



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:

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

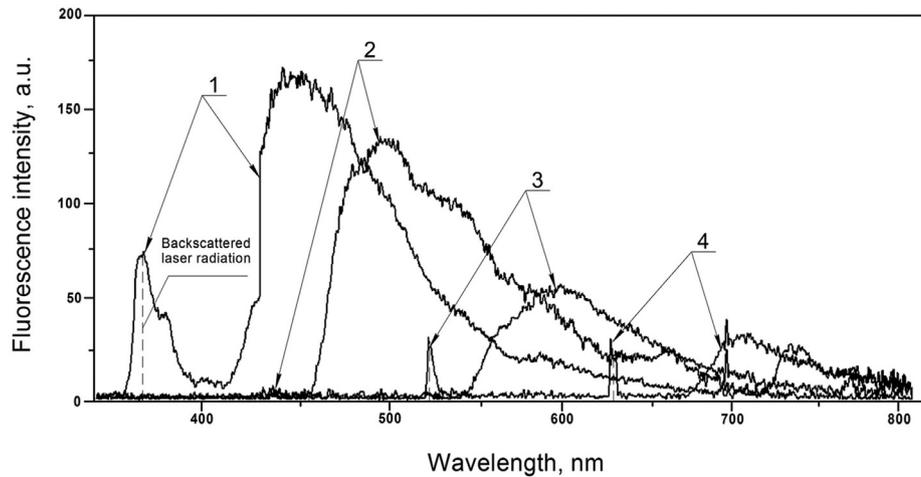


Fig. 1. Fluorescence spectra of endogenous fluorophores in the palmar surface of Caucasian skin type at 365 nm (1), 430 nm (2), 532 nm (3) and 635 nm (4).

Table 2
Reference wavelengths used for tissue fluorophores.

Type of fluorophore	Excitation beam				
	UV – Eq. (1)	UV – Eq. (2)	Blue	Green	Red
Collagen	420	422/433			
Elastin	450	422			
Keratin		466			670
NADH	490	466/494			
Pyridoxine	525				
FAD	550	555	510		
Lipofuscin	570	605		570	
Carotene	608			608	
Porphyrins		635/704		640/680	710

where $I_f(\lambda)$ represents registered fluorescence intensity at wavelength λ ; $I_{bs}(\lambda)$ represents the maximum intensity of the backscattered laser radiation [19]. It can be observed that the coefficient of the fluorescent contrast in this case is within the range of 0–2.

The UV channel required another variant for calculating $k_f^*(\lambda)$ and fluorescence wavelengths [23]:

$$k_f^*(\lambda) = \frac{I_f(\lambda)}{I_f(\lambda) + I_{bs}(\lambda)}. \quad (2)$$

It can be observed that in this case the coefficient of the fluorescent contrast is within the range of 0–1.

The redox ratio was calculated according to Eq. (3):

$$RR = \frac{A_{NADH}}{A_{FAD}}, \quad (3)$$

where A_{NADH} is the amplitude of fluorescence of NADH; A_{FAD} is the amplitude of fluorescence of FAD. Moreover, the A_{FAD} value can be taken from the fluorescence spectrum arising from the UV-excitation channel or from the spectrum using the blue-excitation channel. According to [3,24], using the blue excitation channel allows for a more specialised detection of the FAD fluorescence. In this paper the scatter of the values of RR used values taken from spectra generated only by UV excitation.

The arithmetic mean (M_n) of the parameters was calculated over the course of the study period. The standard deviation (σ) of M_n and the coefficient of variation or relative measurement scatter of the changes in δ taken as a percentage of the average (M_n) were also calculated. To determine the measured values and calculated parameters, distribution functions of the probability density were constructed.

Table 3
The coefficients of variation for the fluorescence intensities $I_f(\lambda)$ and coefficients of the fluorescent contrast $k_f(\lambda)$ and $k_f^*(\lambda)$ for the area with the AVA.

