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Multimodal optical measurement for study of lower limb tissue viability in patients with diabetes mellitus

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Abstract. According to the International Diabetes Federation, the challenge of early stage diagnosis and treatment effectiveness monitoring in diabetes is currently one of the highest priorities in modern healthcare. The potential of combined measurements of skin fluorescence and blood perfusion by the laser Doppler flowmetry method in diagnostics of low limb diabetes complications was evaluated. Using Monte Carlo probabilistic modeling, the diagnostic volume and depth of the diagnosis were evaluated. The experimental study involved 76 patients with type 2 diabetes mellitus. These patients were divided into two groups depending on the degree of complications. The control group consisted of 48 healthy volunteers. The local thermal stimulation was selected as a stimulus on the blood microcirculation system. The experimental studies have shown that diabetic patients have elevated values of normalized fluorescence amplitudes, as well as a lower perfusion response to local heating. In the group of people with diabetes with trophic ulcers, these parameters also significantly differ from the control and diabetes only groups. Thus, the intensity of skin fluorescence and level of tissue blood perfusion can act as markers for various degrees of complications from the beginning of diabetes to the formation of trophic ulcers. © 2017 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.22.8.085003]

Keywords: fluorescence spectroscopy; metabolism; advanced glycation end products; mitochondrial function; laser Doppler flowmetry; diabetes mellitus; diagnostic volume.

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1 Introduction

According to the International Diabetes Federation (IDF), the challenge of early stage diagnosis and monitoring of the treatment effectiveness in diabetes is currently one of the highest priorities in modern healthcare. The medical, social, and economic significance of diabetes is primarily determined by the high prevalence of this disease and the frequency of debilitating and quality of life-reducing effects suffered by affected individuals. The IDF report for 2015 indicated that there are 415 million diabetic patients worldwide, with this figure projected to grow to 642 million by 2040.¹

Over the last several years, multiple studies have indicated that a timely diagnosis and treatment, including an increased level of patient monitoring, reduce the manifestation of various complications, potentially even reversing them at early preclinical stages. Particular attention is given to foot complications such as peripheral arterial disease (PAD), neuropathy, and skin disorders including foot ulcers, which are liable for more hospitalizations than any other complication of diabetes.² Particularly of interest, however, are vascular disorders such as PAD, which lead to the most adverse implications for patients. Macro-

and microcirculatory disturbances, which are often imperceptibly formed, significantly reduce the patient's life quality. This can eventually lead to an early death of the patient. Disorders of microcirculation can occur long before the clinical manifestation of diabetes and play a central role in the development of foot ulcers and their subsequent inability to heal.³⁻⁷

Available diagnostic approaches for the investigation of PAD at the level of macro- and microcirculation offer both advantages and disadvantages inherent to each method. Currently, the commonly employed standards of diabetic foot disorder detection in hospitals or surgery are predominantly thorough medical history examination, visual inspection, and duplex ultrasonography scanning of arteries. The latter methods are capable of determining the level of trophic disorders, specifically the hemodynamics. However, for microcirculatory disturbances, ultrasound Doppler is not efficient enough.⁸ Other methods such as radiocontrast angiography have significant limitations during regular patient control: invasiveness, contrast agent toxicity, and prolonged exposure of the patient to radiation.^{9,10} In addition to the methods, medical device manufacturers offer solutions that allow for a joint diagnosis of macrovascular [measurement of ankle brachial index, toe brachial index, pulse volume recording, and

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segmental pressure (SP) with measurement under ultrasonic or laser Doppler flowmetry control]]^{11,12} and microvascular (transcutaneous oximetry) disorders. Often, this allows the acquisition of qualitative information about PADs and assessment of the wound-healing potential. However, the interpretation of the described parameters of vascular disorders can be somewhat subjective. The measurement results are affected by arterial calcifications as well as the rigidity of the vascular wall.^{13–15} Also, it is known that these methods can underestimate the true prevalence of cardiovascular diseases and, in particular, do not detect an early phase of vascular complications.¹⁶ The method of transcutaneous oximetry (TcPO₂) allows assessment of the oxygen partial pressure in the intercellular liquids.^{17,18} Due to physiological, methodological, and technical obstacles, this approach was not widely used in polyclinic diagnostics. At the same time, the trustworthiness of data obtained by this method could be questioned because of low selectivity to infectious inflammation on the foot, peripheral edema, and other accompanying pathologies.^{19,20} Another conventionally employed approach is thermal imaging, which reveals thermal asymmetry zones on the limb surface. The result of the diagnostics, however, is usually meaningful only during late stages of the limb angiopathy, when the blood circulation is damaged on the level of great vessels.^{21,22}

Additionally, to date, a general drawback of the methods available in the physician's arsenal is the inability to monitor the metabolic processes occurring in biological tissue. Patients with diabetes often display severe disorders of microcirculation, accompanied by significant metabolic shifts. Consequently, oxygen transport, its delivery, and tissue consumption are the most important indicators of the functioning of life support systems, and their adequate evaluation is important for choosing the optimal methods and tactics for treating each individual patient.

