Quantum Electronics **46** (6) 510–514 (2016) ©2016 Kvantovaya Elektronika and Turpion Ltd

DOI: 10.1070/QEL16112

Excitation–emission matrices and synchronous fluorescence spectroscopy for cancer diagnostics in gastrointestinal tract

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Abstract. **We report the development of an improved fluorescence technique for cancer diagnostics in the gastrointestinal tract. We investigate the fluorescence of *ex vivo* colorectal (cancerous and healthy) tissue samples using excitation–emission matrix (EEM) and synchronous fluorescence spectroscopy (SFS) steady-state approaches. The obtained results are processed for revealing characteristic fluorescence spectral features with a valuable diagnostic meaning. The main tissue fluorophores, contributing to the observed fluorescence, are tyrosine, tryptophan, NADH, FAD, collagen and elastin. Based on the results of the Mann–Whitney test as useful parameters for differentiation of gastrointestinal cancer from normal mucosa, we suggest using excitation wavelengths in the range 300–360 nm for fluorescence spectroscopy and wavelengths intervals of 60 nm and 90 nm for SFS.**

Keywords: *excitation–emission matrix, synchronous fluorescence spectroscopy, optical biopsy, colorectal cancer.*

1. Introduction

Colorectal cancer is one of the most common causes of morbidity and mortality worldwide [1]. The incidence of colorectal cancer is increasing [1], and therefore its accurate diagnostics and effective treatment are of primary public health concern. The implementation of more frequent screening has resulted in a decline of the colorectal cancer mortality rate, but in order to achieve a further decrease, improvements of the diagnostic practice are needed [2]. The accuracy of the current standard diagnostic procedure relies entirely on the physician's experience and is limited by a high probability of miss rates and rigorous biopsy protocols, which are costly and time consuming [3]. The potential of the biomedical optical techniques for a detailed minimally invasive or noninvasive analysis of multicomponent substances, such as biological tissues, is being intensively studied for implemention in novel clinical diagnostic modalities for differentiation of cancerous and normal tissues with high sensitivity and accuracy [4].

One of the most intensively investigated optical techniques for an add-on modality to endoscopic gastrointestinal diagnostics is fluorescence spectroscopy [5–9]. Cancerous tissue alterations affect light propagation, absorption properties and fluorophore content in the tissue, to which fluorescence spectroscopy is sensitive. The fluorescence intensity, spectral shape and temporal decay of such endogenous fluorophores as tryptophan, tyrosine, collagen, elastin, nicotinamide adenine dinucleotide (NADH) and flavine adenine dinucleotide (FAD) are very important for diagnostic purposes [10], and so their better understanding and evaluation are necessary for successful introduction of the autofluorescence diagnostics into the clinical practice.

The effectiveness of autofluorescence imaging (AFI) in pinpointing of cancerous tissues in the gastrointestinal tract is implemented in commercially available systems for endoscopic diagnostics [9, 11, 12]. However, there is still a room for improvement of this technique, because the relatively low selectivity and poor image resolution result in a high false positive rate and prevent the AFI technique from its convenient implementation in the clinical practice [5, 13, 14]. The optimisation of available AFI endoscopic systems is mainly directed to noise reduction, colour contrast and evaluation of robust parameters and excitation and/or detection wavelength algorithms [12, 14, 15].

For the standard fluorescence spectroscopy of tissue samples, excitation–emission matrices (EEMs) are a useful tool to provide a fluorescence pattern in a broad spectral range. This three-dimensional fluorescence spectroscopy simultaneously provides information about the excitation wavelength, emission wavelength and intensity of the observed fluorescence. This method allows one to determine the excitation wavelengths inducing fluorescence, whose spectra contain the most valuable data for the clinical diagnostic analysis, and to specify the main endogenous fluorophores in the tissue investigated [16].