Type of fluorophore	λ (nm)	Volunteer no.					
		1			2		
		1	2	3	1	2	3
		$\delta(I_f)$ (%)			$\delta(k_f)$ (%)		
Collagen	420	43.42	40.51	44.93	30.62	31.73	27.52
Elastin	450	35.95	37.79	34.88	19.63	24.75	23.97
NADH	490	27.91	26.39	32.29	20.71	17.11	18.22
Pyridoxine	525	24.84	23.11	25.14	28.60	33.44	30.88
FAD	550	24.09	21.54	23.70	37.96	35.95	36.76
Lipofuscin	570	22.30	19.34	22.61	41.59	41.10	38.85
Carotene	608	21.15	18.99	17.75	44.10	44.37	40.16
		$\delta(I_f)$ (%)			$\delta(k_f^*)$ (%)		
Elastin and collagen	422	35.56	44.13	36.48	26.67	15.00	15.79
Collagen	443	31.02	36.80	29.95	15.52	10.77	12.24
Keratin/NADH	466	30.01	36.58	30.96	15.25	10.61	10.94
NADH	494	26.88	32.54	27.31	17.65	14.04	14.00
FAD	555	22.13	28.73	22.89	34.78	24.00	28.57
Lipofuscin	605	19.39	19.80	19.21	38.46	33.33	31.58
Porphyrins	635	19.17	18.72	18.88	47.50	41.00	27.27
Porphyrins	704	49.24	47.33	49.77	75.00	66.67	67.50

3. Results

3.1. Study of individual variability of parameters

Depicted below are histograms of fluorescence intensity distribution of several fluorophores (Fig. 2a and b) and the coefficients of fluorescence contrast for different fluorophore wavelengths calculated by Eqs. (1) and (2) (Fig. 2c and d), taken from the surface pad of the middle finger of three volunteers. Results of calculating the coefficients of variation (scatter measurements) for fluorescence intensity and the coefficient of the fluorescent contrast in this case are presented in Table 3.

Comparative analysis of the data shows that the relative error (variation) of measurements for three volunteers are similar and, depending on the fluorophore, varies generally between 20 and 40% for the fluorescence intensity, and from 10 to 45% for the coefficient of the fluorescent contrast. Some authors have suggested a 50–70% variation when using a 704 nm excitation source [23], which is not incorporated into the “LAKK-M” data analysis software. Perhaps this increased scatter at the 704 nm wavelength is related to the existence of inhomogeneity in the UV source spectrum, manifested in the form of additional peaks in the red region. This appears to be an instrumental artefact.