Despite this, there is little information on the effect of changes in blood flow in the delivery of oxygen and tissue metabolism of the lower limbs in diabetes mellitus. The influence of the development of diabetes on foot metabolism has not been practically investigated at present.⁴ The main purpose of this article was to show a possible approach in assessing not only vascular disorders occurring in diabetes mellitus but also associated metabolic disorders.

It is known that variations in the respiratory chain enzyme activities are recognized as one of the consequences resulting from a breach in the cell function observable in diabetes mellitus.^{23–25} Currently, a lot of research is being done to study the metabolism of epithelial tissues, including skin, using fluorescence spectroscopy and imaging techniques.^{26–30} Changes in the mitochondrial respiratory chain are recognized as one of the consequences resulting from a breach in the cell function observable in different pathologies. The most likely changes to occur are the accumulation of a coenzyme nicotinamide adenine dinucleotide (NADH) (the reduced form) and flavin adenine dinucleotide (FAD) (oxidized form). Detection of such changes by fluorescence spectroscopy is one of the most promising directions for *in vivo* diagnosis.

A recent review^{31,32} and some other studies show that changes in the fluorescence of the NADH respiratory chain were measured in single cells,^{33,34} tissue slices,³⁵ and organs.^{36–39} Despite the fact that the study of NADH and FAD fluorescence has been successfully held for the past 50 years, relatively little research has been conducted at the organ level. Meanwhile, the results of experiments on a particular organ or area of the

body can be more successfully extrapolated to clinical practice in comparison to the studies at the cellular and subcellular levels.

Furthermore, it has been established in recent years that long-existing diabetes with hyperglycemia results in an increase in protein glycation levels. This is accompanied by an increase in so-called advanced glycation end products (AGEs), which influence the properties of collagen and other structural proteins of the capillary membrane and skin.^{40–43} Among a number of AGE (e.g., N ϵ -carboxymethyllysine, N ϵ -carboxyethyllysine, and pyraline), pentosidine, responsible for the formation of adhesions between collagen fibers, has its own fluorescence.^{44–46}

Thus, the development of new optical diagnostic methods in these latter days has potential to find promising application in diagnosing diabetes disorders. The advantage of these methods lies in their noninvasive properties, good spatial resolution, low cost procedures, and high productivity. One of the promising directions of optical diagnostics development is the establishment of a scientific and instrumental framework to create rapid and real-time *in vivo* analysis methods for determining biological tissue viability.⁴⁷

For a comprehensive diagnosis of the state of microcirculatory-tissue systems, applying several diagnostic technologies simultaneously in one diagnostic volume is currently promising.^{31,48,49} To assess the state of blood flow and detect possible vascular disorders, different combinations of methods are applied. For instance, simultaneous measurements of the intensities of fluorescence and Rayleigh components in human eye lenses allow a good accuracy to separate patients with diabetes from a control group.⁵⁰ Furthermore, diffuse reflectance spectroscopy can be used for correction of the data obtained during fluorescence studies.^{51,52} Another example of combined technologies is a lightguide tissue spectrophotometry (O₂C). It is a combination of LDF and tissue spectrometry. This method allows for the measurement of both relative blood flow by the laser Doppler technique and hemoglobin oxygenation and hemoglobin amount in tissue by spectrometric techniques.⁵³

In this study, we experimentally estimate the potential of coregistering cutaneous blood flow parameters and the intrinsic tissue fluorophore fluorescence to distinguish stages of the complications of lower extremities in patients with diabetes mellitus. From the point of view of the fluorescence analysis, it is logical to consider a complex parameter, which describes the oxidative metabolic processes (changing the contents of NADH and FAD) and the carbohydrate metabolism (AGEs accumulation) in tissue, related to patients with diabetes manifesting as complications of the lower limbs.

2 Material and Methods

2.1 Experimental Equipment

In the experimental studies, the tissue blood perfusion and the tissue fluorescence were assessed by LDF and FS, respectively.⁵⁴ Registered perfusion parameters and the amplitude of the coenzymes' fluorescence were simultaneously evaluated using a specially designed system in the same tissue volume (SPE "LAZMA" Ltd., Russia). The Doppler channel built on the basis of the single-mode laser module used a wavelength of 1064 nm. A 365-nm and 450-nm radiation sources were used for fluorescence excitation. A multi-optical fiber probe was used for delivery of penetrating radiation and registration of backreflected secondary radiation from the tissue [Fig. 1(a)]. The probing fiber of the LDF channel has a diameter of 6 μ m and the

