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Individual variability analysis of fluorescence parameters measured in skin with different levels of nutritive blood flow

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ABSTRACT

Fluorescence spectroscopy 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.

In this study, we aimed to assess individual variability of fluorescence parameters of endogenous markers (NADH, FAD, etc.) measured by fluorescent spectroscopy (FS) *in situ* and to analyse the factors that lead to a significant scatter of results. Most studied fluorophores have an acceptable scatter of values (mostly up to 30%) for diagnostic purposes. Here we provide evidence that the level of blood volume in tissue impacts FS data with a significant inverse correlation. The distribution function of the fluorescence intensity and the fluorescent contrast coefficient values are a function of the normal distribution for most of the studied fluorophores and the redox ratio. The effects of various physiological (different content of skin melanin) and technical (characteristics of optical filters) 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.
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1. Introduction

Fluorescent spectroscopy 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 [3–7]. 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 [8], offering several benefits over traditional diagnostic methods. It is well known that tumours of the skin, mucous membranes of the mouth, gastrointestinal tract and urogenital systems have a number of specific auto-fluorescence (AF) spectra [9]. The high specificity and sensitivity of the FS has been shown in the differential diagnosis of tissue dysplasia, adenoma and adenocarcinoma [10–12]. FS is also used as a tool to monitor the dynamics of the processes occurring in the tissues, during cancer treatments such as radiotherapy [13]. 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, to date the use of FS is limited because of several unresolved issues. Many fluorophores are characterised by similar or overlapping regions of absorption and fluorescence. As a result there are complex fluorescence emission spectra arising from tissue. Therefore, one of the current biggest problems with FS is the inability to delineate and designate individual fluorophores by excitation and emission wavelengths.

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 [14], light pollution at the optical fibre tip and

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Table 1
Number of spectral recordings by wavelength and skin area.

I. Study of individual variability of parameters				
Volunteer no.	Recordings			
	Fingertip (skin area with AVAs)			Forearm (without AVAs)
	UV (365 nm)	Green (532 nm)	Red (637 nm)	UV (365 nm)
1	120	20	50	80
2	50	–	–	40
3	30	–	–	30
Total	200	20	50	150
II. Study effect of skin melanin				
Ethnic skin types	Recordings			
	Skin with AVAs		Skin without AVAs	
	UV (365 nm)			
Middle Eastern	13		13	
Indian	27		27	
African	3		3	
Total	43		43	

instrument errors such as excitation source instability, photodetector limitations, light filter precision, grating precision, CCD performance, etc. [15]. To achieve clinically significant and reliable results, issues of accuracy, convergence and dispersion measurement also need to be addressed.

A number of publications have dealt with these issues. For example, in [16], the random error was 8–10% for healthy tissue (intact) and 30–35% on malignant tissue in a samples size of 30. In [17] the reproducibility of the tissue's optical parameters is reported based on simulated measurements. The relative random error in the measured amplitude of the backscattered radiation on a PTFE phantom was within ±2–3%, and the recorded amplitude error of the fluorescence for a specific phantom and selected wavelength was ±7–8%. The coefficient of fluorescence variability was ±2–3%. No data could be found on lengthy assessments of FS parameter variability for healthy tissue. This study aims to fill this gap and to assess individual parameter variability in laser fluorescence diagnosis, and to analyse its potential sources.

2. Experimental studies

The first part of experimental studies of individual variability of parameters (Table 1) were carried out with the participation of three healthy volunteers (of Caucasian origin): a man of 35 years of age (for 9 months) – volunteer no. 1, a man of 22 years (for 5 months) – volunteer no. 2, and a woman of 24 years (for 3 months) – volunteer no. 3. The measurements were performed at two points on the skin exhibiting different levels of nutritive blood flow: skin pads (palmar surface) of the right middle finger, which is an area rich in arteriolar-venular anastomoses (AVAs) and consequently with great nutritive blood flow [18]; on the outside of the right forearm which is almost devoid of AVAs and dominated by nutritional blood flow (skin area without AVAs), but with a lower nutritive blood flow. 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.

The second part of experimental studies on the effects of skin melanin (Table 1) were carried out with the participation of five healthy volunteers with different skin tones: Middle Eastern (1 volunteer), Indian (1 volunteer) and African (3 volunteers).

Different excitation wavelengths were used to generate the autofluorescence in the two physical skin areas of study.

A multifunctional laser non-invasive diagnostic system (MLNDS) “LAKK-M” (SPE “LAZMA” Ltd, Russia) [19] 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. The system comprises of 5 different lasers and is equipped with an optical probe that has 9 individual fibres – 4 serve as receivers for laser radiation and 5 for secondary radiation from back scatter. The diameter of the optical probe is 2.5 mm, with the separation distance between the source and detector fibres about 1 mm and with the area of the detector (an optical multimode fibre with about NA = 0.22) 0.003 mm^2 [20]. The power of the laser probes within the fluorescence spectroscopy channel are 3–4 mW and the approximate diagnostic volume within tissue is about 1–2 mm^3 [21].

In these studies, involving two measurement channels, FS and TRO data were analysed. A base record on LDF/TRO channels was carried out for 3 min, prior to the registration of the fluorescence spectra, to analyse the impact of changes of the tissue blood volume on the results of FS. The following parameters were recorded: perfusion (I_m, PU), tissue oxygen saturation (S_tO_2, %) and tissue blood volume (V_b, %) [22]. Excitation was carried out using UV (365 nm), blue (430 nm), green (532 nm) and red (637 nm) light sources. These wavelengths initiate fluorescence for elastin, collagen, pyridoxine, keratin, NADH, FAD, lipofuscin, carotene and porphyrins.

Typical palmar surface fluorescence spectra generated from Caucasian skin type by the “LAKK-M” system using the four wavelengths for excitation of endogenous fluorescence are overlaid and shown in Fig. 1.

Using the maximum amplitude of the intensity of the fluorescence spectrum $I(\lambda)$ for different wavelengths allows the calculation of the intensity of the backscattered radiation $I_{bs}(\lambda)$ and two computational parameters – the coefficient of the fluorescent contrast and the redox ratio, RR.

The coefficient of fluorescent contrast $k_f(\lambda)$ was calculated using two different approaches for fluorescence analysis of fluorophores at different excitation wavelengths (Table 2). Presented in the native “LAKK-M” software, the coefficient of fluorescent contrast is calculated as follows:

$$k_f(\lambda) = 1 + \frac{I_f(\lambda) - I_{bs}(\lambda)}{I_f(\lambda) + I_{bs}(\lambda)}, \quad (1)$$

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