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Dynamic Non-Invasive Detection of NADH Based on Blood Flow-Mediated Skin Fluorescence (FMSF) Method

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Abstract

Nicotinamide adenine dinucleotide (NADH/NAD^+) is involved in important biochemical reactions in human metabolism, including participation in energy production by mitochondria. The changes in fluorescence intensity as a function of time in response to blocking and releasing of blood flow in a forearm are used as a measure of oxygen transport with blood to the tissue, which directly correlates with the skin microcirculation status. In this paper, a non-invasive dynamic monitoring system based on blood flow-mediated skin fluorescence (FMSF) technology is developed to monitor the NADH fluorescence intensity of skin tissue during the process of blocking reactive hyperemia. Simultaneously, laser speckle contrast imaging (LSCI) and laser Doppler flowmetry (LDF) were used to observe blood flow, blood oxygen saturation (SOt_2) and relative amount of hemoglobin (rHb) during the measurement process, which helped to explore NADH dynamics relevant physiological changes. A variety of parameters have been derived to describe NADH fluorescence curve based on the FMSF device. The experimental results are conducive to understanding the NADH measurement and the physiological processes related to it, which help FMSF to be a great avenue for in vivo physiological, clinical and pharmacological research on mitochondrial metabolism.

Keywords

Reduced Nicotinamide Adenine Dinucleotide (NADH), Flow-Mediated Skin Fluorescence (FMSF), Laser Doppler Flowmetry (LDF), Blood Flow

1. Introduction

The incidence of cardiovascular diseases (including hypertension, myocardial

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infraction, and stroke) has gradually increased with the changes of diet and lifestyle [1] [2]. Development of methods and tools for diagnosis, treatment, and monitoring of cardiovascular health status, especially priority stressed on the minimally or non-invasive techniques have important significance and value and are highly awaited. Research shows that microvascular dysfunction may precede endothelial impairment in large arteries and clinical manifestations [3] [4] [5]. The skin microcirculation is a readily accessible vascular bed that can be used as a model of generalized microvascular function [6] [7]. In humans, microvascular function in the skin has been extensively studied using non-invasive laser Doppler flowmetry (LDF) or more recently laser speckle contrast imaging (LSCI), which provides a real-time quantification of relative changes in tissue blood perfusion [8] [9] [10]. The conventional approach is to couple these techniques with various dynamic tests such as post-occlusive reactive hyperemia (PORH). Indeed, peripheral microvascular dysfunction assessed by LDF and PORH has been shown to be an independent predictor of atherosclerotic damage [11] [12]. However, quantitative analysis of changes in flowmotion caused by different pathologies is not always reliable, due to the own shortcomings of LDF and LSCI measurements, which makes their scope of application is also limited [9].

Current microvascular techniques quantify skin blood flow. However, skin microcirculatory function and efficiency of blood supply to the skin can impact mitochondrial activity and the changes of nicotinamide adenine dinucleotide (NADH) fluorescence in the reduced form [13]. NADH/NAD^+ participates in glycolysis, the citric acid cycle and the mitochondrial respiratory chain, beta-oxidation, reduction of pyruvate to lactate, as well as the modification of RNA together with regulation of transcription [14] [15]. Mitochondrial function can be indirectly evaluated by NADH fluorescence that has been measured in animals and humans at rest and under other conditions [16] [17] [18] [19]. It was claimed that the monitoring of the NADH level in tissue provides important information about the mitochondrial metabolic state (energy production, amount of intracellular oxygen) [16].

Skin, the largest organ of human body, is characterized by a unique metabolism [20]. As the epidermal layer of the skin is not directly vascularized, oxygen and nutrients are transported from the dermis by diffusion processes. Epidermal cell metabolism may therefore be regarded as a unique and sensitive marker of early dysfunction in the vascular circulation and metabolic regulation. NADH fluorescence is the strongest component of overall fluorescence emitted from human skin [2] [6]. The NADH content can be measured in several ways. Some of the examples include fluorometric [21], spectrophotometric [22] and bioluminescent enzyme assays [23] [24]. Interestingly, exciting NADH with ultraviolet (UV) light in the 320 - 380 nm range produces autofluorescent light emission in the 420 - 480 nm range with peak intensity at 450 - 460 nm [22]. This optical feature has been widely used for measuring NADH concentration or content in solutions, cells and tissues [24].