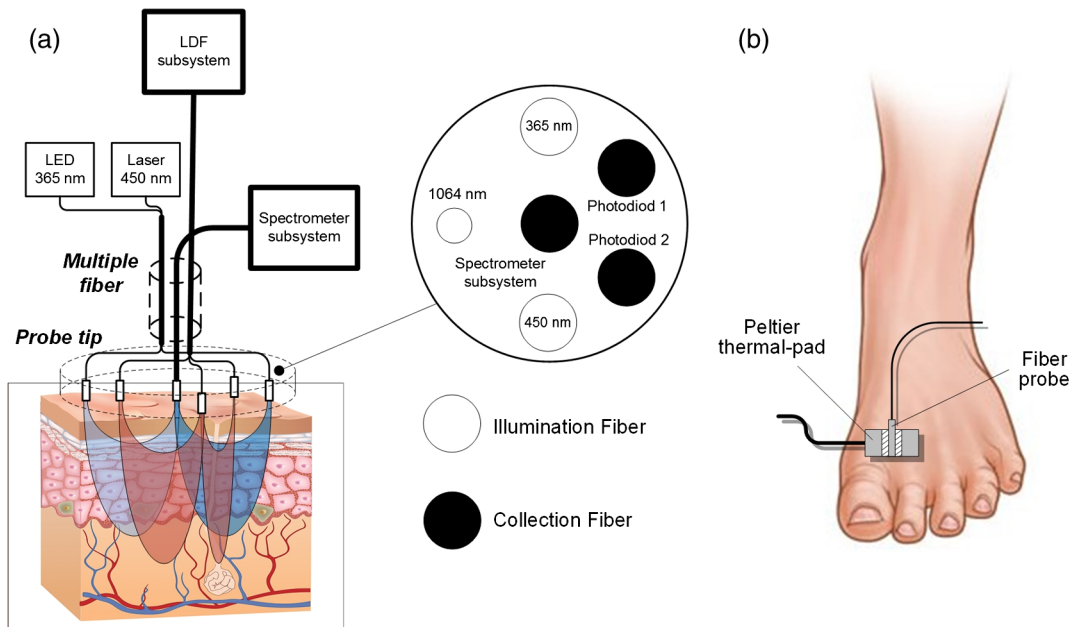


Fig. 1 (a) The general scheme of the measuring system and (b) localization of the fiber optic probe with Peltier thermal-pad.

receiving fiber has a diameter of $400\ \mu\text{m}$.⁵⁵ The source-detector spacing for the LDF channel is 1.5 mm. In the FS channel, diameters of all probing and receiving fibers are $400\ \mu\text{m}$. For safety reasons, as well as to keep photobleaching of the tissue at an acceptable level, probe radiation power of the 365-nm excitation wavelength at the output of the fiber probe did not exceed 1.5 mW. To assess the safety of the probe, coupled with the LED, we used documentation of the International Commission on Nonionizing Radiation Protection (ICNIRP).⁵⁶ The calculation gives the effective skin irradiance of $1.4\ \text{W}/\text{m}^2$. According to the cited guideline document, the limiting UV exposure duration per day for the level of effective irradiance is at least 10 s. During this protocol, the actual time of the UV-irradiation did not exceed 1 s. Therefore, from the point of view of the ICNIRP recommendations, the proposed protocol of using the probe was deemed to be safe. The output power for the 450-nm excitation wavelength did not exceed 3.5 mW. The source-detector spacing for the FS channel was 1 mm. The numerical aperture of the fibers was 0.22.

Specialized software was developed to work with the system. This allowed for real-time control of the course of the experiment and analysis of the recorded parameters. It further allowed the control of the unit responsible for realizing thermal functional tests.

2.2 Experimental Study

The experimental study involved 76 patients with Caucasian skin type and with type 2 diabetes mellitus at the Endocrinology Department of the Orel Regional Clinical Hospital (Orel, Russia). The study did not include volunteers with cardiovascular, bronchopulmonary, or neuroendocrine system disease comorbidities, nor with diseases of the gastrointestinal tract, liver, kidneys, blood, and any other serious chronic diseases, which could have an impact on the diagnostic results. Volunteers with a history of alcoholism, drug addiction, and drug abuse were also excluded. Laboratory parameters were measured

according to standard laboratory procedures. The measurement of blood pressure was carried out after 5 min of rest, with the patient in a sitting position. Fourteen people from the patient's group were considered to have a more severe course of the disease (diabetic group 2). The decision on the degree of severity in each case was taken based on the presence of trophic disorders in the form of ulcers, analysis of anamnesis, and consultation with the attending physician. Thus, diabetic group 2 consisted of all diabetics with the presence of visible trophic ulcers, and diabetic group 1 included patients without trophic ulcers. Patients were examined on the first day of admission to the clinic. Prior to hospitalization, patients took medication to reduce high blood glucose levels. The control group consisted of 48 healthy volunteers (mean age 46 ± 6 years). The participating volunteer group was collected through an internal university advertisement circulated by physical posters and notification e-mail. The experiments were conducted under established protocols. The study protocol was approved at a meeting of the ethics committee at Orel State University named after I.S. Turgenev from March 11, 2015 (minutes of the meeting No. 7). After receiving the description of the protocol, all participating volunteers signed an informed consent form.

The main characteristics of the studied groups are presented in Table 1. With a specified probability of 0.01, we can consider that the compared groups are statistically homogeneous with a difference in fasting glucose level and body mass index. The table shows that diabetic group 2 had a longer duration of diabetes and higher values of creatinine and urea (which may indicate kidney function is decreased), but these parameters did not reach statistically significant levels ($p < 0.01$). However, the percentage of complications in this group (diabetic polyneuropathy, diabetic retinopathy, diabetic nephropathy, and diabetic microangiopathy of the lower limbs) is statistically significant compared to diabetic group 1.