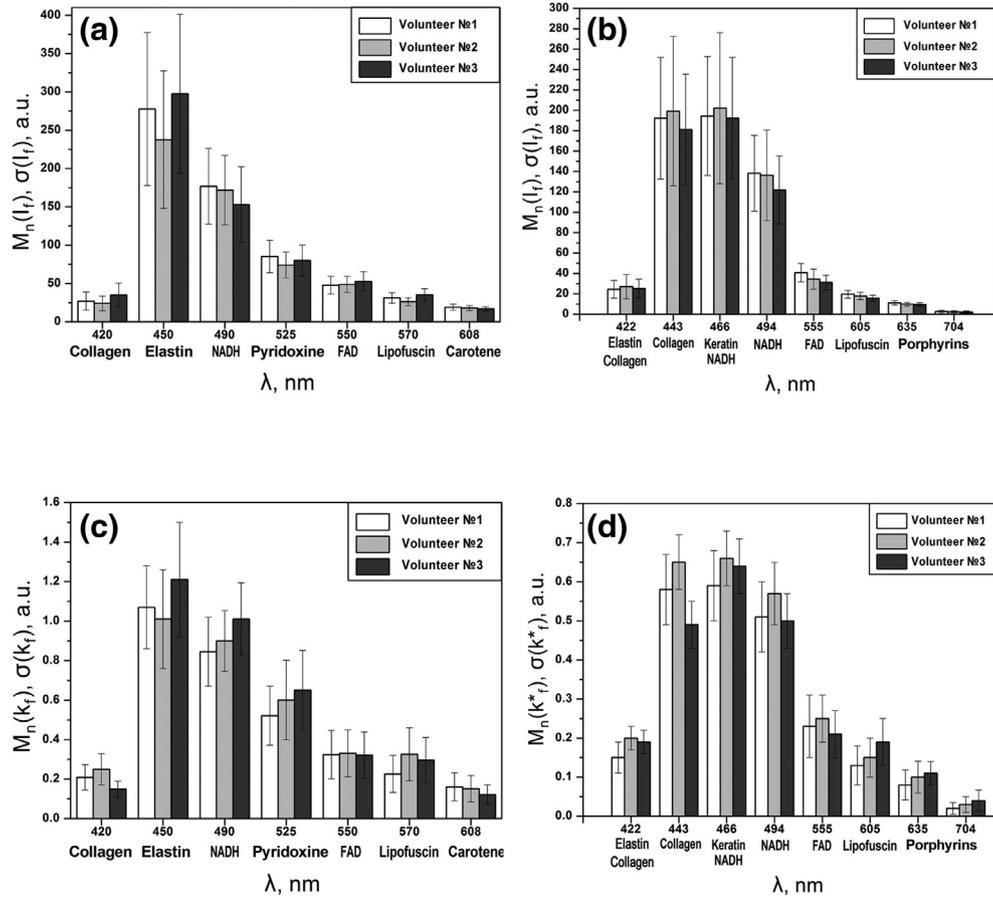


Fig. 2. Histogram plot of the distribution of average intensities of fluorescence ((a) and (b)) and the coefficient of the fluorescent contrast ((c) and (d)) with error bars for the area with the AVA.

The results of calculation of the consumption of oxygen reflected in the redox ratio for three volunteers were: $RR1 = 3.58 \pm 0.31$ AU for volunteer no. 1, $RR2 = 3.4 \pm 0.29$ AU for volunteer no. 2, $RR3 = 3.1 \pm 0.21$ AU for volunteer no. 3. The scatter of the values obtained for all three volunteers did not exceed 10%. It should be noted that these values are inversely proportional to the redox-ratio by which the respiratory redox processes in the mitochondria is judged, and thus evaluate the energy status of the cell. There are a number of different methods for performing redox-ratio calculations. Thus, in [25] it is proposed to use the ratio of the fluorescence intensity of FAD and that of NADH, in [26,27] redox ratio was defined as the ratio of the fluorescence intensity of FAD and the sum of the fluorescence intensities of FAD and NADH. But ultimately, the main issue remains the wavelengths of excitation and fluorescence of the appropriate fluorophores.

The following histograms depict distributions of fluorescence intensities (Fig. 3a and b) and coefficient of the fluorescent contrast (Fig. 3c and d) for the skin area without AVAs on the forearm of the three volunteers, also calculated in two ways (by Eqs. (1) and (2)), respectively. Results of calculation of the corresponding coefficients of variation (scatter measurements) for fluorescence intensity and the coefficient of the fluorescent contrast for the forearm are in Table 4.

The relative scatter of the measurement results for the forearm was 1.5–2.0 times less than that for surface pad of the finger. This confirms the influence of the degree of tissue blood saturation on the variability in the optical properties of the tissue. Obviously, the skin blood circulation is one of the main factors affecting the fluorescence measurement.

Also, the example of volunteer no. 3 (Fig. 3) clearly shows the advantage of using a relative parameter (the coefficient of the

Table 4

The coefficients of variation for the fluorescence intensities $I_f(\lambda)$ and coefficients of the fluorescent contrast $k_f(\lambda)$ and $k_f^*(\lambda)$ for the area without the AVA.

