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BIOLOGICAL AND TECHNICAL FEATURES AND PROBLEMS OF THE FLUORESCENCE SPECTROSCOPY METHOD

Fluorescence spectroscopy (FS) has recently become more common in clinical medicine. However, there are still many unresolved issues related to the methodology and implementation of instruments with this technology.

Here we provide evidence that the level of blood volume in tissue impacts FS data with a significant inverse correlation. The effects of various physiological (different content of skin melanin) and technical factors on the measurement results were additionally studied.

The data on the variability of the measurement results in FS should be considered when interpreting the diagnostic parameters, as well as when developing new algorithms for data processing and FS devices.

Keywords: non-invasive diagnostics, fluorescence spectroscopy, fluorophores, metrological support.

Introduction

Fluorescent spectroscopy (FS) is becoming more widely used in chemistry, biology, in various fields of medical technology and medicine in general. These methods are highly sensitive and provide a unique opportunity to study the excited states of molecules, photochemical reactions, dynamics of fast molecular processes, structures, and properties of complex biochemical and cellular systems [1, 2]. The FS provides effective and non-invasive optical diagnostics, primarily in medical areas such as oncology, transplantation, cosmetology and surgery [3]. The FS method is based on exciting fluorescence from tissue endogenous and exogenous fluorophores and recording the emission in the visible spectral region. FS is a reliable method to differentiate benign and malignant tumours of various origins. FS is also used as a tool to monitor the dynamics of the processes occurring in the tissues, during cancer treatments such as radiotherapy. Many purulent wounds, burns and other destructive inflammatory processes are accompanied by changes in the fluorescent activity of the tissues, which occurs due to a misbalance in accumulation of natural fluorophores: FAD, NADH, lipofuscin, porphyrins, structural proteins, etc.

However, fluorescence spectroscopy of biological tissue is a complicated technique that depends on the temperature, topological heterogeneity, different properties of each sample, etc. Therefore, the reliability of FS is affected by multiple factors, including the availability of data concerning the scattering and absorbing properties of specific tissues in specific conditions, light pollution at the optical fibre tip and instrument errors such as excitation source instability, photodetector limitations, light filter precision, grating precision, CCD performance, etc. This study aims to fill some gaps FS method.

Experimental studies

Experimental studies (Table 1) were carried out with the participation of eight healthy volunteers with different ethnic type of the skin: Caucasian (3 volunteers), Middle Eastern (1 volunteer), Indian (1 volunteer) and African (3 volunteers). The measurements were performed at two points on the skin: skin pads (palmar surface) of the right middle finger; on the outside of the right forearm. All measurements were performed daily at 11:00 am to avoid any influence of circadian rhythms on the blood circulation. The measuring fibre was positioned in the same place, without applying any pressure. Local light pollution and other environmental factors that could cause possible errors were mitigated.

Table 1 – Number of spectral recordings

Ethnic skin types	Fingertip	Forearm
	UV (365 nm)	
Caucasian	200	150
Middle Eastern	13	13
Indian	27	27
African	3	3
Total	243	193

A multifunctional laser non-invasive diagnostic system (MLNDS) “LAKK-M” (SPE “LAZMA” Ltd, Russia) [4] was developed for research and diagnostics in various fields of biomedicine (cardiovascular diseases, diabetes, skin disorders, cancer, cosmetic surgery, etc.) and was used in this research. This system includes 4 channels in one device: tissue reflectance oximetry (TRO), laser Doppler flowmetry (LDF), pulse oximetry and fluorescence spectroscopy. In these studies, involving all two measurement channels, FS and TRO data were analysed.

Results

Effect of the tissue blood volume level

The intensity of the backscattered radiation parameter was analysed. This depends on the blood supply to the skin and may change during pathological processes in tissues, thus bearing additional diagnostic information. To assess the nature of this effect on volunteer, consistent measurements were carried out over 30 days on the two study areas of the skin, recording intensity of backscattered radiation $I_{bs}(\lambda)$ and the level of the tissue blood volume V_b (TRO-channel).