A relatively new approach for fluorescence investigations of biological tissues is synchronous fluorescence spectroscopy (SFS). Its application and basic theory have been presented by Lloyd [17] and implemented in biomedical research by Vo-Dinh [18]. The SFS method is based on simultaneous scanning of both the excitation and emission wavelengths, while a constant wavelength interval is kept between them. As a result, in the detected spectrum for a chosen wavelength interval, a relevant spectral feature of the investigated sample will be much more pronounced and spectrally resolvable due to the reduced spectral overlapping. The method enables a greater sensitivity and especially selectivity and, therefore, is a promising instrument for improving the current AFI technique [19].

The SFS application for cancer diagnostics through tissue discrimination and biofluids' analysis has been investigated

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Received 21 April 2016
Kvantovaya Elektronika **46** (6) 510–514 (2016)
Submitted in English

for breast, cervical and thyroid gland cancers, where it proved worthwhile and promising for valuable implementation in the clinical diagnostic practice [18–25]. However we failed to find any evidence for detailed studies of the application of the SFS technique in colorectal autofluorescence cancer detection.

The objective of the present work was to evaluate the potential of SFS for colorectal cancer detection and to determine feasible diagnostic parameters suitable for devising a robust diagnostic parameter.

2. Materials and methods

The investigated tissue samples were excised during a standard surgical procedure for removing gastrointestinal lesions, performed at the University Hospital "Tsaritsa Yoanna – ISUL" (Sofia). All patients received and signed written informed consent approved by the Ethics Committee of the University Hospital. The tissue samples were kept in a modified Krebs safe-keeping solution (NaCl, KCl, glucose, taurine, hepes, piruvic acid, calcium chloride dehydrate) in isothermal conditions and were transported as soon as possible to the Biophotonics Laboratory at the Institute of Electronics. The fluorescence of the safe-keeping solution was evaluated as significantly negligible in comparison with the investigated tissue fluorescence. The shape and dimensions of the samples varied depending on the size and pattern of the excised tumour; however, the thickness of the investigated samples did not exceed 7 mm.

A Fluorolog-3 spectrofluorometer (HORIBA Jobin Yvon, France) was used for the measurements. The system's light source was a Xenon lamp with a power of 300 W, the excitation and emission wavelengths were defined with two double monochromators, and the detector was a photomultiplier. Since our samples varied in shape and dimensions, their fluorescence was investigated with an additional F-3000 fibre-optic adapter (HORIBA Jobin Yvon, France) used with the FluoroLog-3, which allows investigation of samples outside of the sample chamber. During the experiments, the optical fibre of the used fibre-optic module was positioned perpendicularly to the sample and the tip of the fibre was brought in contact with the tissue; therefore, the illumination area of the sample corresponded to the surface of the tip of the optical fibre probe.

We performed fluorescence investigations through EEMs with excitation wavelengths in the range of 280–440 nm with an increment of 10 nm. Fluorescence was detected in the range of 300–800 nm with a scanning step of 1 nm. The EEM data acquisition took approximately 27 min.

The second approach applied was SFS measurements of the tissue samples – comparative measurements of cancerous and normal tissue samples. In our investigations we applied synchronous fluorescence measurements with excitation in the spectral range of 280–720 nm and the wavelength interval $\Delta\lambda$ between excitation and emission wavelengths in the range of 10–280 nm with an increment of 10 nm. A single SFS scan took around 2 min, and since the offset parameter was manually changed for 28 values the SFS matrix scan acquisition time was around 60 min.

Point-by-point spectroscopy measurements were performed for the cancerous part and healthy part of every of the investigated 14 tissue samples of colorectal cancer with similar histopathology diagnosis originated from 11 patients (three tissue samples originated from one patient and two other samples also originated from one patient). Point-to-point variations in signal intensity and band shape for each tissue sample were checked during the experiment. We consider the observed differences between scans to be related to a visually observable higher blood content or a finely furrowed surface of the sample. An average of 3-to-5 points of measurement was used for a sample.