The local heating stimulation was selected as a provocative action on the blood microcirculation system, allowing assessment of the local regulatory mechanisms of blood flow. Using a special

Table 1 Statistical difference between patient groups and data for controls.

Characteristics	Diabetic group 1	Diabetic group 2	Control group	P value
Sex (M/F)	20/42	8/6	30/18	0.08
Age (y)	54 ± 10	53 ± 13	46 ± 6	0.89
Systolic BP (mmHg)	136 ± 16	144 ± 22	128 ± 9	0.29
Diastolic BP (mmHg)	83 ± 8	85 ± 8	80 ± 5	0.12
Body mass index (kg/m ²)	31.9 ± 6.3	32.0 ± 6.2	24.2 ± 2.5	<0.001 ^a
Fasting glucose (mmol/l)	9.3 ± 4.8	9.1 ± 2.5	4.8 ± 0.4	<0.001 ^a
Diabetes duration (y)	11 ± 7	18 ± 12	—	0.03 ^b
HbA1c (%)	8.6 ± 1.5	8.6 ± 0.7	—	0.28 ^b
HbA1c (mmol/mol)	70 ± 9	70 ± 4	—	0.31 ^b
Total cholesterol (mmol/l)	4.9 ± 1.2	5.1 ± 1.3	—	0.76 ^b
Creatinine (μmol/l)	81.5 ± 28.7	90.3 ± 16.9	—	0.02 ^b
Urea (mmol/l)	7.7 ± 6.8	8.2 ± 2.3	—	0.09 ^b
ALT (IU/L)	34.2 ± 20.3	28.4 ± 19.3	—	0.25 ^b
AST (IU/L)	31.0 ± 24.8	24.1 ± 13.4	—	0.13 ^b
The presence of complications (% of the total number of patients)	15	50	—	0.007 ^b

Note: Data are mean ± SD unless stated otherwise.

Note: Reference values of the laboratory: HbA1c 4.0% to 6.0%, total cholesterol 3.5 to 5.0 mmol/l, urea 2.5 to 7.5 mmol/l, creatinine 70 to 110 μmol/l, ALT 10 to 38 IU/L, and AST 10 to 40 IU/L.

^aControl group versus diabetic group 1 and diabetic group 2.

^bDiabetic group 1 versus diabetic group 2. Nonparametric data of the three studied groups were analyzed by a Kruskal–Wallis analysis of variance (ANOVA). A Mann–Whitney U-test was used to identify differences between the two groups.

block “LAZMA-TEST” (SPE “LAZMA” Ltd., Russia), consecutive local cold (25°C) and heat (35°C and 42°C) provocations were applied for 4 min each. The first stage included registration of microcirculatory parameters in the basal conditions for a 4-min period and the recording of a pair of fluorescence spectra. Thus, the duration of one study was ~16 min.

In this study, the probe was secured on the dorsal surface of the foot to a point located on a plateau between the first and second metatarsal bones [Fig. 1(b)]. We investigated the area of nonglabrous skin, where changes in blood flow are mediated by the vessels themselves and by two mechanisms of the sympathetic nervous system: noradrenergic vasoconstrictor nerves and a cholinergic active vasodilator system.⁵⁷ It is due to this that the bulk of the literature^{58,59} is focused on controls of the nonglabrous skin with less focus on the glabrous skin of the palms, soles, and digits. Moreover, some research stated that perfusion in the sites with arteriovenous anastomoses (with glabrous or nonhairly skin) may fluctuate substantially,^{60,61} so the microcirculation assessment in this area may not be a very sensitive indicator of disease severity.⁶² Particular attention was paid to minimize local pressure of the probe on skin, as the parameter strongly influences the measurement results of skin blood perfusion and fluorescence intensity.⁶³ All studies were performed in the supine position. The volunteers were asked to refrain from food and drink 2 h before the study to exclude the influence of these factors on the change of microhemocirculation

and metabolic processes. Room temperature was maintained at a steady 24°C to 25°C. All volunteers underwent preliminary adaptation to these conditions for at least 10 min. Furthermore, skin temperature change of volunteers was recorded during the study. In the basal conditions, subjects had different skin temperatures. In this regard, to unify measurements, we pre-cooled the study area on the foot to 25°C.

2.3 Validation of Diagnostic Depth of the Probe

Proper evaluation of fluorescence intensity measurements in healthy tissue and areas of pathology will be possible if the penetration depth of the radiation, as well as diagnostic volume, was known. It is also important for the researcher or practicing physician to understand the depth of penetration of probing radiation in laser Doppler measurements for a specific measuring device.⁶⁴ This would provide information on the blood vessels involved in the formation of the signal.