Type of fluorophore	λ (nm)	No. volunteer					
		1	2	3	1	2	3
		$\delta(I_f)$ (%)			$\delta(k_f)$ (%)		
Collagen	420	28.07	31.25	29.94	17.27	15.47	19.08
Elastin	450	22.90	26.66	24.79	10.18	10.01	10.64
NADH	490	20.62	21.43	23.10	10.48	10.69	12.34
Pyridoxine	525	15.14	19.32	18.67	12.75	14.55	14.46
FAD	550	15.44	18.34	16.88	14.84	15.23	16.44
Lipofuscin	570	12.92	16.68	11.99	20.56	22.91	20.90
Carotene	608	10.24	14.39	9.037	29.16	30.74	27.65
		$\delta(I_f)$ (%)			$\delta(k_f^*)$ (%)		
Elastin and collagen	422	23.21	22.15	26.57	14.33	15.91	17.93
Collagen	443	18.96	20.31	20.47	10.51	10.76	13.09
Keratin/NADH	466	16.43	17.17	18.20	10.19	10.94	10.27
NADH	494	15.26	15.56	15.12	14.12	13.83	14.49
FAD	555	13.32	14.98	14.35	18.21	17.00	17.41
Lipofuscin	605	11.38	10.31	12.34	23.58	22.00	22.58
Porphyryns	635	9.94	13.38	10.95	28.57	31.43	27.57
Porphyryns	704	42.45	48.38	46.92	45.87	46.33	49.24

fluorescent contrast) compared to the absolute values of the fluorescence intensities. Normalisation of the fluorescence intensities using the recorded value of the backscattered radiation can neutralise the influence of the tissue blood volume and allow comparison of the relative levels of fluorophores for a variety of biological tissues and in different patients.

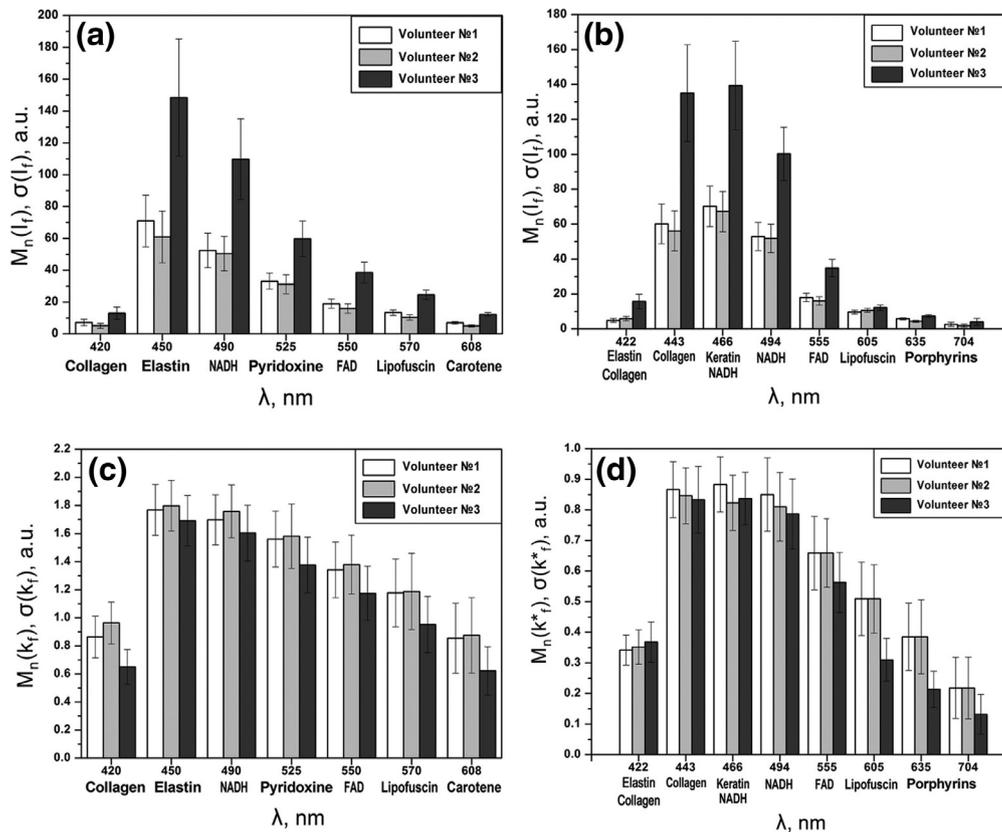


Fig. 3. Histogram plot of the distribution of average intensities of fluorescence ((a) and (b)) and the coefficient of the fluorescent contrast ((c) and (d)) with error bars for the area without the AVA.

Table 5

Statistical evaluation of the results of measurements for the green and red excitation wavelengths.