The signal related to the excitation light was manually cleared for the EEM measurements; as regards to the SFS measurements, detection was performed in the regime of correcting the detected signal with the lamp intensity, available in the standard software of the used spectrofluorometer.

3. Results and discussion

Figure 1a shows the EEM of normal colon mucosa and Fig. 1b shows the EEM of a cancerous tissue sample. For better comparison of the EEM results, the same intensity scale is applied in the both cases. In order to evaluate the suitability of SFS for tissue differentiation, we present the results in 3D graphics with two axes presenting the excitation wavelength and the maintained wavelength interval $\Delta\lambda$ during the scan, and colour contour map scheme, which represents the intensity of the observed fluorescence (Fig. 2).

From the spectral data observed, by comparing the intenisty maxima parameters in the coordinates λ_{ex}, λ_{em} and

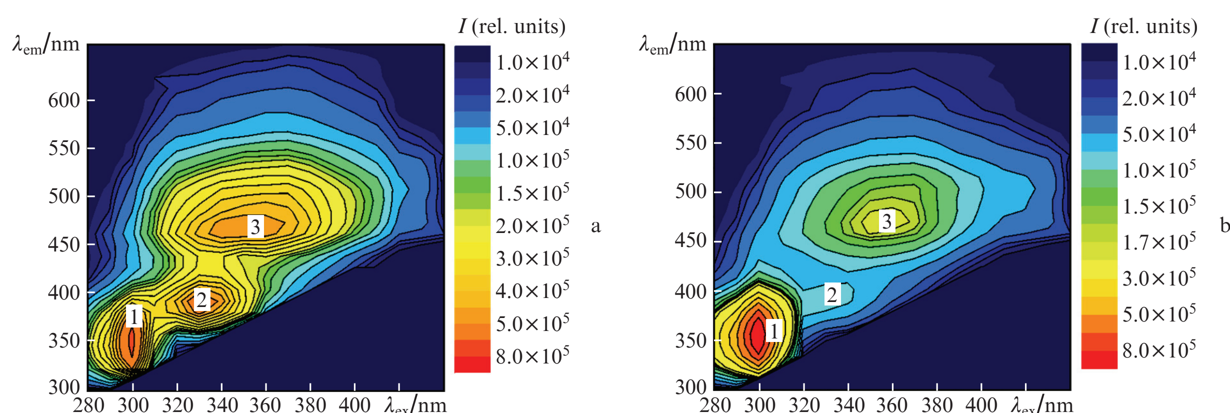

Figure 1. (Colour online) EEM of (a) normal colon mucosa and (b) colon carcinoma. Fluorescence islands are addressed as (1) amino acids (tyrosine and tryptophan), (2) structural proteins (collagen and elastin), and (3) co-enzymes (FAD and NADH); I is the fluorescence intensity; and λ_{ex} and λ_{em} are excitation and emission wavelengths, respectively.

