Polarization-sensitive autofluorescence spectroscopy of non-melanoma cutaneous tumours

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I*n vivo* and ex vivo point-by-point measurements of excitation-emission matrices (EEM) from skin tumours using linear polarizer and analyzer for excitation and emission light were carried out. The fluorescence spectra obtained reveal differences in spectral intensity, related to general attenuation, due to filtering effects of used polarizer/analyzer couple. Significant spectral shape changes were observed for the complex autofluorescence signal detected, which correlated with collagen and protein cross-links fluorescence, that could be addressed to the tissue extracellular matrix and general condition of the skin investigated, due to morphological destruction during lesions' growth.

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1. Introduction

Fluorescence polarization measurements could be applied to estimate various parameters of the fluorophore environment, and they have the potential role to discriminate between normal and malignant tissues for the needs of biomedical diagnostics. At polarized light excitation, the emission from a fluorophore in a non-scattering media becomes depolarized because of the random orientation of the fluorophore molecules and the angular displacement between the absorption and emission dipoles of the molecules [1]. The level of light depolarization in the tissues depends from the number of scattering steps of the excitation and fluorescence photons in their way to and from the given fluorophore in the sample investigated. In this way both – the excitation and the emission are depolarized in strongly scattering media, such as human skin. Nevertheless, the normal and diseased tissues could have different scattering coefficients, and respectively – different depolarization levels could be obtained. In malignant skin the extracellular matrix is changed and the scattering due to the structural proteins, such as elastin and collagen is different from normal to diseased tissue, and these changes give diagnostically important information about the tissue condition [1, 2].

Many investigators work on polarization fluorescence applications for skin cancer detection. Several groups already reported newly developed imaging systems for polarization-sensitive fluorescence for tissue analysis with very promising results [3-6]

Our group has significant experience on autofluorescence investigations of skin tumours and polarization technique could allow us to improve the diagnostic accuracy achieved up to now (93 %) for such severe pathologies, as malignant melanoma lesions [7-9]. We are interested to analyze the feasibility of fluorescence polarization technique for the clinical needs of primary diagnosis of skin neoplasia, without adding of contrast agents, due to the patient convenience.

2. Materials and methods

In our study we measured excitation – emission matrices (EEMs) for normal human skin in vivo, and ex vivo non-melanoma cutaneous tumours. The tumours are received after surgical excision during standard cancer treatment procedures. Ethical approval for our investigations of human skin was received from the Ethical Committee of University Hospital "Queen Jiovanna-ISUL" – Sofia, in the frames of research project DMU-03-46.2011.

We used spectrofluorimeter FluoroLog 3 (HORIBA Jobin Yvon, France) with fiber-optic module - F-3000 with fiber optic probe - 1950-1M that allows measuring the fluorescent properties of samples which cannot be put in a standard cuvette. Using this system could perform measurements of the excitation and fluorescence spectra, excitation-emission matrix and time-resolved regime of the fluorescence signal of biological tissues including in vivo. Three different situations were evaluated and corresponding excitation-emission matrices were developed – with parallel and perpendicular positions for linear polarizer (for the excitation) and analyzer (for the emission), and without polarization of excitation and fluorescence light detected.

Autofluorescence spectroscopy measurements, using different excitation wavelengths for the needs of EEM development of fluorescence data, were carried out. Excitation applied was in 280-440 nm region. The fluorescence emission was measured between 300 nm and

650 nm for all three situations of polarizer positions in the spectrofluorimeter set-up. Analysis of the spectra on intensity and spectral shape changes for the three variants of polarization, as well as evaluation on the principal fluorescent maxima observed of the polarization P are carried out.

Ratio of the maxima at 450 and 400 nm, related to the emission of co-enzyme NADH and collagen fluorescence, is also evaluated. We found that this ratio could be used as indicator of structural changes in the human skin in vivo and could be useful indicator for the forthcoming needs of development of a polarization fluorescence spectroscopy modality for cancerous skin diagnosis.

3. Results and discussion

The fluorescence spectra obtained reveal differences in spectral intensity, related to general attenuation, due to filtering effects of used polarizer/analyzer couple. Significant spectral shape changes were also observed for the complex autofluorescence signal detected, which correlated with collagen and protein cross-links fluorescence, that could be addressed to the tissue extracellular matrix and general condition of the skin investigated.