To study the dependence of the fluorescence on blood supply, measurements have been performed in an area with subcutaneous hematoma. Here, the basic absorber is haemoglobin, the absorption maxima of which are in the range of 400-550 nm. This range falls in the recorded fluorescence under UV excitement. The fluorescence of normal (intact) tissue was measured along with the fluorescence of the hematoma, at a distance of not more than 5 mm from the object. Registered spectra are shown in Fig. 1.

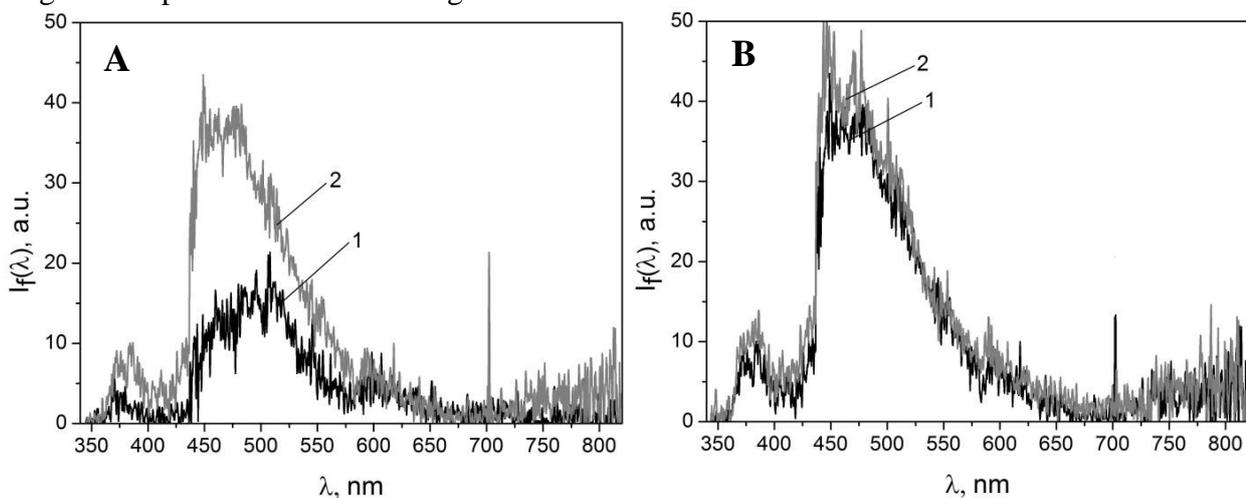


Figure 1 – Examples of the fluorescence registering the third day (a) and eighth day (b). 1 – hematoma, 2 – intact tissue

Effect of the skin melanin

Additionally, as is known, fluorescence is dependent on the pigmentation of the skin. Thus, this part of the research studied the effect of the skin melanin on the measured parameters in skin areas on different ethnic skin types including Caucasian, Indian, Middle Eastern and African (with a range of melanin content approximately from 1% to 43%) [18]. Using the maximum amplitude of the intensity of the fluorescence spectrum $I_f(\lambda)$ NADH and FAD and computational parameter – the redox ratio, RR. The results are shown in Table 2.

Table 2 – Results of the study of effect of the skin melanin

The laser wavelength, nm	Ethnic skin types	$M_n \pm \sigma$, AU					
		Fingertip			Forearm		
		NADH	FAD	RR	NADH	FAD	RR
UV	Caucasian	175.2±48.9	49.2±11.8	3.6±0.3	57.1±11.8	21.3±3.3	2.7±0.2
	Indian	77.0±22.3	25.2±6.6	3.1±0.5	35.9±14.2	15.4±5.1	2.3±0.5
	Middle Eastern	99.3±47.7	31.5±12.6	3.1±0.3	14.9±3.9	8.9±2.5	1.7±0.4
	African	60.4±30.5	22.3±17.5	2.7±0.4	→ 0	→ 0	–

Also separately research was carried out on a 25 year old female with a high level of melanin. Measurements were carried out, as previously, on two skin zones: a weakly pigmented finger pad and forearm area with a high content of melanin. The results are shown in Fig. 2.

