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AUTOFLUORESCENCE SPECTROSCOPY OF MALIGNANT TISSUE

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Diagnosis established by means of fluorescence spectroscopy is currently used in the field of urology and bronchology. Its major advantage is that it allows the diagnosis of epithelial dysplasia or malignant proliferation even if routine diagnostic endoscopy fails to reveal any macroscopic changes. We report the *ex-vivo* investigation of colorectal tissue by autofluorescence spectroscopy. Wet microscopic mounts of healthy colon mucosa were examined and compared with those prepared from colon mucosa affected by adenocarcinoma. The diagnosis of adenocarcinoma was verified by clinical and histology means. Fluorescence spectra of tissue samples, excited by means of 488 and 514.5 nm lines of Ar ion laser and/or by 632.8 nm line of He-Ne laser, have been studied. This study demonstrated differences in both the spectral shape and in the signal intensity (at unchanged spectral shape) of photoluminescence spectra emitted by tissue affected by adenocarcinoma as compared to that of healthy colon mucosa. The study is aimed at development of the diagnostic system and the methodology usable in the clinical practice.

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1. Introduction

Carcinogenesis is a multi-step process driven by the accumulation of mutations resulting in errors in key regulatory mechanisms. Due to those errors the altered cells, initially pre-malignant, then malignant acquire new characteristics that give them advantage over the neighbours and allow for uncontrolled proliferation. It is thus obvious that both metabolism and structure of such cells are different in comparison with the cells in a tissue of their origin. All the molecules constituting a cell can interact with light and their optical properties are determined by both their nature and by properties of their microenvironment. Thus the alterations due to the neoplastic transformation will result in changes of some optical characteristics of transformed cells. Hence, the scattering, absorption or emission of light can yield information about a presence of transformed cells.

The fluorescence spectroscopy provides a unique and sensitive tool to reveal changes in the physical and chemical properties that occur in healthy and abnormal cells in tissues. There are well known intrinsic fluorophors bound to proteins within cells that fluoresce in a wide visible and near infrared spectral region, after being excited by suitable external laser source. The return of excited molecules of the tissue to their equilibrium state is accompanied by emission of corresponding optical radiation. The energy or wavelength of emitted photons roughly corresponds to the energy difference between excited and equilibrium states of the tissue molecular system. The emitted spectral shape is sensitive to the microenvironment such as pH level, redox potential, bonding sites, polarity, ion concentration etc. Natural fluorophors within cells emit luminescence and display a unique set of spectral features which characterise the state of cells making up the tissues. Depending on the environment, flavins and porphyrins are known to fluoresce in 510-530 nm and 590-640 nm regions, respectively. The

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experiments proved the higher concentration of porphyrins in malignant tissues in comparison with that in the normal ones. The excited porphyrins produce the fluorescence in red part of the spectrum [1,2].

Fluorescence techniques are well known for their sensitivity that can be optimized by a proper selection of the excitation and detection wavelengths. Using ultraviolet or visible light it is possible to excite the fluorescence of many biomolecules. Since the biological sources of this fluorescence are endogenous to the tissue, this type of tissue fluorescence is called autofluorescence (AF). The AF response of transformed tissue differs from that of the normal one [3-5]. After illumination by blue light bronchial mucosa emits fluorescent light with a major peak at 520 nm (green) and minor peak around 630 nm (red). In the dysplastic areas or carcinoma *in situ* the fluorescence can be reduced almost ten- folds and a change in proportion of green to red light is observed. Precise mechanism of this effect is not completely elucidated, but most important factors are epithelial thickening, tumor tissue redox changes, increased hemoglobin concentration and reduced fluorophore concentration [3, 4].