The laser wavelength (nm)	λ (nm)	The recorded fluorescence intensity $I_f(\lambda)$ (AU)			The coefficient of fluorescent contrast $k_f(\lambda)$ (AU)		
		M_n	σ	δ (%)	M_n	σ	δ (%)
532	570	35.21	13.45	38.20	1.87	0.54	28.89
	608	55.76	16.18	29.02	1.92	0.44	22.92
	640	38.85	9.12	23.48	1.89	0.36	19.05
	680	18.66	4.73	25.35	1.78	0.39	21.91
637	670	0.84	1.14	134.92	0.03	0.05	143.85
	710	25.41	15.34	60.37	0.81	0.35	43.21

Similar studies on the variability of the green and red fluorescence excitation wavelengths with the calculation formula of Eq. (1) were conducted on volunteer no. 1. The results of statistical processing of the data obtained are shown in Table 5.

This data indicates that the relative variation is minimal at certain wavelengths. The considerable variability of values at the 670 nm wavelength for analysis of keratin makes its application problematic. Perhaps, these data should be considered when standardising spectrometers in MLNDS. The analysis of the distribution of the measurement results in FS for all three volunteers showed that the distribution function of the deviation of the fluorescence intensity and fluorescence contrast ratio for UV excitation is generally normal. This distribution is not consistent with the measurement for the wavelength of 704 nm, for which there was the greatest range of results.

3.2. 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

no. 1, 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 [28]. 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. 4.

3.3. 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 with and without AVAs as recorded on volunteers of different ethnicities including Caucasian, Indian, Middle Eastern and African (with a range of melanin content approximately from 1.3% to 43%) [29]. The results are shown in Table 6.

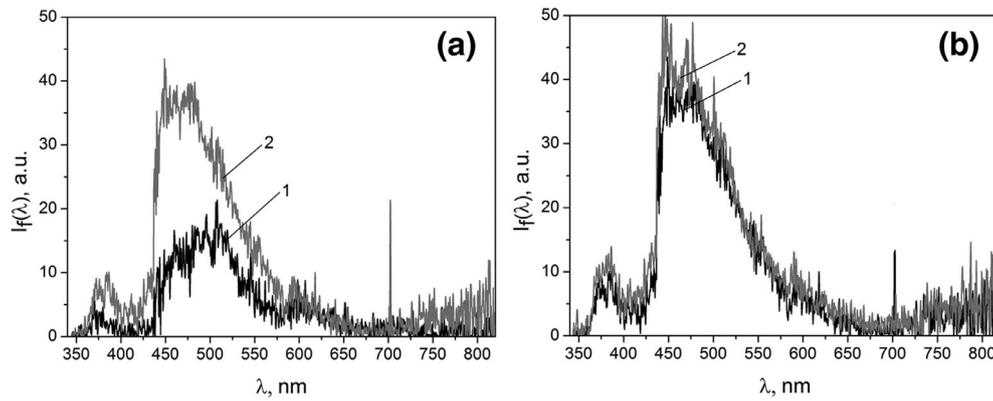


Fig. 4. Examples of the fluorescence registering the third day (a) and eighth day (b). (1) hematoma, (2) intact tissue.

Table 6
Results of the study of effect of the skin melanin.

The laser wavelength (nm)	Ethnic skin types	$M_n \pm \sigma$ (AU)					
		Skin area with AVAs			Skin area without AVAs		
		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	–