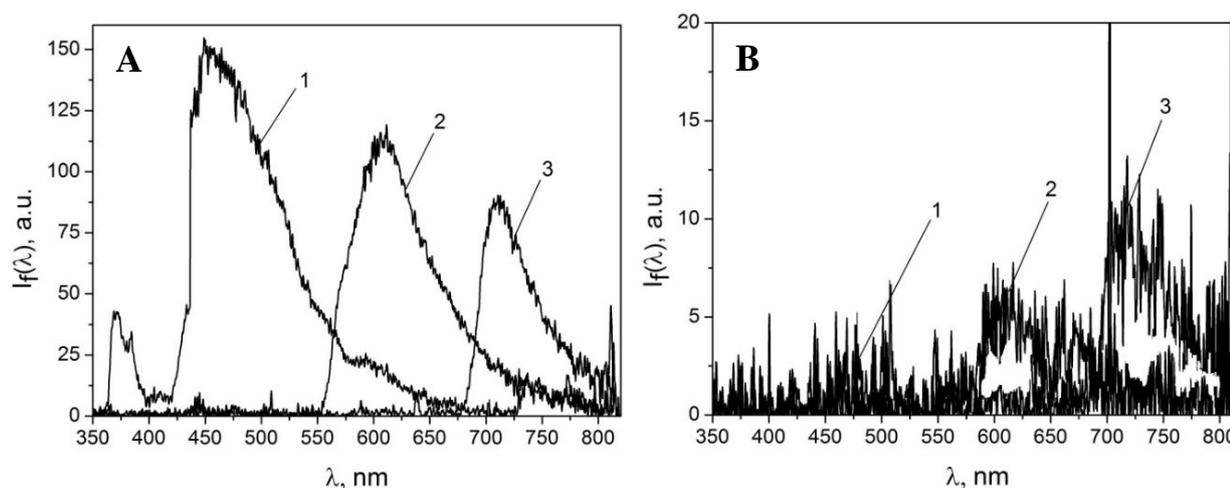


Figure 2 –The fluorescence spectra of skin with high levels of melanin, a – fingertip; b – forearm: the UV (1), green (2) and red (3) light excitation wavelengths

Effect of technical errors

Experiments were conducted to assess the performance of 2 identical FS-channels. Measurements were conducted on a solution of porphyrin in PBS solution. The cuvette with the biomarker was placed on a white PTFE reflection standard. A concentration of 0.25 mM was used. The depth of immersion of the fibres in the cuvette was about 5 mm. Figure 3 shows some

porphyrin fluorescence spectra (Figure 3a and 3b for green and red fluorescence excitation lines respectively).

