In our investigations we did not observed yet significant level of endogenous porphyrins fluorescence in the GIT tumours investigated. However, with the broadening of the spectral database and new samples investigated we could expect such fluorescence to be detected.

The feasibility of different wavelengths (namely 337, 375, 405 and 460 nm) for an excitation of autofluorescence of colonic cancer is investigated recently [10]. This group acknowledged that the autofluorescence spectra obtained under 337 nm excitation could provide more diagnostic information. In our own investigations, we observe significant changes in the level of amino acids autofluorescence in the region of 320-350 nm. The autofluorescence intensity for normal mucosa is about 2.5 times higher vs. tumour, when excitation at 280 nm is applied and about 2 times higher for the 290-300 nm excitation. Significant changes in the spectral shape of the emission for normal and cancerous mucosa are observed for all cases investigated when 310-330 nm excitations is applied. This region is appropriate to be used for diagnostic differentiation not only due to intensity changes occurred, but due to rapid changes in the spectral shape for both tissue states.

The results obtained from polarization autofluorescence measurements for the normal colon and carcinoma reveal the influence of collagen anisotropy in the autofluorescence features of the normal and abnormal tissues. On fig.2 are compared EEMs of normal colon mucosa (2a and 2b) and carcinoma (2b and 2d) for both modalities – parallel (2a and 2c) and perpendicular (2b and 2d) linear polarization of the excitation and emission light from the tissue samples detected.
Figure 2. Comparison between EEMs of normal colon mucosa with parallel polarization (a) and perpendicular polarization (b) of the excitation and emission light beams; Comparison between EEMs of colon carcinoma with parallel polarization (c) and perpendicular polarization (d) of the excitation and emission light beams. Excitation is in the region of 280-440 nm, autofluorescence emission detected is in the region 300-800 nm.

Some of the biologically important fluorophores are anisotropic by their nature, due to the specific structure of chirality. Typical example of scattering and fluorescence anisotropic molecule in the human tissues is its major structural compound – collagen, due to its fiber structure. The structure of collagen fibers could be responsible for the higher degree of polarization effects observed for the fluorescence signal obtained from this fluorophore as well.

The most significant changes are observed in the region of 380-420 nm, where the collagen type I fluorescence maximum is situated, as well as in the region of 480-520 nm, where the protein cross-links fluorescence has place.

In the longer wavelength spectral region, for excitation range longer than 360 nm, when the coenzymes, such as NADH and flavins are excited to fluoresce, no significant spectral shape changes for the parallel and perpendicular positions of polarizer/analyzer couple were observed. Only the fluorescence maxima related to structural proteins and their cross links reveal spectral shape differences in that comparison.

The slight spectral shift of the maxima between the parallel and cross-polarized fluorescence spectra observed for thick tissue layers (∼2 mm), or as in our case of ex vivo study, could be associated to the absorption properties of the tissue investigated. [11] The observed spectral peculiarities could be used as indicators for a discrimination of the tissue health condition.

Other investigators observed that the main difference between fluorescence spectra of normal and cancerous tissue is the lower intensity in the maxima of 380-400 nm, which is due to collagen fluorescence. [10] The lower intensity of this maximum is discussed to be caused by increasing of the mucosa resulting from neoplastic transformation so that lower the fluorescence quantum yield of collagen mainly distributing in the submucosa. Other researchers comment that the most pronounced difference in the autofluorescence properties of normal colon vs. colorectal carcinoma is related to the NADH signal. [8] Its maximum at 460 nm is observed to be significantly lower for the pathologic tissue. We also observe such decrease of the collagen and NADH autofluorescence, which allow us to compare our results with these of other research groups and to develop summarized picture of the endogenous fluorophores observed in the normal and malignant GIT mucosa.

The reduction of the quantity of the structural proteins affects the fluorescence spectra of cancerous tissue by lowering the intensity of fluorescence maxima of structural proteins,
comparison with the same maxima in the spectra of healthy tissue. In the autofluorescence spectra of cancerous tissue, additional difference, in comparison with the spectra of normal tissue is the spectral shift of the NADH and FAD maxima in the range of the FAD fluorescence, which we interpreted like increasing of FAD contained in the cancerous tissue.

4. Conclusions

GIT cancer diagnostics could be developed based on the difference in metabolism and structure for normal and diseased tissues. The fluorescence anisotropy measurement of the tissues shows its high sensitivity to the structural and morphological changes, related to health condition. The correlation between such small changes, related to the extracellular matrix decrease of structure and integrity could be more pronounced in the case of tumour lesions, where such integrity and structural matrix are partially demolished due to the lesion growth. The polarization autofluorescence measurements may provide a noninvasive method for cancer detection. In order to further demonstrate the feasibility of this method, studies need to be carried out to measure the fluorescence anisotropy of GIT benign and malignant lesions. Our preliminary investigations on measurements of healthy mucosa and colon carcinoma ex vivo allow finding the most appropriate excitation wavelengths and the specific fluorophores, which could contribute to the diagnostic discrimination of normal and cancerous GIT.

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