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Synchronous autofluorescence spectroscopy of gastrointestinal tumours – tool for endogenous fluorophores evaluation

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Synchronous autofluorescence spectroscopy (SFS) using excitation in the range of 280-440 nm and varying delta lambda from 10 to 200 nm were applied on lower GIT tumours obtained after surgical excision. Due to the improved efficiency of SFS for the signals, where the delta lambda is optimal, we could obtain higher spectral resolution for the detection of the set of endogenous fluorophores. Major spectral features are addressed and diagnostic discrimination algorithms based on lesions' emission properties are proposed.

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1. Introduction

Fluorescence spectroscopic investigations of gastrointestinal tissue samples of normal and cancerous tissue are performed in terms of identification of spectral characteristics in their emission spectra that can be used for differentiation between normal, dysplastic and malignant tissue. Fluorescence signal is obtained in two modalities – EEM – excitation –emission matrix and SFS – synchronous autofluorescence spectroscopy of the tissue. In the first case EEM is 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.

Second approach for the fluorescence signal detection – synchronous autofluorescence spectroscopy appears to provide more information, in comparison with EEMs, based on its greater selectivity. Investigating fluorescence through the method of SFS is performed by maintaining constant wavelength interval between excitation wavelength and emission wavelength through the spectrum. This allows optimal excitation of the emission maxima, which result in narrower emission peaks. That is the main reason for the greater sensitivity of SFS in comparison with EEMs [1]. Narrower peaks in the obtained fluorescence spectra allows decrease the extent of spectral overlaps and this effect is useful in investigating multi-component samples which consist mixture of fluorescence compounds, like biological tissues [2].

SFS high sensitivity, non-invasive character, relatively fast performing and lower cost are main factors for the rapid widespread exploitation of the method for qualitative and quantitative analysis of food and beverages [3, 4]. Its application in biomedical researches has emerged as a promising modality for investigation of blood and urine samples for cancer diagnostic purposes and for differentiation between normal and pathologically diseased cells and tissues [5, 6]. The diagnostic potential of SFS for identification and localization of dysplastic tissues has been investigated for breast, cervical and thyroid gland cancers. Conclusions of those investigations support the superiority of SFS in sensitivity and specificity for differentiating cancerous and healthy tissue on the basis of their fluorescence spectra [7, 8].

One of the main drawbacks of autofluorescence diagnostic techniques is the lack of specificity, which arises from the similar metabolic alterations between cancerous and inflammation pathologies [9]. Implementation of SFS among fluorescence diagnostic techniques can lead to improvement in specificity of the fluorescence diagnostic techniques.

2. Materials and methods

SFS was performed, along with EEM, over pairs of cancerous tissue and healthy tissue from the 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 tissues obtained in EEMs were performed with applied excitation in 280-440 nm spectral region and emission observed between 300 nm and 800 nm. SFS measurements were performed with excitation wavelength in the spectral range of 280-440 nm with increment of 10 nm and wavelength interval (offset) in the range of 10-200 nm with increment of 10 nm. After the performing of both spectroscopic measurements for healthy and cancerous tissue the samples were in formalin solution, for safe-keeping.

3. Results and discussion

Main differences observed between the fluorescence spectra of healthy and cancerous colon 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 Fig. 1 are presented the results in form of contour maps for excitation-emission matrices and raw autofluorescence spectra for the excitation in the range of 280 nm to 440 nm of healthy and cancerous tissues.

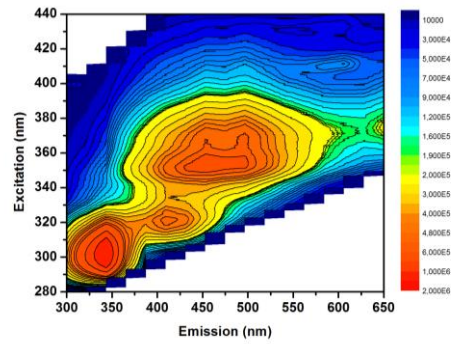
Coenzymes NADH and FAD are rapidly demolished in the tissue samples after their surgical excision, therefore the fluorescence arising from those endogenous fluorophores could be detected only in the freshly excised samples. Other reason for the differences in the co-enzymes fluorescence signals arise from the different metabolic rate and structural characteristic of cancerous cells to healthy cells. 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 – the region around 440-460 nm of the emission. The problem here is that we could not easily distinguished the effect of excision of the tissue form the body and the metabolic condition of the tissue on the level of the NADH and flavins fluorescence, as these factors formed a complex response on the autofluorescence intensities level for the co-enzymes.

The higher metabolic rate of the cancerous tissues results in intensive production of the amino acids tyrosine and tryptophan, hence we observe higher intensity of their fluorescence. Abnormal oversized growths of cancerous cells results in lack of structural proteins in unit volume 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 – region of 400 nm emission max for the collagen type I, and respectively emission region at 460-500 nm for the protein cross-links.

This is the major difference between autofluorescence of the normal and tumour tissue - 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 [9, 10].

Therefore, the structural proteins and amino acids fluorescence signals are better indicator for the tumour appearance, when surgically excised samples are investigated than the co-enzyme autofluorescence.