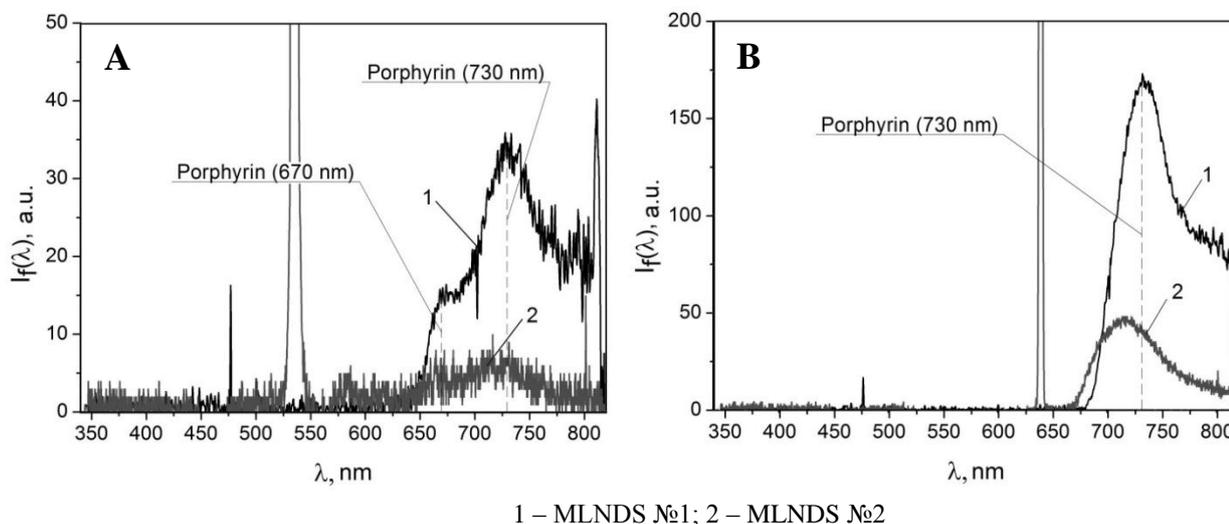


Figure 3 – Examples of fluorescence spectra: for porphyrin on the green (a) and red (b) lines excitation

In addition to physical errors, there were also a number of technical errors in the method. Thus, even the minor parameter changes in optical radiation of the device, differences between separate receiver sites and individual nodes of the optical system, may visibly influence the shape of the spectral curve and, consequently, the final diagnostic result when measuring the fluorescent spectra.

Discussion

Effect of the tissue blood volume level

In order to analyse this type of data, it is necessary to apply non-parametric methods of correlation analysis. This is due to the nonlinear coupling between the features, the lack of data on the distribution, as well as a small number of observations ($n=30$). Therefore, the direction and strength of correlation was found using the Spearman rank correlation coefficient. The results were $r_1 = -0.672$ for the surface of the finger, and $r_2 = -0.479$ for the forearm, indicating a moderate inverse correlation. The significance of the correlation coefficient was evaluated using the Student t-test, giving value of 0.05 and indicating that the relationship is significant. The true value of the correlation coefficient for the finger surface and the forearm is in the interval between $(-0.84; -0.5)$ and $(-0.72; -0.24)$, respectively. Thus, the data obtained on the strong influence of the level of the tissue blood volume on the registered signal in the FS should be considered in the formulation of medical and technical requirements for devices of this type.

Also, as seen from Fig. 1, by day 8 the reduction of the hematoma resulted in increased tissue fluorescence. It can be concluded from this that the level of blood volume of tissue affects not only the intensity of the backscattered radiation, but, in general, the fluorescence signal itself.

Effect of skin melanin

Table 2 shows the results of the measurements strongly dependent on the level of melanin present in skin. This results in strongly pigmented skin areas, such as the forearm of tested African volunteers, to exhibit next to no fluorescence signal and the redox ratio becomes impossible to calculate. Furthermore, it is important to note that the different levels of melanin in the skin of Middle Eastern, Indian and African volunteers (the finger pad compared to the forearm) appears to be an additional factor influencing the physiological variability of recorded parameters. This indicates the need for assessing the feasibility of using the FS method on patients with a high level of skin melanin.

As can be seen in Figure 2, the fluorescence in the UV and green excitation wavelengths are virtually absent, which confirms the fact that melanin is a strong attenuator of fluorescence in the ultraviolet and visible range. For the red excitation wavelength, fluorescence is more pronounced than in the first two cases.