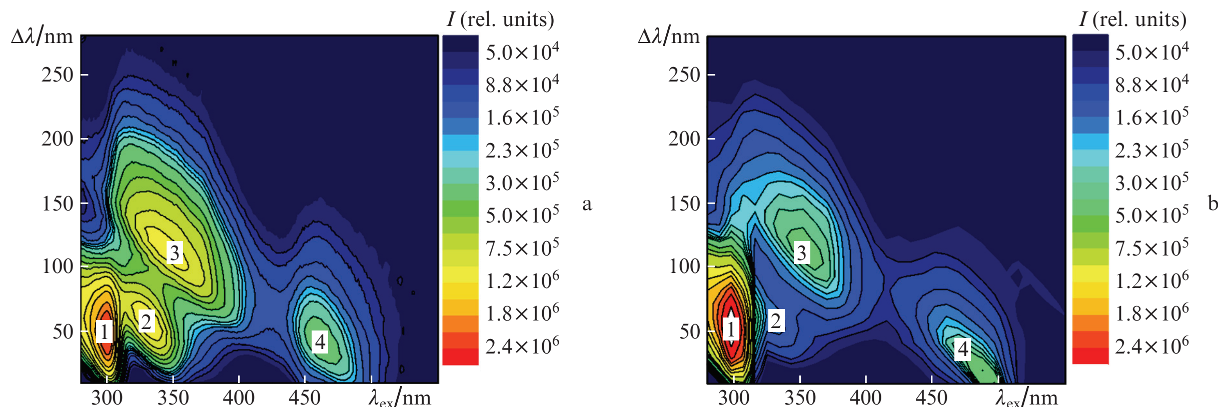

Figure 2. (colour online) SFS maps of (a) healthy and (b) cancerous colon mucosa for $\Delta\lambda$ from 10 nm to 280 nm and excitation in the range of 280–720 nm. Fluorescence islands are addressed as (1) amino acids (tyrosine and tryptophan), (2) structural proteins (collagen and elastin), and co-enzymes (3) NADH and (4) FAD.

λ_{ex}, $\Delta\lambda$ (for SFS data only) with the data for the fluorescence characteristics of the main endogenous fluorophores, presented in the work of Ramanujam et al. [26], we can determine several endogenous fluorophores, as follows: amino acids (tyrosine and tryptophan), co-enzymes (NADH and FAD), and structural proteins (collagen and elastin), as well as their cross-links, as main sources of fluorescence in the investigated tissues. Their major spectral features are presented in Table 1.

Table 1. Excitation and emission maxima of the main endogenous fluorophores, observed in the gastrointestinal tract tissues under study.

Endogenous fluorophore	Excitation maximum/nm	Emission maximum/nm
Tryptophan Tyrosine	280–300	320–400
NADH	340–380	450–500
FAD	460–500	530–550
Collagen Elastin	320–360	400, 460–500

Main differences observed between the fluorescence spectra of healthy and cancerous tissue are in the intensity levels of the fluorescence from amino acids, tyrosine and tryptophan; enzymes and coenzymes, NADH and FAD; and structural proteins, elastin and collagen. The observed increase in the intensity of the fluorescence of the amino acids (tyrosine and tryptophan) arises from the extensive production of proteins, constructed from amino acids, due to the higher metabolic rate of cancerous cells [27]. One of the functions of the NADH coenzyme, which is altered in cancer cells [28, 29], is to be an electron carrier in the cellular respiration process. This alteration could be one of the reasons for the observed lower intensity of the fluorescence maxima of NADH in cancerous cells. Other fluorophores, whose fluorescence intensity is reduced in cancerous cells, are structural proteins – collagen and elastin. The most likely cause for this reduction is the abnormal oversized growth of cancerous cells, which causes disruption of the extracellular matrix, build by structural proteins and their cross-links [30].

The superior sensitivity and reduction of overlapping, which is a characteristic for SFS data, revealed more detailed 3D spectra of the investigated tissues. Comparing Figs 1a and 2a, as well as Figs 1b and 2b, one can clearly see that more spectral details are observed in the SFS regime and fluorescence intensity islands, which are correlated to a given type of the endogenous fluorophore, are better manifested. The SFS technique allowed us to observe distinctive maxima of collagen's fluorescence at λ_{ex} = 350 nm and λ_{em} = 400 nm, whose intensity is lower in the cancerous tissue's fluorescence in comparison with healthy tissue's fluorescence. In the SFS spectra one can also see two separate maxima of the fluorescence of FAD and NADH, which are observed as one common broad maximum in EEM maps.

The fluorescence of fluorophore observed with SFS is better pronounced than the fluorescence of other present fluorophores, when the used wavelength interval matches the difference between the absorption and the emission wavelength maxima. By choosing the wavelength interval near the difference between excitation and emission maxima of the major endogenous fluorophores detected, we obtain SFS spectra of healthy and cancerous tissues with the most significant differences.