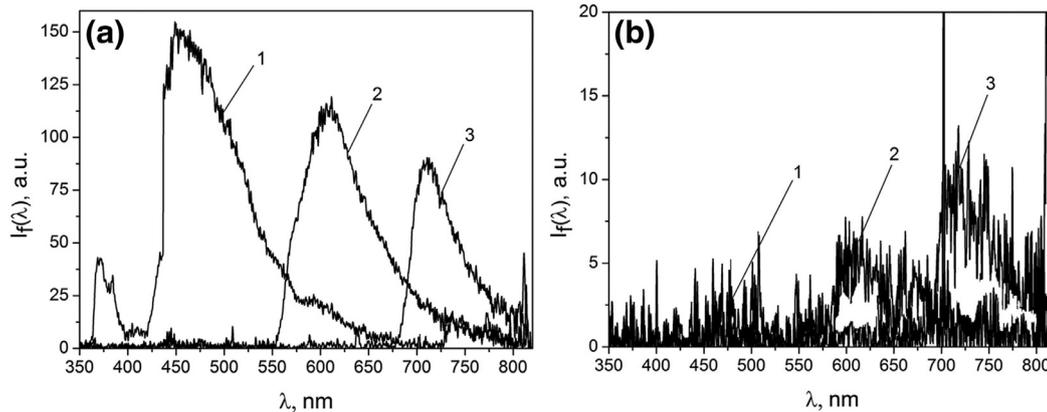


Fig. 5. The fluorescence spectra of skin with high levels of melanin, (a) fingertip; (b) forearm: the UV (1), green (2) and red (3) light excitation wavelengths.

Separate 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. 5.

3.4. Effect of technical errors

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.

4. Discussion

Prior to beginning the analysis of factors affecting the individual variability of fluorescence parameters (effects of the tissue blood volume level, skin melanin and technical errors), we consider the

spatial distribution of the fluorescence excitation in skin based on the example of the FS channel of the MLNDS “LAKK-M” (in the case excitation by UV radiation). It is important to note that the spatial distribution of the fluorophores in the skin is one of the essential factors affecting the registered fluorescence signals [30–32], and therefore has a direct impact on the variability of the parameters in FS. For this purpose, a model was tested with the aid of software implementing a version of the Monte Carlo method, in which the multilayered nature of skin tissue, the effects of reflection, absorption and refraction of light at the layer boundaries are taken into account [33,34]. The Henie Greenstein function was used as the phase function of the scattering.

As is known, the skin autofluorescence spectrum excited by light in the UV range is determined by the fluorescence of epidermal (NADH, keratin, etc.) and dermal (collagen) fluorophores, the absorbance of exciting radiation and fluorescence by the epidermis (melanin) and dermis (blood haemoglobin) and the scattering properties of skin tissue at excitation and fluorescence wavelengths. Optical characteristics of the skin based on literature sources are presented in Table 7.

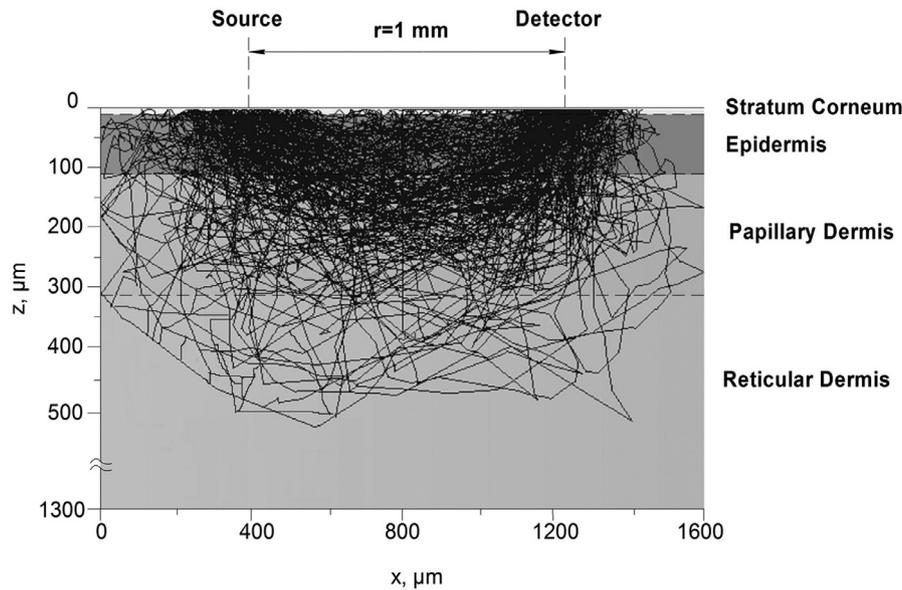


Fig. 6. The total spread of fluorescence and exciting rays inside tissue.

Table 7
The four-layer optical model for skin.