Due to the complexity of the actual sensing conditions, as well as a complex composite structure of human skin, it is not possible to obtain a general analytical solution to evaluate the diagnostic volume. In this regard, the method of stochastic Monte Carlo (MC) simulations was used. Currently, there are some known realizations of this approach used to simulate the propagation of optical radiation in organic tissues.^{65–67} One of the more advanced approaches is the use of an object-oriented

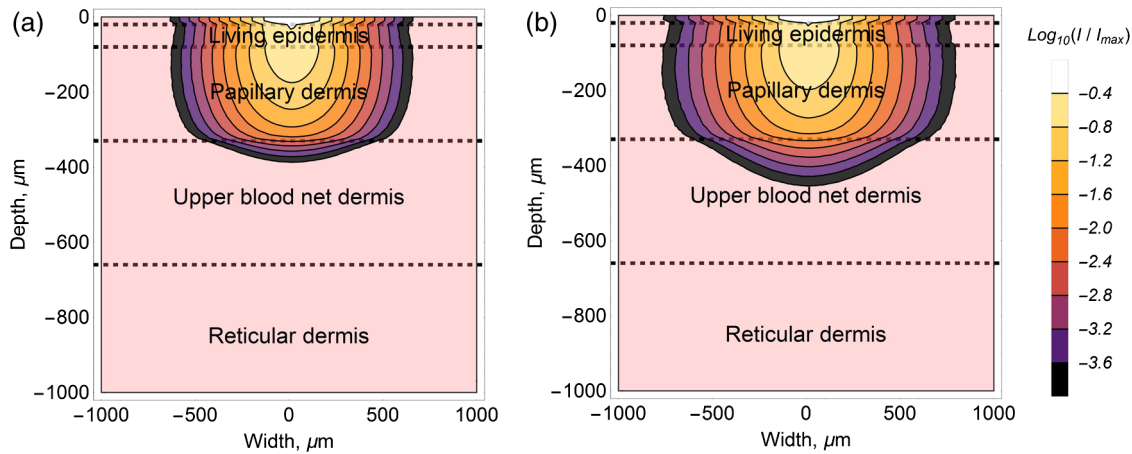


Fig. 2 Result of the modeling of diagnostic volume for the fluorescence measurements for the excitation wavelength of (a) 365 and (b) 450 nm for the parameters of the used optical fiber probe: the distance between the fibers: 1 mm, fiber diameter: 400 μm , receiving aperture angle: 0.22.

MC model,^{68–71} which allows for the description of the photons and the structural components of the tissue as independent objects interacting with each other. For acceleration of simulation performance, parallel computing was implemented on the used algorithms. Parallel computing technology CUDA (Computer Unified Device Architecture, NVIDIA Corporation) was employed as a framework of the calculations.

For both the LDF measurements and the fluorescence measurements, the diagnostic volume was assessed through an evaluation of the probe radiation distribution. For fluorescence measurements, the validity of this approach is based on the fact that, according to the rule of Stokes shift, the fluorescence spectrum is shifted into the long-wave region. It is also known that the absorption of light in most of the biological tissues for wavelengths of visible spectrum falls as wavelength increases. Thus, to get a lower-bound estimate of the diagnostic volume of the measurements, assessing the sampling volume of the radiation for a wavelength of fluorescence excitation is sufficient. One of the possible ways to formalize calculation of the sampling volume is calculating the distribution of rays reaching the detector using the MC method.

A seven-layer tissue model was used to simulate the diagnostic volume and depth of penetration of the probe radiation. This model was originally proposed in an earlier article.⁶⁸ Figure 2(a) shows an example of the distribution obtained for the above simulation parameters for a wavelength of 365 nm. Thus, diagnostic depth of the conducted diagnostics is at least several hundred microns and includes the epidermis, papillary dermis layer, and small part of upper blood net dermis [Fig. 2(a)]. Diagnostic volume, estimated by simulation, is in the order of 0.2 mm^3 .

For an excitation wavelength of 450 nm, diagnostic depth reaches 450 to 500 μm and penetrates the epidermis, papillary dermis, and most of the upper blood net dermis [Fig. 2(b)]. In this case, the diagnostic volume is approximately estimated by the simulation as 0.35 mm^3 .

The performed simulation shows that the proposed probe allows for the registration of fluorescence in the epidermis, mainly contributed to by NADH and FAD, and the dermis with a significant contribution from collagen.

The results of diagnostic volume modeling for the LDF probe show that the value is about 1.8 mm^3 . This confirms that this probe of the developed device is highly sensitive to the

variations of blood flows in the papillary dermis and upper blood net plexus and is able to cover the top part of the reticular dermis (Fig. 3).

2.4 Data Analysis

The recorded and subsequently averaged amplitudes AF_{460} of the fluorescence at 460 ± 10 nm with excitation at 365 nm and AF_{525} of the fluorescence at 525 ± 10 nm with excitation at 450 nm (normalized to the intensity of the backscattered excitation radiation), as well as the average blood tissue perfusion I_m at each stage of the functional test, were analyzed. The wavelengths were selected to maximize the probability of detection of NADH and FAD signals. Taking into account the relatively small sample sizes, nonparametric methods were used to confirm the reliability of differences in the results even in small groups of observations, namely the Mann–Whitney U-test and Kruskal–Wallis ANOVA test. Values of $p < 0.01$ were considered significant.