Many scientists have made efforts to accurately measure the content of NADH and have obtained some positive achievements. Among these studies, Piotrowski *et al*. developed a non-invasive optical technique called Flow Mediated Skin Fluorescence (FMSF) measures the changes of NADH fluorescence intensity from the skin of forearm as a function of time in response to blocking and releasing of blood flow in the forearm [7] [20]. This method modulates tissue ischemia and reactive hyperemia, changes the local blood oxygen supply of skin tissue, and monitors the dynamic change characteristics of NADH fluorescence spectra to obtain microcirculation and metabolic regulation. Compared with conventional detection methods, NADH in vivo detection based on FMSF will not cause any trauma to the human body, and does not require any other reagents. It can continuously monitor the human body from the cellular and molecular level. It is simple, fast, and allows real-time, dynamic and non-invasive measurements. Since the content of NADH is closely related to the state of mitochondria in human cells, the dynamic detection of the fluorescence intensity of NADH in the human body can promptly screen out related acute or chronic diseases, thereby realizing in vivo monitoring of the human body's pathophysiological state, which has high application prospects and social value. So far, the FMSF method has been applied in several studies, including both healthy people and patients with various pathologies and disorders (e.g., coronary artery disease (CAD) [6] [7], type 1 diabetes mellitus (DM1) [25], systemic lupus erythematosus (SLE) [26], COVID-19 [27] and so on), as well as in sport physiology [28].

According to available literatures, there is a lack of studies describing changes of skin tissue physiological parameters and their effect on changes of NADH fluorescence during FMSF. Therefore, in this study, we developed a skin NADH fluorescence measurement system to evaluate the changes of NADH fluorescence in the skin based on FMSF method, synchronously record the physiological parameters including blood flow, venous oxygen saturation (SO_2) and relative amount of hemoglobin (rHb) during the measurement process by joint use of LDF and LSCI. Furthermore, we have clarified the reaction process that occurs in the skin tissue via parameters derived to describe NADH fluorescence curve based on the FMSF device.

2. Experiment and Method

2.1. Instrument

NADH fluorescence was measured by a homemade FMSF system designed as in **Figure 1(a)**. The main components of this system are: a UV excitation source (LED with wavelength of 340 nm, Shanghai Ideaoptics Corp., Ltd., China), an optical fiber probe (customized, Beijing Scitlion Technology Corp., Ltd., China), a blood pressure pneumatic cuff (Jiangsu Yuyue Medical Equipment & Supply Co Ltd., China), an optical fiber spectrometer (Ocean Optics, USA), a special forearm support bracket (customized, Wuhan kaifry Manufacturing Co., Ltd., China) and a computer control system. The forearm support bracket provides

support and stability of participants to minimize recording noise throughout the process. Figure 1(b) shows the face of the probe, from which a central fiber collects fluorescence light and the surrounding twelve source fibers (core radius of 100 μm) deliver exciting light. The radius of the central detection fiber was 300 μm, the center-to-center separation of the source and the detection fibers is 550 μm. The numerical aperture of all the fibers is 0.22. In the measurement, the probe was vertical and soft touched on the medial forearm skin and then the signal was recorded. The whole system is controlled and operated by a home-written computer software in LabVIEW (National Instruments, USA).

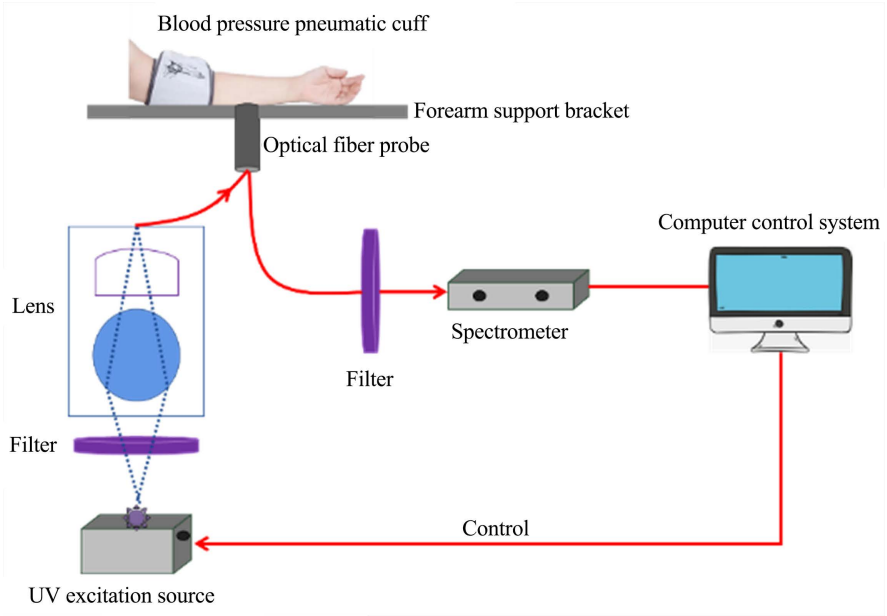

Figure 1. Schematic diagram of FMSF system.