Synchronous fluorescence spectroscopy is well established and widely used method for analysis of multicomponent systems, as it could enhance the spectral resolution in the analysis of complex mixtures of fluorophores. The biological tissues could be investigated as such complex systems with several fluorophores included for each type of tissue condition, or excitation wavelength applied. Therefore the SFS technique could be useful for better discrimination of endogenous chromophores, as well as to be applied as a diagnostic tool, comparable to the standard steady-state fluorescence spectroscopy.



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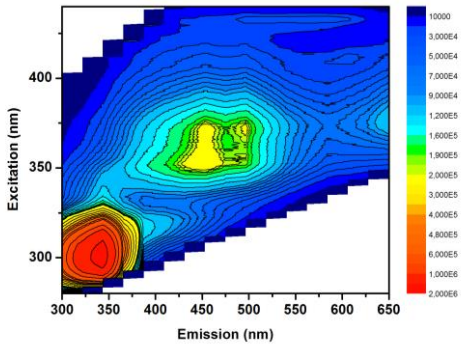

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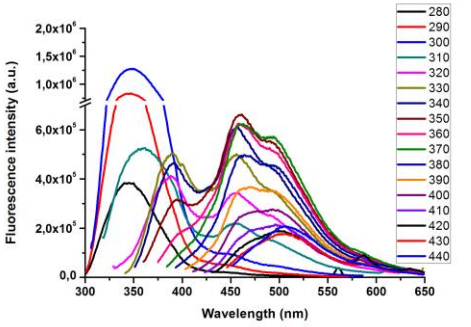

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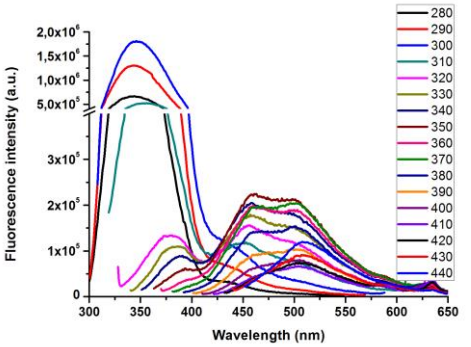

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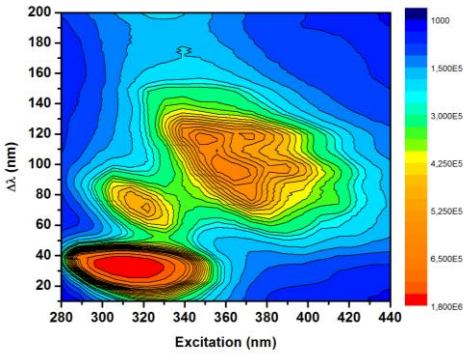
Fig. 1. Comparison between a) EEM of normal tissue sample from colon mucosa and b) EEM of colon carcinoma; c) autofluorescence spectra of normal colon mucosa for different excitation wavelengths applied (in the region of 280 nm to 440 nm, with step of 10 nm) and d) autofluorescence spectra of a colon carcinoma for the same patient on different excitation wavelengths applied (in the region of 280 nm to 440 nm, with step of 10 nm). Breaks are applied for better visualization of the fluorescence spectra detected for longer excitation wavelengths.



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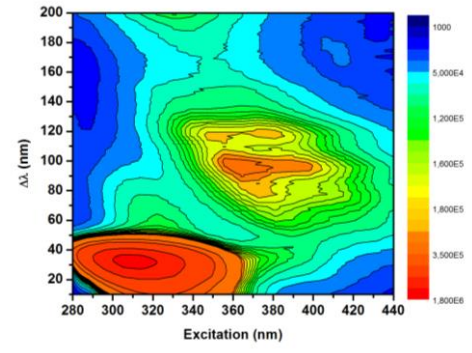

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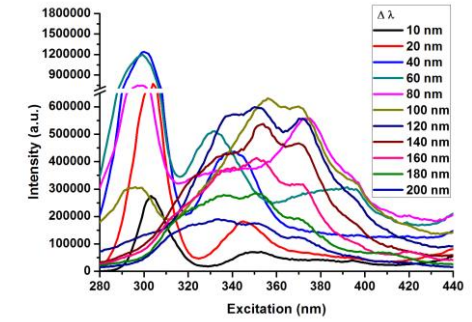

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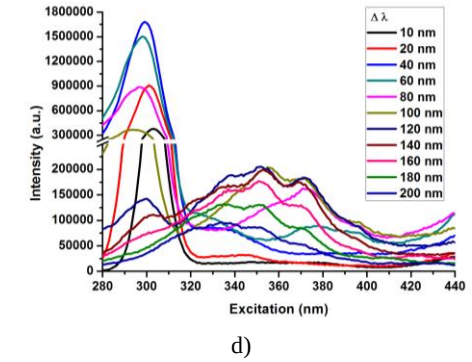

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Fig. 2. Comparison between a) SFS maps of normal tissue sample from colon mucosa and b) SFS maps of colon carcinoma; c) synchronous fluorescence spectra of normal colon mucosa for different delta lambda applied (in the region from 10 to 200 nm, with step of 10 nm) and d) synchronous fluorescence spectra of a colon carcinoma for the same patient on different delta lambda applied (in the region from 10 nm to 200 nm, with step of 10 nm). Breaks are applied for better visualization of the fluorescence spectra detected for longer excitation wavelengths.