The sensitivity of AF spectroscopy enables to discriminate normal mucosa from moderate dysplasia and every more serious lesion. AF spectroscopy, sometimes called optical biopsy, is a method deeply rooted in a traditional molecular spectroscopy and consists in recording an autofluorescence spectrum from a small area of tissue of interest excited with a narrow beam of exciting light. Thus the optical biopsy could be viewed as point measurement technique. Within the framework of this approach it is expected that it could be possible to select such conditions of excitation and collection that the normal and the neoplastic cells would emit different autofluorescence spectra. However, with growing experience it became clear that collected AF spectra may reflect more subtle experimental factors. Consequently various approaches were suggested [4] to correct for such factors and to ensure a sensitive and proper differentiation between normal and diseased tissues. An algorithm based on ratios of the AF intensity in properly selected spectral bands seems to be best suited for practical applications. Thus the optical biopsy may assist in assessing the character of lesions by facilitating the correct decision. It should be noted that in the case of optical biopsy technique, similarly to classical biopsy, it is the examiner who decides on the selection of suspicious areas, and real-time characterization by AF spectroscopy is of great importance.

Ex vivo studies showed that the spectroscopic properties of the overall fluorescence emission of malignant, pre-malignant, and non-neoplastic tissue are sufficiently different to ensure a reliable differentiation in oncological diagnosis [2]. However, the complexity of the biological substrates makes it difficult to ascertain the relationship between the spectroscopic evidence and the biochemical and histogical features of the tissues that could guide the choice of the experimental parameters of fluorescence spectroscopy suitable for an optimal diagnostic scheme.

A quantitative analysis detects intensity changes of fluorescence. A number of reports [6] relay on the observation that under the same excitation and collection geometry the fluorescence intensity of lesions is almost always lower than that of the surrounding normal tissue. Consequently most of the imaging techniques concentrate on the proper calibration technique to correct the effects of autofluorescence signal caused by the varying illumination/collection geometry across the imaged tissue surface. We believe that quantitative analysis, in general, is not as reliable as the analysis and understanding of spectral changes seen in the autofluorescence due to precancerous and cancerous changes in the tissue. To be able to detect small changes in the spectral shape we need not only highly sensitive photoluminescence (PL) spectrometer but also optimal transfer of both exciting and fluorescence radiation to the tissue and the spectrometer, respectively.

In the case of *ex-vivo* investigation the ordinary laboratory equipment for photo-luminescence spectroscopy could be used. However, ***in-vivo*** spectroscopy, particularly diagnostic endoscopy, would require the use of suitable optical fibres for bringing excitation light to the tissue and for collection of tissue fluorescence. Since the duration of *in-vivo* diagnosis should be minimised the spectrum analyser equipped with fixed grating and combined with CCD or diode array detection element should be used.

Lung cancer and colorectal carcinoma are the major candidates for fibre optic application of AF spectroscopy for the early detection of cancerous changes in the tissue.

Lung cancer is a major cause of tumor death in most industrialized countries, claiming an estimate of 1,000,000 lives each year. Its epidemic continues unabated since its single and most important risk factor- smoking remains almost unchanged despite continuing effort of prevention specialists and others. Despite novel combination therapies lung cancer kills over 85% of those it afflicts within five years. The close inverse correlation between tumour stage and survival is well documented. Even in the case the tumour is radically extirpated the risk of developing a second lung cancer is approximately 4% per patient per year. When lung cancer is diagnosed in early stages, the survival is excellent. After resection of early small lesions it can be more than 80% after 5 years. That means that detection of lung

cancer at early stages improves prognosis, yet early disease is asymptomatic and hence difficult to uncover. Only 15% of lung cancers are discovered when the disease is still localized. With our current diagnostic technology , by the time lung cancer reaches a point at which it is clinically detectable, the disease is already in the late stages of its natural history and it is only a couple of doublings from reaching a lethal tumour burden. Lung cancer tumour burden typically exceeds 1,000,000,000 cells at the time of diagnosis (a volume of 1 cm^3).

Optic fibre bronchoscopy has not been consistently able to identify pre-neoplasias. 30% detection rate in carcinoma *in situ* (CIS) was reported [2, 7]. This is because of the predominant intraepithelial growth pattern of most early lung cancers. Recently autofluorescence bronchoscopy has been shown to be able to increase the detection rate two or three-fold [8].