Thus, it is possible to conclude that the technique of FS can be potentially useful in the assessment of melanin driven pigmentation changes in the skin. Currently, the red region of the spectrum is used only for porphyrin fluorescence studies. The fluorescence signal, excited in the red region of the spectrum, can be particularly useful for non-invasive diagnosis of pathologies such as malignant melanoma, which is difficult to distinguish from other pigmented skin lesions using UV excitation. As shown by the experiments, the fluorescent properties of melanin are very dependent on the wavelength of excitation, which in turn requires further detailed study. It is assumed that at a longer wavelength (from the near-infrared range and above) it will be possible to register a significant expression of melanin fluorescence. Latest results in multiphoton melanin excitation (780-820 nm) have revealed significant differences in eu- and pheomelanin fluorescence ratio in melanoma lesions and normal melanocytes which may become a diagnostic criteria for melanoma detection.

Effect of technical errors

In the figures, dotted lines represent the peaks of fluorescent biomarkers.

As seen in Figure 3, for the two compared FS channels fluorescence intensity values differ greatly in amplitude. The reason for this may be a different spectral sensitivity of the transfer function and resolution of different diagnostic instruments, since cell CCD arrays have unequal sensitivity, i.e. even if the signal is homogeneous, illumination produced is different. The magnitude of this heterogeneity is typically less than 5%. However, this does not eliminate the problem of reducing CCD noise itself, as fluorescent applications often work with the signals in the tens of photons per cell. For this reason, in systems which require high photometric accuracy such as this, it is necessary to apply the same non-uniformity correction algorithms as well as adjusting dark current. Spectral characteristics of the CCD, however small, affect the quality of the signal.

Often, the output electronic cascades are an even more intense source of noise. In this regard, attention should be paid to the rational matching of a receiver and a follow-up system [5].

As mentioned above, the different nodes of the opto-electronic system appear to be the primary sources of noise, interference and other distortion. Thus, a set of interchangeable filters is installed on the input of the devices polychromator, damping the radiation at given source wavelengths to required levels. As studies have shown, utilized filters affect the sensitivity of the measurement channel. More specifically, they weaken not only the backscattered radiation, but the fluorescence emission of various fluorophores at 3-5 dB, which affects the whole recorded spectra and calculated parameters.

One may infer the need for more precise filters with a narrow band filter and a high percentage transmittance of other wavelengths.

Based on the preliminary results, we can conclude on the need to develop methods of metrological monitoring for FS devices. Metrological provision should cover both the spectral calibration of the instrument readings and the normalisation of quantitative fluorescence, based on modelling the endogenous fluorescence of the investigated fluorophores. Creation of new FS hardware also requires new approaches to software development for the processing and analysis of diagnostic information to develop practical methods for its application in applied clinical medicine. It should be emphasized that addressing the issues of metrological support for the FS method can bring this diagnostic technology to a new level.

Conclusion

The data obtained should be considered when developing new integrated medical and biological parameters of measurement techniques and algorithms, as well as addressing specific diagnostic problems in clinical practice. The presented results demonstrate the relevance of metrological support of the technology in general and in particular the implementation of the instrument. Ultimately, further problem solving of the outlined issues will bring fluorescence spectroscopy closer to standardized diagnostic technologies, which comply with the requirements of modern medicine.

REFERENCES

1. Lakowicz, J.R. Principles of Fluorescence Spectroscopy. New York: Kluwer Academic Publishers; 2006.
2. Mycek, M.A. Pogue BW. Handbook of Biomedical Fluorescence: Taylor & Francis; 2003.
3. Tuchin, V.V. Handbook of optical biomedical diagnostics. Washington: SPIE Press, Bellingham; 2002.
4. Rogatkin, D.A., Sokolovski, S.G., Fedorova, K.A., Sidorov, V.V., Stewart, N.A., Rafailov, E.U. Basic principles of design and functioning of multifunctional laser diagnostic system for non-invasive medical spectrophotometry. Photonics West. San-Francisco: SPIE Proceedings 2011; 78901H1.
5. Yakushenkov, Y. Electro-optical devices: Theory and design: Mir; 1983.

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