The SFS spectra for the 14 samples were normalised so that the area under the curve was equal to unity. This eliminates inter- and intra-patient variations [31]. The averaged normalised SFS spectra of normal and cancerous tissue for $\Delta\lambda$ = 60 and 90 nm are presented in Fig. 3. The SFS fluorescence maxima for the wavelength interval of 60 nm arise from tyrosine and tryptophan as well as from collagen and elastin. Tryptophan and NADH are addressed as the fluorophores, whose maxima are the most intense in the SFS data for the wavelength interval of 90 nm [32–34]. The analysis applied to the SFS spectra is similar to that performed for the SFS spectra of cancerous and healthy breast tissue, proposed by the group of Dramicanin et al. [22]. Two areas of interest were defined: area 'a' ranging from 340 to 380 nm at $\Delta\lambda$ = 60 nm and from 385 to 420 nm at $\Delta\lambda$ = 90 nm, and area 'b' ranging from 380 to 420 nm at $\Delta\lambda$ = 60 nm and from 420 to 500 nm at $\Delta\lambda$ = 90 nm. For the spectral quantification, we used the areas under the normalised spectra in the selected subregions, which donated the parameters S_{a60} (the area under the curve in region 'a' at $\Delta\lambda$ = 60 nm), S_{b60} (the area under the curve in region 'b' at $\Delta\lambda$ = 60 nm), S_{a90} (the area under the curve in region 'a' at $\Delta\lambda$ = 90 nm), and S_{b90} (the area under the curve in region 'b' at $\Delta\lambda$ = 90 nm). Additional six area ratios were included in the analysis: S_{a60}/S_{b60}, S_{a90}/S_{b90}, S_{a60}/S_{b90}, S_{b60}/S_{b90},

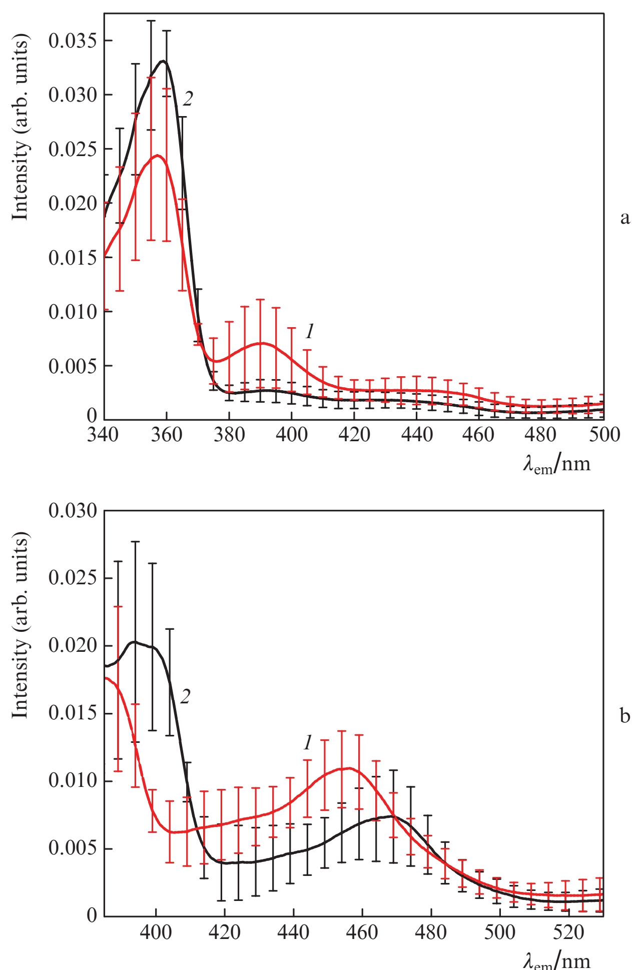

Figure 3. SFS spectra of (*1*) healthy and (*2*) cancerous tissues for $\Delta\lambda$ = (a) 60 and (b) 90 nm.