FMSF enables recording of the changes in NADH fluorescence as a function of time in response to ischemia and reperfusion in forearm skin cells. NADH molecules have autofluorescence capability at a wavelength of 460 nm. The changes in fluorescence intensity observed during the examination are produced in the skin epidermis cells, which is due to very shallow skin penetration by excitation light at the wavelength of 340 nm. About 90% of the recorded signal comes from the skin depth up to 0.5 mm [7] [22].

Calibration procedures involve establishing baseline fluorescence intensity using a standard fluorophore solution, calibrating the probe against a known fluorescence standard to account for variability in the optical fiber probe, and monitoring and compensating for environmental factors such as ambient light and temperature to ensure consistent measurements.

2.2. Study Population and Measurement Protocols

In this experiment, we recruited 6 female and 7 male healthy volunteers. All the subjects gave written informed consent prior to participation. None of them had

a chronic disease or was undergoing treatment. Their average age was 27 ± 4 years, their average systolic and diastolic blood pressures were 116 ± 12 and 78 ± 10 mmHg, respectively, and their body mass index (BMI index) was 21.5 ± 2 kg/m^2. Protocols 1 and 2 were repeated three times for each participant.

Protocol 1: Using FMSF system to measure the dynamic changes of NADH fluorescence.

The whole experiment process was carried out in a temperature-controlled room (25˚C ± 1˚C). Participants sat on a chair with his/her arm resting on the measuring device. Immediately before examination, systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured using the Yuwell sphygmomanometer (Jiangsu Yuyue Medical Equipment & Supply Co Ltd., China) device. After a 20-minute acclimatization period, skin fluorescence intensity was recorded for 1 min on the forearm after acclimatization period for baseline NADH fluorescence, then blood flow in the brachial artery was occluded for 3 mins by inflating a cuff placed on the left upper arm to 50 mmHg above SBP of participants. The cuff was then released for reperfusion and return to normal for 3 mins. During the baseline, occlusion and reperfusion period, the NADH fluorescence was continuously measured in the same area of the forearm.

Protocol 2: Using the LDF and LSCI methods to measure the physiological parameters.

The same subjects who participated in protocol 1 also participated in the experiment of protocol 2 using a high frame rate LSCI (PeriCam PSI System, Perimed, Järfälla, Sweden) and LDF (O2C, Version 2424, LEA Medizintechnik GmbH, Germany) devices. The subjects' measurement sites are randomly chosen on the ventral surface of the medial forearm which are close to the site of measuring NADH, and hair, broken skin, areas of skin pigmentation and visible veins were avoided. The blood perfusion during rest, blocking ischemia, and reactive hyperemia was monitored and evaluated by LDF and LSCI at the same time. Furthermore, SO_2 and rHb were monitored by LDF. In addition, we monitored the blood flow changes of the medial forearm skin and fingers under the same conditions for comparison.

In the experiment, the two instruments ran at the same time with the NADH measurement. The system of LDF transmits continuous wave laser light (830 nm and 30 mW) and white light to tissue where it is scattered and collected on the skin surface at fibers in the probe. The collected light is split into its spectral components by charge-coupled device array and converted into an electrical signal. The single-point probe uses a transparent film to fix it on the arm skin to follow the movement of the arm and reduce the artifacts caused by the movement. The system of LSCI uses a divergent laser beam at a wavelength of 785 nm to create a speckle pattern in the illuminated area. And it uses two cameras, one that captures the speckle contrast image, and the other that captures a conventional color image of the area. The size of the image was set to an area of 2 × 1.5 cm, the exposure time was 5 ms, the distance between the camera and the skin

was 10 cm, and the acquisition rate was set to 21 frames/s. The spatial resolution of the perfusion image was 0.2 mm/pixel.

3. Results and Discussion

3.1. Measured NADH Fluorescence and Physiological Parameters

The simultaneous recording of FMSF, LSCI and LDF during the occlusion and PORH procedures according to the procedures described in Section 2.2. The signals are synchronized according to the beginning of baseline and end of reperfusion. Figures 2(a)-(j) show a typical original image of the tested forearm/finger and the reconstructed blood perfusion maps during the occlusion and PORH procedures by LSCI.