Layer	z (μm)	n	μ_a (cm^{-1})	μ_s (cm^{-1})	g
Stratum corneum	10	1.55	300	2200	0.9
Epidermis					
Melanin content: 1%	100	1.4	24.2	1000	0.72
Papillary dermis					
Blood content: 0.2%	200	1.37	4.7	460	0.73
Reticular dermis					
Blood content: 0.2%	1000	1.4	4.7	460	0.74

The transport parameters (μ_a , μ_s , g) shown are for 365 nm only. The optical parameters for other wavelengths do not appear in this table, but were derived in a similar fashion using published skin optical properties by Refs. [28,29,35].

NADH and collagen were used as test fluorophores for epidermis and dermis respectively. The values of parameters $r = 1$ mm (separation distance between the source and detector fibres) and $d = 0.06$ mm (diameter of the fibre core) were used for MLNDS “LAKK-M” fibres to analyse the passage of excited photons between the source and receiver of radiation. The resulting image on Fig. 6 displays the spread of both the exciting (365 nm) radiation rays backscattered through the tissue as well as the fluorescence rays picked up by the detector.

The results obtained show that, under excitation by UV radiation, the signal registered by the “LAKK-M” system mainly consists of fluorophore radiation from the epidermis and upper dermis layers (containing NADH and collagen). It appears that the collagen signal from the lower dermis is heavily screened by haemoglobin fractions. Thus, the presented analysis suggests the need to take into account the characteristics of optical fibres during qualitative evaluation of layers of registered fluorophores as well as during the evaluation of individual variability of the recorded signals in the FS as a whole.

4.1. Study of individual variability of parameters

Analysis of the data leads to several conclusions regarding the distribution law of the measurement results as well as allowing us to analyse the reasons for the large variability of the measurement results and calculated parameters in the FS.

Fig. 7 shows an example of the resulting density values of the fluorescence intensity and the coefficient of the fluorescent contrast of NADH fluorescence at a wavelength of 494 nm for the volunteer no. 1. Distribution for NADH displays a truncated normal distribution. This may be due to the limited range of possible values. Both criteria with a confidence level of 0.95 reiterated the commitment of selection distributions [36,37]. In general we can conclude that the distribution

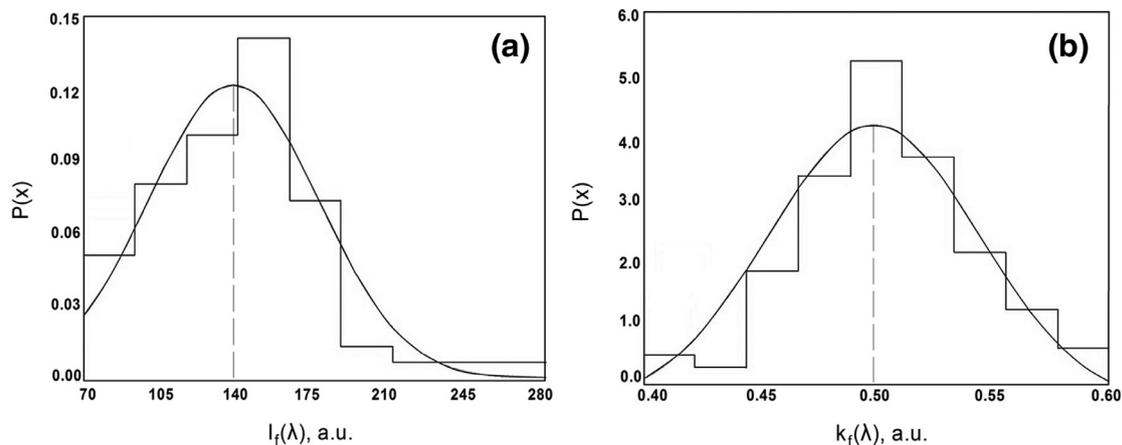


Fig. 7. The probability density distribution $P(x)$ values of fluorescence intensity (a) and coefficient of the fluorescent contrast (b) for NADH.