Considering the former division of experimental data into classes (two patient and one control), a linear discriminant

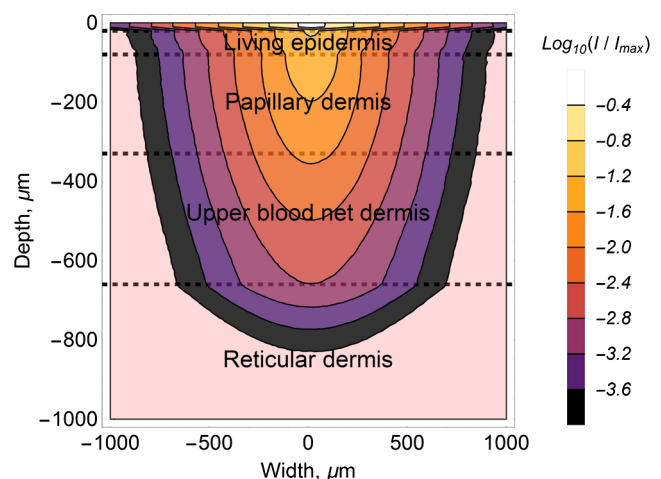


Fig. 3 The result of modeling the diagnostic volume for the LDF measurements for the wavelength of 1064 nm, using the following optical fiber probe parameters: distance between the fibers: 1.5 mm and receiving aperture angle: 0.22.

Table 2 The results of experimental studies.

Parameter	Control group	Diabetic group 1	Diabetic group 2
Fluorescence amplitude AF ₄₆₀ , AU	2.1 ± 0.8	2.7 ± 0.8 [*]	3.8 ± 0.7 ^{**}
Fluorescence amplitude AF ₅₂₅ , AU	1.2 ± 0.4	1.8 ± 0.7 [*]	2.5 ± 0.6 ^{**}
Perfusion I_m , PU	5.0 ± 1.9	5.3 ± 2.2	3.8 ± 1.6
Perfusion $I_m^{25^\circ\text{C}}$, PU	4.4 ± 1.6	4.7 ± 2.0	3.6 ± 1.4
Perfusion $I_m^{35^\circ\text{C}}$, PU	8.7 ± 3.1	6.6 ± 2.4 [*]	5.0 ± 1.4 ^{**}
Perfusion $I_m^{42^\circ\text{C}}$, PU	19.9 ± 4.6	12.3 ± 3.5 [*]	9.2 ± 4.6 ^{**}

^{*}Confirmed statistically significant differences between control group and diabetic group 1 ($p < 0.01$).

^{**}Confirmed statistically significant differences between diabetic group 1 and diabetic group 2 ($p < 0.01$).

analysis was employed to determine the discriminant function, leading to the synthesis of the desired decision rule. The obtained classifiers allow for a reappearing object to be assigned to one of the above classes by measured parameter values. Evaluation of the discriminant analysis quality was performed using the receiver operating characteristic (ROC) curve.

3 Experimental Results and Discussion

3.1 Experimental Study

Experimental studies have shown that diabetic patients have elevated values of normalized fluorescence amplitudes, as well as a lower perfusion response to local heating (up to 35°C and 42°C). At the same time, in the group of diabetic patients with trophic ulcers (diabetic group 2), these parameters also significantly differ from the control and diabetes only groups (Table 2, Fig. 4).

As already mentioned, AGEs contribute to the mechanisms responsible for the development of complications in diabetes. Formation of intracellular AGEs promotes violation in protein function, with their accumulation acting as an objective marker for the tissues glycation. The level of skin fluorescence observed in our studies is related to the degree of conventional glycation marker HbA1c as determined *in vitro*. It is important to note, however, that the standard measure of glycation using HbA1c characterizes the glycation processes that occur only in the short term (around 3 months). Changes in AGE content reflect long-term processes in the diabetic skin. Considering the long molecular lifetime of collagen and the stability of AGEs, it is possible to use skin fluorescence as a measure of the total impact of hyperglycemia throughout the course of life.

It is known that diabetes can lead to tissue hypoxia.⁷²⁻⁷⁴ In the event of hypoxia, the aerobic cellular respiration pathway is disrupted, and the mitochondrial NADH oxidation is slowed down. Additionally, the glycolysis pathway for NADH formation is activated. In this context, the accumulation of NADH may act as a sign of tissue hypoxia, with its contribution to the total fluorescence signal serving as a marker of total oxygen deficiency in tissues. Meanwhile, the processes of oxidative phosphorylation exhibit changes in the concentrations of NADH and FAD, which are very complex, and the dynamics of these changes may be nonlinear throughout the cell's lifetime.^{38,39} This should be taken into account when interpreting the data received and further researching in this area.