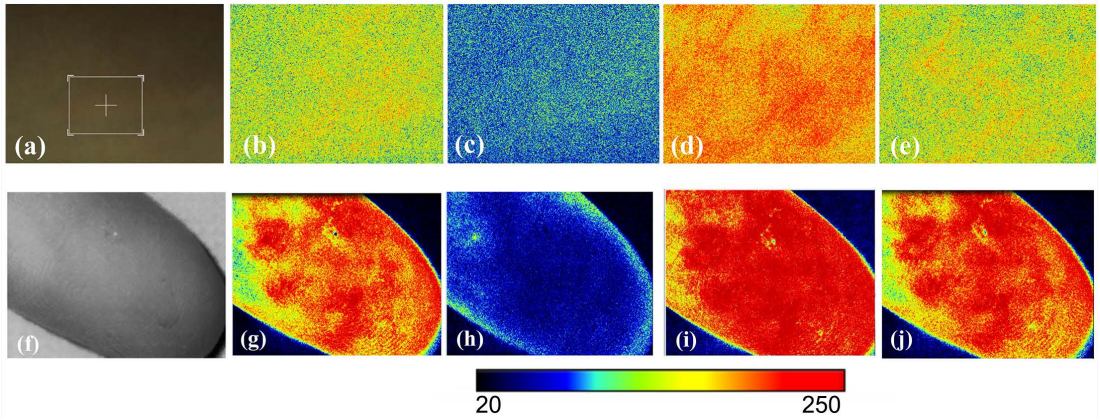

Figure 2. Measurement of skin blood volume by laser speckle contrast imaging (LSCI) on the medial forearm (a) - (e) and index finger (f) - (j). Colors range from dark blue (no perfusion) to red (high perfusion). (a) and (g) intensity image representing backscattered light; (b) - (e) and (g) - (j) skin blood perfusion map before (b) (g), during (c) (h), after vascular occlusion (d) (i) and at the end of the test (e) (j).

Figure 3(a) shows a typical trace of NADH fluorescence and its dynamical change of a healthy individual, recorded using the FMSF method. Within 0 - 60 s, without any cuff pressure applied to the subject, the fluorescence intensity of NADH in the body presents a dynamic and stable state. During ischemia from 60 s to 240 s, there is a gradual increase of the 460 nm fluorescence intensity until approaches the plateau, which called ischemic response (IR). When the blockage is released at 240 s, the restoration of blood flow leads to a rapid decline in the strength of NADH fluorescence intensity followed by its gradual recovery to the reference values. This is known as the hyperemic response (HR). In fact, two distinct phases can be identified in HR. The first phase of about 20 - 30 s is associated with a sharp drop in NADH fluorescence, and can be linked to hyperemia. The post-hyperemic phase is followed by reperfusion, when NADH fluorescence returns to the baseline. Some grossly visible oscillations of the 460 nm fluorescence are present on the baseline and the hyperemic (reperfusion) part of the FMSF trace but not during ischemia, the internal mechanism is unclear and under investigation. Researches declared that the oscillations known as

flowmotion are a well-recognized characteristic of cutaneous blood flow [20]. And a pilot study that focused on an analysis of the flowmotion for various diseases and pathologies including diabetes and cancer as well as aging was performed [29].

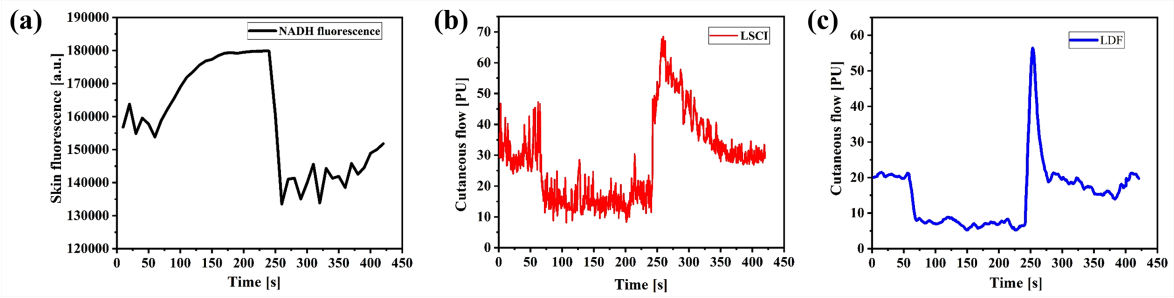

Figure 3. Schematic diagram of change of NADH fluorescence (a) and skin blood flow measured by LSCI (b) and LDF (c) devices on medial forearm. PU: perfusion unit, AU: absorbance unit.