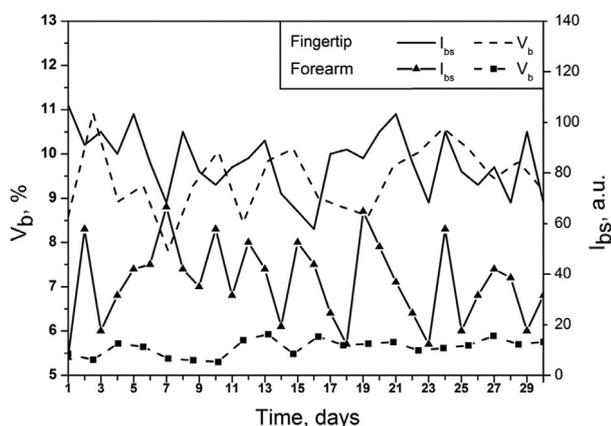


Fig. 8. Graphs of parameter changes $I_{bs}(\lambda)$ and V_b for the two zones of the skin for volunteer no. 1.

of the fluorescence parameter may also depend on parameters such as the individual characteristics of the studied tissues, the specific type of fluorophore, etc. These are possibly related to the uneven sensitivity of the photodetector measuring channel in the reception range of endogenous fluorescence spectra.

As is known, the larger the coefficient of variation, the greater the scatter of values and lower the uniformity of the investigated values. It is believed that if the coefficient of variation measures less than 33%, then the set is homogeneous [37]. Data analysis of individual variability of fluorophore fluorescence indicates that most commonly studied fluorophores have an acceptable scatter of values for diagnostic purposes.

4.2. Effect of the tissue blood volume level

Graphs of the study results are shown in Fig. 8. As can be seen, the intensity of the backscattered radiation is inversely related to the tissue blood volume for both areas of the skin – with and without AVAs.

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 [37]. 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 [20].

Also, as seen from Fig. 4, 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.

4.3. Effect of skin melanin

Table 6 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 ration

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 Fig. 5, 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 [38]. 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 [39]. 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 [40] 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 criterion for melanoma detection [41,42].

4.4. Effect of technical errors

For the two compared FS channels fluorescence intensity values may 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 [43].

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 [44].

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, utilised 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. An example of such attenuation is shown in Fig. 9.

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 [45]. Creation of new FS hardware also requires new

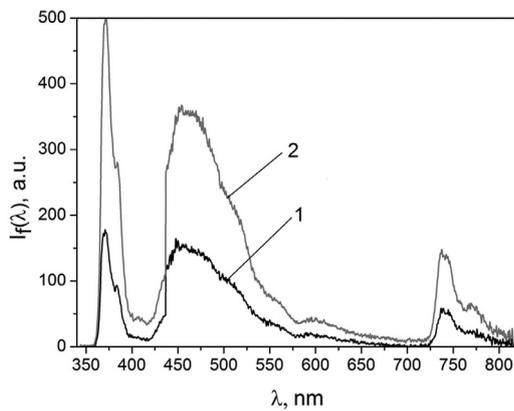


Fig. 9. Filter effect on the fluorescence intensity, (1) with or (2) without filters.

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 emphasised that addressing the issues of metrological support for the FS method can bring this diagnostic technology to a new level.

5. Conclusion

As a result of experimental studies, we have provided values of individual variability (mostly up to 30%) of directly measurable endogenous fluorescence intensities and the calculated relative parameters: the coefficient of the fluorescent contrast and redox ratio. One of the main factors affecting the scatter of the results of measurements in the FS is the levels of the tissue blood volume and content of the skin melanin. Results also established that the probability density function of the intensities of fluorescence and the fluorescent contrast ratio for most investigated fluorophores are normal.

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 standardised diagnostic technologies, which comply with the requirements of modern medicine.

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Ethical approval

This research was conducted on healthy volunteers according to Health and Safety practice of the University of Dundee. The protocol on which this study was based was approved by the University of Dundee Research Ethics Committee (Application number: UREC Ref. 12007), and all subjects provided informed consent to the collection and use of data reported in the manuscript. Standard measurements were performed on permitted equipment (“LAKK-M” system) for use in diagnostics according to the user manual.

Conflict of interest

The authors confirm that there is no conflict of interest in relation to this work.

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