It is also shown that the accumulation of AGEs may suppress the synthesis of NO in endothelial cells.⁷⁵⁻⁷⁷ This may also explain the differences in perfusion response to various stimuli. Basal level does not statistically differ among the compared groups. However, the proposed functional stimulation in the form of thermal tests can detect and distinguish between microvascular abnormalities in diabetic patients. The perfusion increase during a local heating test occurs mainly due to two mechanisms. The heating of skin to 34°C to 35°C results in activation of peptidergic nerve fibers. This happens due to the activation of thermosensitive TRPV1 receptors.⁷⁸ The vasodilation caused by heating skin to 42°C is associated with a release of NO from vascular endothelium.^{79,80} Topographically, both

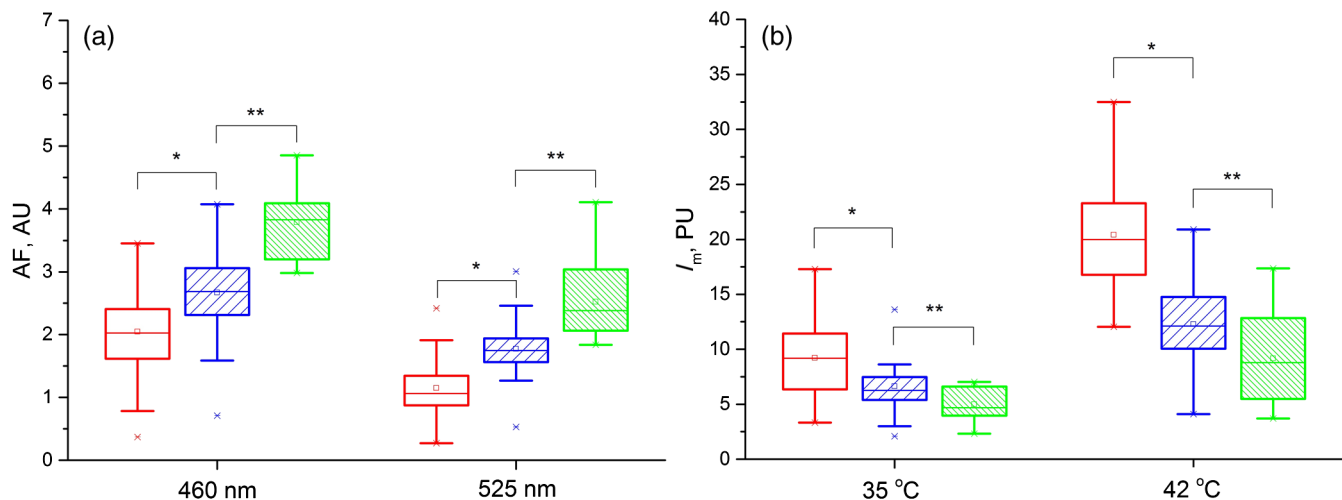


Fig. 4 Comparison of parameters among control (red empty bars), diabetic (blue loose shading), and diabetic with ulcers (green tight shading) groups: (a) the normalized fluorescence amplitude and (b) the average perfusion in the stages of heating to 35°C and 42°C. In each box, the central line is the median of the group, while the edges are the 25th and 75th percentiles.

Table 3 Sensitivity and specificity for the first classification rule.

Parameter	AF ₄₆₀	AF ₅₂₅	$I_m^{35^\circ\text{C}}$	$I_m^{42^\circ\text{C}}$	AF ₄₆₀ , $I_m^{35^\circ\text{C}}$	AF ₄₆₀ , $I_m^{42^\circ\text{C}}$	AF ₅₂₅ , $I_m^{35^\circ\text{C}}$	AF ₅₂₅ , $I_m^{42^\circ\text{C}}$
Sensitivity	0.64	0.77	0.77	0.88	0.75	0.92	0.87	0.90
Specificity	0.7	0.79	0.62	0.81	0.77	0.90	0.77	0.83

arterioles and capillaries are involved in local heating. All aspects of the microvasculature and tissue systems undergo dysfunction during diabetes, including the vascular endothelial and perivascular nerve fibers. Therefore, the thermal test is pathogenically justified for diagnosing these disorders during diabetes. A reduction in perfusion growth when skin is heated to 35°C acts as an objective criterion of sensory nerve fiber dysfunction, which in turn serves as a component of diabetic neuropathy. The reduction of perfusion growth in people with diabetes compared with the control group during skin heating to 42°C reflects a deficit of endothelium-dependent vasodilation mechanisms (presumably NO-induced).

3.2 Data Analysis

The analyzed parameters of normalized skin fluorescence amplitudes and perfusion are proposed for the synthesis of the decision rule. As stated above, these parameters satisfy the principles of statistical independence, as well as the significance of the differences of their values, calculated for the patient and control groups. The discriminant function is synthesized in such a way as to provide high sensitivity while providing excellent specificity. Table 3 summarizes the sensitivity and specificity for the first classification rule (the control group is compared with a group of patients without ulcers) for a different combination of measured parameters. As can be seen from the table, the lowest level of error is obtained with the combination of fluorescence intensity at an excitation wavelength of 365 nm and the level of stimulation of microcirculatory perfusion at 42°C.

For the second classification rule for the separation of the diabetic group and diabetic group with ulcers, a sensitivity and specificity of 0.86 and 0.85, respectively, were obtained.