The EEM matrices are developed for parallel and perpendicular positions of the polarizer/analyzer couple. When polarized excitation is applied the fluorescence signal is also polarized [1, 2]. The depolarization depends from the number of multiple scattering processes in the tissue during the light transport – of the excitation and of the induced fluorescence. In biological tissues, such as skin, where strong scattering has place the depolarization effects are very strongly revealed. However, non-scattered excitation and fluorescence part could be evaluated in a comparison of the fluorescence signals detected in parallel and perpendicular positions of the polarizer-analyzer couple, fig. 1. Decreased fluorescence intensity is related to the losses of non-depolarized fluorescence component coming from the fluorophore emitting in the given spectral range.

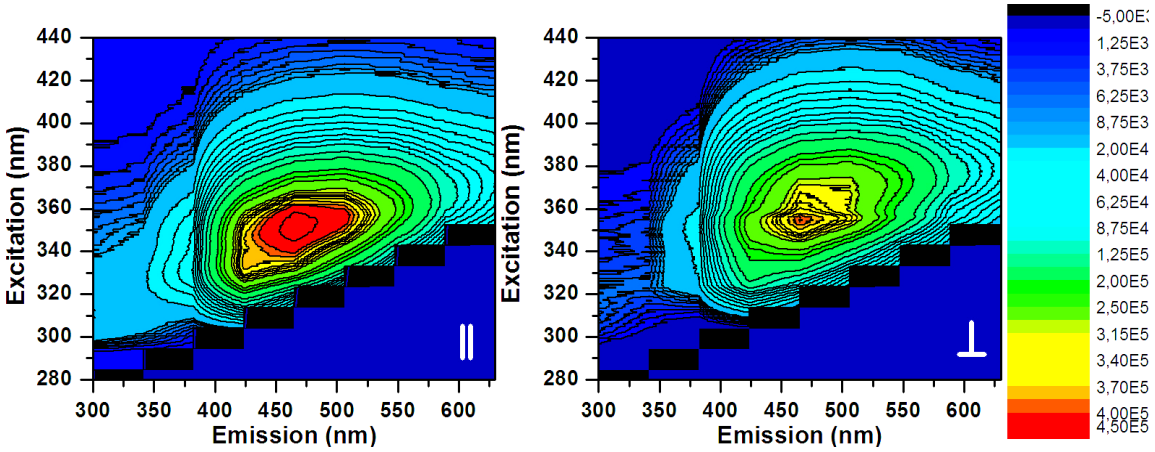

Fig. 1. Excitation-emission matrix of human skin in vivo when the excitation and emission are detected after passing of parallel (left) and perpendicular (right) positioned polarizer and analyzer plates.

Fluorescence maxima observed in our measurements are addressed according our knowledge and the previous reports of different investigators, working in the field of autofluorescence detection of skin tissues. In general, the fluorescence intensities depend from the skin photo type of the person investigated [10].

Significant differences are observed in comparison of EEMs data of normal skin and BCC lesions fluorescence, which difference is even more pronounced in the case of polarizers' application. We observed general decrease of long-wavelength part of the fluorescence in the case of tumour vs. normal skin and rapid increase of amino acids emission due to neoplastic changes in the tissue.

On fig.2 are presented comparisons of excitation – emission matrices and autofluorescence spectra of normal skin and basal cell carcinoma lesion from one patient for better comparison of deviations between healthy and diseased tissue fluorescence emission for whole set of excitation from 280 to 440 nm.

Significant differences in the autofluorescence signals obtained for the cross-polarized cases on volunteers with normal skin were observed, as the maximum at 400 nm, corresponding to the collagen autofluorescence, depends strongly from the ages of the person investigated. The fluorescence signal at 400 nm, obtained from older persons after excitation at 280-320 nm region, is higher by intensity that for the younger ones. In the longer wavelength spectral region, for excitation range longer than 360 nm, when the co-enzimes, 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.

When the polarized fluorescence signals are compared, the polarization ratio $P = (I\parallel - I^{\perp})/(I\parallel + I^{\perp})$ could be calculated for the parallel and cross-polarized signals detected from the different patients.