S_{a60}/S_{a90}, and S_{b60}/S_{a90}. The significance of the chosen parameters for gastrointestinal tissue differentiation was evaluated in two steps. Initially the parameters for every spectrum were calculated and Shapiro–Wilk normality test with the level of significance 0.05 was applied. Some of the parameters failed to meet the criteria of the normality test and, as a result, a nonparametric algorithm for hypothesis testing had to be used. Therefore, we applied the Mann–Whitney test with the standard threshold probability value of 0.05 and the accepted null hypothesis underlying the fact that there is no difference between the parameters for cancerous and healthy tissue. The results of the test are presented in Table 2.

The test shows that five parameters are extremely significant ($p < 0.001$), three are very significant ($0.01 < p < 0.001$) and two are significant ($0.01 < p < 0.05$) in differentiation between cancerous and healthy tissue. Note also that among the chosen parameters to be analysed, not significant parameters ($p > 0.05$) are absent. The results show that the chosen wavelength intervals of 60 nm and 90 nm give enough elements with statistically significant difference to be applicable for tissue discrimination with high specificity.

However the results concern *ex vivo* tissue investigation and in their translation to the *in vivo* case we should take into account a few considerable remarks: 1) the contribution of the haemoglobin will cause a minimum in the fluorescence spectra due to re-absorption at λ_{ex} = 425 nm.; 2) the fluorescence of NADH is expected to be higher, since this coenzyme decays rapidly after tissue excision; and 3) the effect of the temperature of the sample, which is lower than the body temperature at the moment of the measurement [35, 36].

Table 2. Mann–Whitney test results for the evaluated spectral parameters with a probability value p and decision on the level of significance.

Spectral parameter	Probability p	Decision*
S_{a60}	2.1448×10^{-4}	ES
S_{b60}	2.10858×10^{-5}	ES
S_{a90}	7.34006×10^{-6}	ES
S_{b90}	0.04072	S
S_{a60}/S_{b60}	2.10858×10^{-5}	ES
S_{a90}/S_{b90}	0.00405	VS
S_{a60}/S_{b90}	0.00715	VS
S_{b60}/S_{b90}	0.03251	S
S_{a60}/S_{a90}	0.00101	VS
S_{b60}/S_{a90}	7.36123×10^{-6}	ES

*Decision on statistical significance was made on the basis of the probability value for null hypothesis, namely, for $p > 0.05$ – not significant (NS), 0.01 to 0.05 – significant (S), 0.01 to 0.001 – very significant (VS), and $p < 0.001$ – extremely significant (ES) [22].

4. Conclusions

In this study for human cancerous colon tissue classification with fluorescence spectroscopy we suggested excitation wavelengths in the range of 300–360 nm and wavelength intervals of 60 nm and 90 nm for performing diagnostically specific EEMs and SFS, respectively. SFS performed with the suggested wavelength intervals results in the most significant differences, with diagnostic meaning, between the fluorescence spectra of healthy and cancerous tissues.

The parameters evaluated here will be used as a base for the algorithm, whose robustness for colorectal cancer detection, would be tested after gathering more fluorescence spectral data.

Further investigations of the potential implementation of the SFS fluorescence technique for tumour detection in the family of optical diagnostic modalities are necessary, as the initial results showed the feasibility of SFS to receive diagnostically important spectral features with a high potential for the development of discrimination procedures between normal and cancerous colon mucosa.

Acknowledgements. This work was supported by the National Science Fund of the Bulgarian Ministry of Education and Science (Grant Nos DFNI-B02/9/2014 and DMU-03-46/2011). E. Borisova and Ts. Genova personally acknowledge BM1205 COST Action, WFS National Scholarship Programme support and Programme for Support of Young Scientists in the Bulgarian Academy of Sciences for funding within the grant 'Macroscopic and microscopic fluorescence spectroscopy for investigations of tumours in the gastrointestinal tract'.

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