For better combinations of sensitivity and specificity, Fig. 5(a) shows the scatter plot of the experimental data with applied discriminant lines that divide the experimental points into three groups (healthy, diabetic, and diabetic with ulcers). The immediate diagnostic criterion, with the aid of the discriminant functions (D_1 and D_2), allows for the relation of a newly measured subject to one of the three groups:

$$\begin{cases} D1 = -2.55 - 0.45AF_{460} + 0.23I_m^{42^\circ\text{C}}, \\ D2 = -1.8 + 1.16AF_{460} - 0.18I_m^{42^\circ\text{C}}. \end{cases} \quad (1)$$

It can be seen from Fig. 5(a) that the shift toward the top-left characterizes the deterioration of the patient's condition and increase in the risk of development of foot ulcers.

Figure 5(b) shows the ROC curves calculated for the obtained discriminant functions. To compare the quality of different classifying rules, it is convenient to use the integral characteristic AUC—area under curve. In our case, for both classification rules—AUC = 0.93. This indicator indicates a high level of quality of the classifier.

Thus, the skin fluorescence and level of tissue blood perfusion during a local heating test can act as markers for various stages of diabetes, beginning with early disease, to the advancement and development of trophic ulcers.

4 Conclusion

The obtained experimental results confirm the prospects of the development of new methods for evaluating metabolic shifts in diabetes mellitus. The proposed method could be a marker of diabetic complications. It can also be used to assess therapeutic

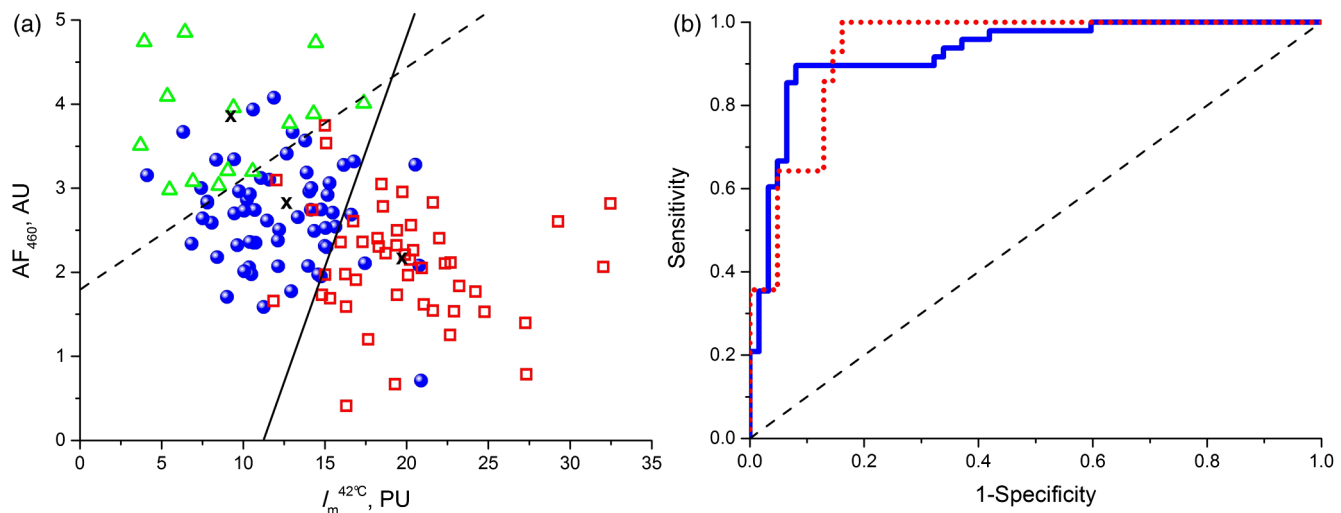


Fig. 5 (a) The scatter plot with applied discriminant lines, obtained by linear discriminant analysis method and (b) ROC-curves for assessing the effectiveness of the classifiers. The healthy group is shown by squares, the diabetic group by circles, and the diabetic group with ulcers by triangles. For the ROC curve: dot line: control group versus diabetic group 1; solid line: diabetic group 1 versus diabetic group 2.

interventions aimed at preventing or reversing the progression of these diabetic complications.

Modeling and experimental results of this study confirm the high sensitivity of optical noninvasive methods (laser Doppler flowmetry and fluorescence spectroscopy) when detecting violations of biological tissue in type 2 diabetes mellitus. Both approaches, either individually or in combination, may have important clinical implications as they may help to identify patients at risk of developing future problems within their lower limbs.

Improvement of methodological support of optical noninvasive diagnosis (convenient and completely safe for the patient) will make a significant contribution to the fight against diabetes, which is very relevant given the prevalence of the disease.

There is also further potential to expand the number of clinical investigations and improve the methodology. This will eventually allow the methods in question to be introduced into the practice of the attending physician.

A promising direction for future research within this field is the differentiation of AGEs and NADH and FAD contributions to the resultant fluorescence signal. This would allow for separate studies into the possible pathways of biological tissue violation: by oxidative phosphorylation and carbohydrate metabolism.

Disclosures

No potential conflicts of interest relevant to this article were reported.

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