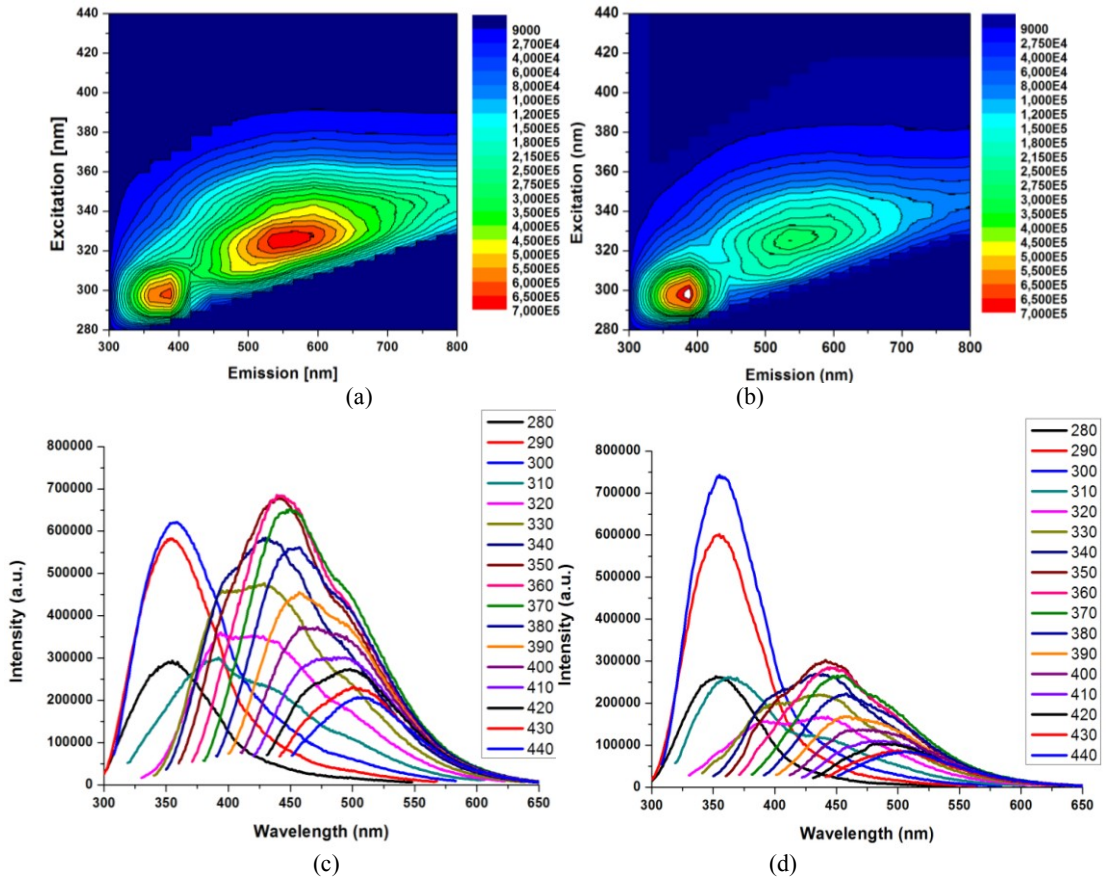

Fig. 2. Comparison of excitation-emission matrices of normal skin (a) and basal cell carcinoma (b) and comparison of raw autofluorescence spectra of normal skin (c) and basal cell carcinoma (d) for excitation wavelengths in the region from 280 nm to 440 nm

The polarization is decreased with increase of the fluorescence wavelength for the patients below 40 years old and for the higher ages the polarization is increased with the wavelength of the fluorescence detected. These relations were observed for the different excitation wavelengths applied. Polarization is calculated for the principal fluorescence maxima observed, addressed as follow: tryptophan - 360 nm, collagen- 400 nm, NADH – 450 nm and collagen cross-links – 490 nm.

This correlation to the structural proteins influence on the polarized measurements is observed in the case of non-melanoma tumours investigated as well, namely for basal cell carcinoma lesions, see Fig. 3. Here, the strong influence on the fluorescence level of the signal is observed on the maxima, related to structural proteins and their cross-links as well, namely maximum at 400 nm, related to collagen I type fluorescence and maximum at the region of 480-500 nm, related to protein cross-links signal.

Other significant difference observed in such comparison for couples normal-abnormal tissue with parallel positions of the excitation-emission polarizers and normal-abnormal tissue with perpendicular positions of the excitation-emission polarizers is related to the significant decrease (about 2 times) of the protein cross-links fluorescence. This change is not a result of the application of different geometry of the polarizer and analyzer, because it appeared in both positions of the polarization elements. The observed decrease is a result of extracellular matrix demolition and partial destruction in the case of tumour development.

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 skin 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.

Other researchers reported that all compounds - collagen, elastin, co-enzymes NADH and flavins contribute to the polarized fluorescence spectra and the spectra received at a longer wavelength of 460 nm. The contribution of NADH dominates with excitation at 340 nm and different forms of flavins dominate with excitation at 460 nm [11].