At present the colorectal carcinoma is the most frequent type of the digestion tract cancer in the Czech Republic (CR). The number of newly emerging cases of this type of disease reaches almost 7,000 a year and what is even more important the increasing trend does not seem to change in the nearest future. The data clearly demonstrate that within 40 years (1960-1999) the absolute incidence (men + women) of colorectal carcinoma increased roughly 3.5 times. The increase is 4 times in the case of men and 3 times in the case of women. It is also seen from available data that the increase of newly emerging cases in time could well be approximated by linear dependence. Let us describe the absolute incidence of colorectal carcinoma as *I* Thus the absolute incidence (men + women) of colorectal carcinoma as a function of time could be described by the following expression: $I = 1{,}376 + 144 * (Y-1960)$, where *Y* describes the year following 1960. The parameter 144 (the rate of increase) characterises the current situation and the trend in the near future, here in the CR.

Similar curves that describe trends of the disease development in other countries show the tendency to saturate or even the decrease in the rate of absolute incidence. Unfortunately the situation in the CR is still characterised by steady linear increase with the rate given by 144.

In this paper we report the investigation of colorectal tissue by measuring the autofluorescence emission in the range 450-800 nm excited by various laser sources. Measurements by both the laboratory PL spectrometer and by the portable spectrometer equipped by the optic fibre connector are reported and compared. In view of the fact that measurements are performed in the visible and near inrared region, plastic fibers have been used. However, the AF in the infrared (IR) is expected to be equally important [9] but its exploitation is hampered at this time by poor detection in IR region. When this limitation is overcome the importance of fibres drawn from special glass materials, such as chalcogenide glasses, will become evident.

Another example of the urgent need of fibres transparent in the IR has been reported recently [10]. It was the measurement of the temperature difference of cancer and normal tissue in the tracheobronchial tree by contact thermometer monitoring. This supports the hypothesis that tumour tissue has significantly higher temperature compared to normal mucosa. The temperature difference between cancerous and normal tissues has been found to be 1-2°C. Thus the thermal emission of tumour tissue could be one of the target values in the non-invasive lung cancer diagnostics.

In view of this, the imaging of spontaneous thermal emission from bronchial tissue would be a valuable diagnostic technique. However, the spontaneous emission from human tissue falls into the IR spectral range with maximum intensity around 10 μm and consequently fibres or bungles transparent in IR must be used in order to reach compatibility with bronchoscopy. Thus optical fibres on the base of special multicomponent glass systems, such as chalcogenide glasses, must be implemented to effectively transmit thermal spontaneous radiation out of the bronchial tree for detection and analysis.

2. Experimental

Our study was aimed to verify the theoretical premises and to compare sensitivities of laboratory photoluminescence spectrometry with the portable spectrometer compatible with optic fibres. The laboratory photoluminescence spectrometer based on the 1 m focal length monochromator cooled with the liquid nitrogen. Ge detection system or cooled GaAs photo-multiplier enables sensitive and high resolution measurements in the spectral range 400-1700 nm by using the lock-in technique and the computer controlled data collection. He-Ne and Ar ion lasers are available for excitation. Avantes portable spectrometer AvaSpec-2048 with fixed grating, fibre connector and CCD linear array detector with 1,024 elements was used for comparison to simulate conditions in clinical practice. In view of the fact that the intensity of the tissue autofluorescence is relatively weak, the major goal was to assess the

influence of the fibre optics and low sensitivity portable spectrometer by using the same excitation sources.

Autofluorescence spectra emitted by normal colon mucosa and other tissue components have been compared with those emitted by tissue affected by colorectal carcinoma. The probes were taken during radical surgeries performed in order to treat the colorectal carcinoma. First of all, both the histology of healthy mucosa and histology of adenocarcinoma were examined by using quick biopsy. The final histological examination followed. The wet probes were taken from tumour centre and from oral edge of the cut piece that should have consisted of healthy mucosa. The induced fluorescence produced by glass slides, cover glasses and all materials used within the probes testing was measured in order to avoid possible luminescence from this material

Two groups of tissue samples have been measured. One group consisted of samples that had been preserved in the fridge at the maximum temperature of 5°C before they were examined. Native samples that were immediately after the surgical operation transferred into the optical laboratory for characterization fall into the second group.

AF spectra collected by laboratory PL spectrometer equipped with collecting lens of large diameter are compared with spectra collected by using portable Avantes spectrometer and optics fibres for both excitation and collection of AF. Plastic fibres with a diameter of 600 μm have been used.