Investigating fluorescence through the method of SFS is performed by maintaining constant wavelength interval between excitation wavelength and emission wavelength through the spectrum. This allows optimal excitation of the emission maxima, which result in narrower emission peaks. Narrower peaks, from their side, in the obtained fluorescence spectra allows decrease the extent of the spectral overlaps. That effect is the main reason for the greater sensitivity of SFS in comparison with classic EEMs [1].

On Fig. 2 are presented SFS maps for the normal and abnormal colon mucosa, as well the synchronous fluorescence spectra for different delta lambda applied – from 10 to 200 nm. Synchronous fluorescence spectra are received by plotting the intensity as a function of wavelength interval and excitation wavelength.

In comparison between EEMx data and SFS maps, one could see the increased number of spectral details, related to an appearance of spectral fluorescence intensity islands in the spectral maps and increased number of pronounced autofluorescence maxima of the emission in raw spectra presented on Fig. 1 (EEM data) and Fig. 2 (SFS data).

In the case of SFS spectra of cancerous tissue, additional difference, in comparison with the SFS 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 or it's a result of the lower NADH fluorescence in combination with the superior sensitivity of the SFS to EEMs.

In EEM technique we detected the whole set of internal fluorophores which excitation band consists the applied excitation wavelength. In SFS we detect a signal only when the chosen wavelength interval matches the difference between the absorption and the emission maxima, which results in observing the fluorescence of a particular fluorophore in a multicomponent sample. By choosing wavelength interval near the difference between excitation and emission maxima of the major fluorescence sources observed, we could present SFS spectra of healthy and cancerous tissues with significant differences.

From our previous investigation we found that SFS of normal and cancerous tissue for $\Delta\lambda$=60 nm, $\Delta\lambda$=90 nm and $\Delta\lambda$=120 nm could give maximum differentiation for tumour diagnostics needs [11].

In Table 1 are presented all observed excitation wavelengths and delta lambda applied for normal and cancerous colon mucosa and the addressed endogenous fluorophore, which correspond to this couple – excitation wavelength – delta lambda excitation-emission. In this way we could found all compounds in a current multicomponent system, such as biological tissues investigated.

Table 1. Maxima of excitation and delta lambda of the emission of the tissue endogenous fluorophores observed in the normal colon mucosa and colon carcinoma exhibited under synchronous fluorescence spectroscopy of GIT malignancies.

	Normal colon mucosa	**Colon carcinoma**		
Delta lambda [nm]	**Excitation [nm]**	**Excitation [nm]**	**Endogenous fluorophores**	**Additional comments**
10	303, 348	303	Amino acids – tryptophan, tyrosine	Increased emission for carcinoma lesions vs. normal mucosa
20	304, 345	303, 348		
40	300, 348	299, 340	Amino acids, collagen type I	About two times lower fluorescence of collagen for tumour vs. normal
60	300, 330, 396	298, 329, 378, 398	Amino acids, Collagen type I, NADH	
80	300, 340, 375, 395	295, 357, 372	Collagen type I, NADH, NADPH, collagen-cross links	Higher fluorescence of the normal tissue vs. tumour
100	300, 360, 371, 397	295, 356, 371, 397	Collagen, NADH, NADPH, collagen cross-links	
120	300, 338, 351, 372, 396	300, 338, 352, 370, 396	Elastin, NADH, NADPH, protein cross-links, flavins	Increased ratio NADH/flavins for the tumour sites
140	330, 350, 370	300, 352, 371, 397	Elastin, NADPH, protein cross-links, flavins	
160	337, 351, 372	302, 337, 351, 372	Protein cross-links, flavins, FAD	Higher fluorescence of the normal tissue vs. tumour
180	337, 351, 370	322, 335, 352, 371	Protein cross-links, flavins, FAD, lipids	
200	320, 337, 352, 372	322, 337, 352, 371	Flavins, FAD, lipids, endogenous porphyrins	Appearance of low endogenous porphyrins fluorescence signal in tumour sites

4. Conclusions

In SFS regime of detection we detect a signal only when the chosen wavelength interval matches the difference between the absorption and the emission maxima, which results in observing the fluorescence of a particular fluorophore in a multi-component tissues' sample. By choosing wavelength interval near the difference between excitation and emission maxima of the major fluorescence sources observed, we present SFS spectra of healthy and cancerous tissues with significant differences. Optimal SFS signals of normal and cancerous tissue are found for $\Delta\lambda$=60 nm, $\Delta\lambda$=90 nm and $\Delta\lambda$= 120 nm, respectively In terms of finding the wavelength $\Delta\lambda$ for performing SFS, which results in fluorescence spectra with diagnostically significant differences between fluorescence spectra of healthy and cancerous tissues, the presented $\Delta\lambda$ shows potential for further investigations of the potential implementation of SFS fluorescence technique in the family of optical diagnostic modalities.

Acknowledgements

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