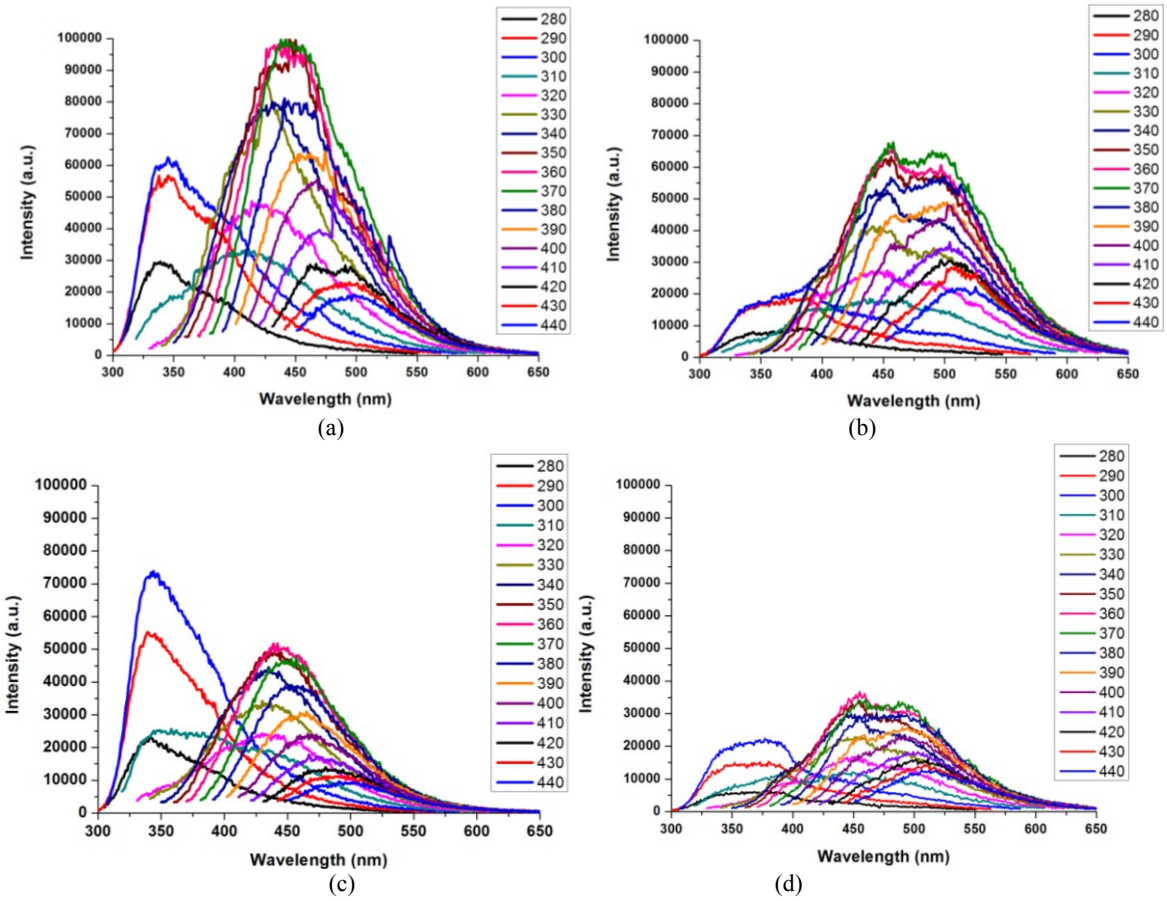

Fig. 3. Comparison of autofluorescence spectra of normal skin (a) and basal cell carcinoma (c) using parallel positions for the excitation and emission polarizers and comparison of the autofluorescence spectra of normal skin (b) and basal cell carcinoma (d) for perpendicular positions of the excitation and emission polarizers. Autofluorescence spectra are received using excitation wavelengths in the region from 280 nm to 440 nm, step 10 nm.

The slight spectral shift of the maxima between the parallel and cross-polarized fluorescence spectra was observed for thick tissue layers (≈ 2mm), or as in the case of in vivo measurements, that could be associated to the absorption properties of the tissue investigated. In these measurements, the most significant alterations observed between parallel and cross-polarized fluorescence were for the short wavelength spectral region of excitation lambda applied – 280-360 nm, where the structural proteins excitation spectra have place.

4. Conclusions

Skin cancer diagnostics could be developed based on the difference in metabolism and structure for normal and diseased tissues. The fluorescence anisotropy measurement of skin shows its high sensitivity to the structural and morphological changes, related to aging of the skin. The correlation between such small changes, related to the extracellular matrix decrease of structure and integrity could be more pronounced in the case of skin lesions, where such integrity and structural matrix are partially demolished due to the lesion growth. The polarization fluorescence 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 skin benign and malignant lesions. Our preliminary investigations on measurements of healthy human skin *in vivo* allow detecting the correlation between polarization vs. wavelength of the fluorescence detected and age of the skin samples investigated.

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