3. Results and discussion

Only thick samples (of the order of 1-2 mm) turned out to be useful in order to supply a measurable luminescence signal. Thin samples routinely used for optical histology analysis could not be measured even on highly sensitive laboratory PL spectrometer. AF spectra measured by the laboratory PL spectrometer are reported first. In this case mirrors and the lens have been used to direct the excitation source to the measured sample and to collect the laser induced fluorescence, respectively. Figs. 1 and 2 show PL spectra of samples when carcinoma is manifested by spectral changes as compared to control normal tissues. Samples whose spectra are shown in Fig. 1 were kept in the fridge while spectra on Fig. 2 were measured on fresh samples. Fluorescence spectra of human colon tissue taken at room temperature and excited by He-Ne laser are shown in Fig. 1. Several samples of normal and malignant tissues have been measured. The He-Ne laser has been used for excitation so that only that part of tissue autofluorescence (photoluminescence) exceeding 632.8 nm could be observed. In view of the fact that the fluorescence signal is relatively weak it cannot be observed simultaneously with the scattered laser one, when the two signals are not sufficiently separated. Thus in the case of normal tissue which does not generate any fluorescence in the studied range, we can see just the tail of the scattered exciting laser signal. In the case of malignant tissue we can see a broad luminescence curve centred around 670 nm that is superimposed on the tail of scattered laser signal. It is known that fluorescence bands at about 500, 550, 640 and 680 nm are important for fluorescence diagnostics of the tissues. When He-Ne laser is used, only the band at 680 nm could be observed. Our observation supports the opinion that the autofluorescence spectral shape enables to distinguish malignant from normal colon tissue.

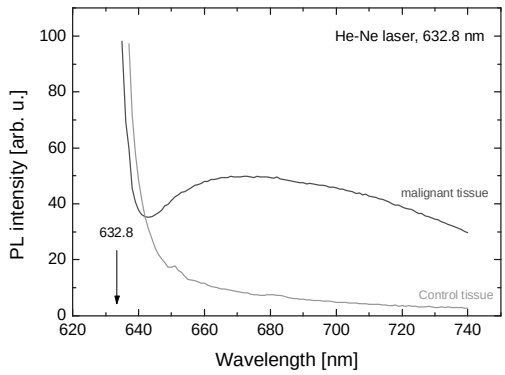

Fig. 1. Fluorescence spectra of human colon tissue taken at room temperature and excited by He-Ne laser at the power density of 200 mW/cm^2. The arrow indicates the wavelength 632.8 nm of the exciting laser. In the case of control sample (normal tissue) only the tail of scattered laser signal is detected; there is no luminescence from the sample. In the case of malignant tissue we can see a broad luminescence curve centred around 670 nm.

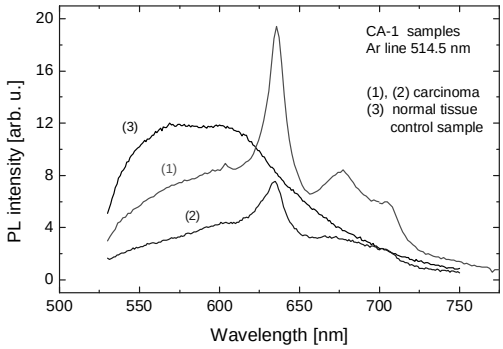

Fig. 2. Autofuorescence spectra of native human colon tissue taken at room temperature and excited by 514.5 nm line of Ar ion laser at the power density 100 mW/cm^2 are shown. Curves (1) and (2) correspond to tumour tissues while the curve (3) corresponds to normal – control tissue. A considerable increase in fluorescence intensity at 630 and 680 nm bands is observed for tumour tissue samples.

When Ar ion laser has been used for excitation, the biggest differences were measured at the autofluorescence band of 590 nm, as shown in Fig. 2. It can be seen from Fig. 2 that new PL bands appear at 630 and 680 nm in the case of malignant tissue. This roughly coincides with changes observed in the case of excitation by He-Ne laser line at 632.8 nm.

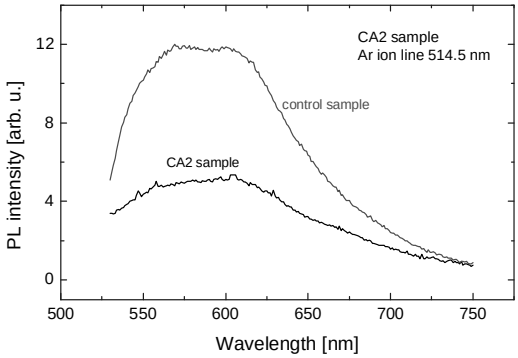

Fig. 3. AF from control and malignant colon tissues which shows only the decrease in intensity but little change in the spectral shape.

However, the presence of carcinoma does not necessarily leads to spectral change. In Fig. 3 are shown PL spectra of samples where the presence of carcinoma does not change the spectral shape but considerable decrease of luminescence signal is observed on malignant samples. Spectra shown on Fig. 3 are taken on native samples.

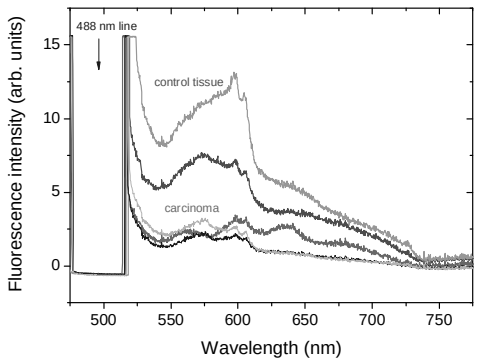

Fig. 4. AF spectra collected from normal and malignant part of colon tissue are shown. Spectra were single fibre collected and detected by Avantes spectrometer.

Ar ion laser (488 nm line) coupled to the optical fibre has been used for excitation of native samples transported in the fridge from the hospital to the lab. A system of two fibres coupled at one end has been used for the excitation of the tissue and for the collection of AF signal from the same spot. We did not have a suitable interference long-pass filter to block the exciting laser light from entering the fibre connector of the spectrometer and thus the measured spectra are distorted by laser tail, since the spectral range of interest is too near to 488 nm laser line. Spectra collected by single fibre and analysed by Avantes fixed grating spectrometer are shown in Fig. 4. Spectra show qualitatively the same behaviour as those in Figs. 2 and 3, namely a considerable decrease in intensity in the range 530-620 nm and a relative increase in intensity in the range exceeding 620 nm. However, some regions of the measured tissue show only decrease in green part of the spectra without any increase in the blue part. It turns out that it is important to incorporate into the optical path a low-loss optical element properly coupled to fibres that would enable to insert suitable long-pass filters.

4. Conclusions

The Ar ion and He-Ne lasers seem to be suitable sources for tissue excitation. The alternative source could be the He-Cd laser using the wavelength of 442 nm in combination with the He – Ne laser. (GaN diode laser at 405 nm could also be used for optic fibre excitation) More experiments and careful comparison of luminescence spectroscopy with optical histological examinations are needed to understand the changes in the tissue that are manifested by considerable decrease of AF intensity (when going from healthy to tumour tissue) without significant change in the spectral dependence. On the other hand, the observed spectral differences are believed to be attributed to the transformation of local environment surrounding the fluorophors assigned to flavins and porphyrins in the normal and cancerous tissues. However, conditions when cancerous and precancerous tissues yield different AF spectra as compared to normal tissue and when AF signals differ only by intensity, remains to be clarified. Experiments with laboratory PL spectrometer show that AF spectroscopy is a promising technique that might help to identify the early steps of displasia. The endoscopic *in-vivo* system would improve the surgical treatment of rectal carcinoma due to potential precise determination of resection line. In addition, it could allow routine control of anastomoses resulted from low resections of the rectal carcinoma as well as accurate biopsy focusing. Further work is needed to be able to detect the malignant colon tissue and differentiate it from the healthy colon tissue by using AF spectroscopy coupled to endoscope. The optic fibre system compatible with the endoscope requires suitable semiconductor laser diode or other source properly coupled to the fibre and optimal blocking of the exciting radiation from entering the spectrometer.

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