Figure 3(b) and Figure 3(c) also respectively shows the blood flow of the medial forearm measured by LSCI and LDF devices. It can be more intuitively found from Figure 3(b) that under the baseline conditions before the vascular occlusion taken by LSCI device, the skin blood flow of medial forearm is in the range of 30 to 40 PU. As the blood vessel is occluded, the skin blood volume drops to <15 PU. After the occlusion is released, the skin blood volume increases to >60 PU due to hyperemia and then resumes its baseline value. It can be seen from the figure that the baseline values of blood flow measured by LSCI and LDF are not inconsistent, which is mainly related to the measurement mechanism of LSCI and LDF. LDF measures the blood flow at a single point, while LSCI measures the blood flow in a regional area. In the baseline phase, the average blood perfusion volume of LDF and LSCI are PU_{LDF} = 22 and PU_{LSCI} = 32, respectively. In the ischemic state, the values of LDF and LSCI both decrease rapidly in about 20 s and then it stabilized again, at where, PU_{LDF} = 5 and PU_{LSCI} = 15 relative to the plateau. At last, when the brachial artery cuff pressure is released, the blood perfusion rises rapidly and reaches the highest point (PU_{LDF} = 56.5 and PU_{LSCI} = 62). After that, the blood flow drops rapidly and re-reach the state of dynamic equilibrium.

We assessed microcirculatory parameters SO_2 and rHb during arterial occlusion of the arm in LDF presented in Figure 4. The SO_2 is determined by the color of blood. The rHb value is determined by the amount of light absorbed by tissue. This measurement represents a hemoglobin concentration per tissue volume and is independent from the vessel density (microvessels), vessel lumen (only vessels < 100 μm), and hemoglobin quantity in the blood. Assessing these parameters simultaneously and in the same skin site enables studying the true interrelationship between the parameters. This real-time analyze method may provide new insight into the microcirculation of the tissue and aid in a more comprehensive assessment of microvascular status. The SO_2 fluctuates at around

40%, and the rHb is about 65. With the blood volume fraction dropping rapidly after the occlusion of blood vessels, the SO_2 and rHb decreased to 8% and 30, respectively. When the occlusion was removed, the tissue became congested rapidly, and the SO_2 and rHb increased to maximum value more than 80% and 75, respectively. Then both gradually decreased, and finally returned to near the baseline value. Studies have shown that in the entire FMSF process, there is a certain correlation between the changes in the content of oxyhemoglobin/deoxyhemoglobin and the fluorescence of NADH.

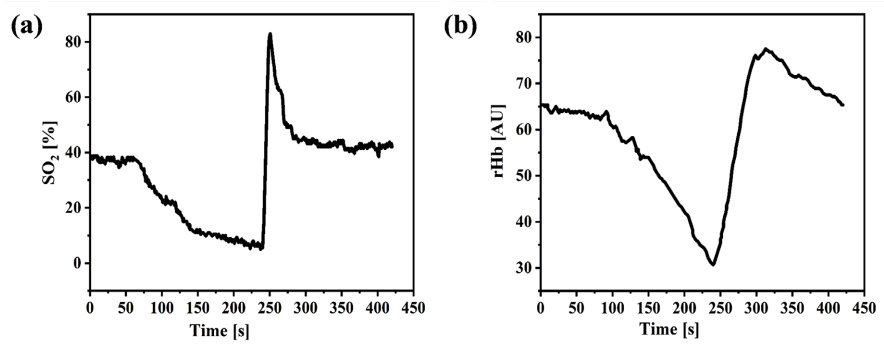

Figure 4. Changes in blood oxygen saturation SO_2 (a) and relative amount of hemoglobin rHb (b) of medial forearm. AU: absorbance unit.

In Figure 5, we compared the blood flow of the medial forearm and finger measured by LSCI, there is not much disparity exist in blood flow between medial forearm and finger at the initial and the ischemic stages. However, in the PORH stage, the maximum blood flow of the finger was higher than that of the medial forearm, and then both returned to a normal state. The blood flow of the finger is greater than that of the skin of medial forearm, it may be because the finger itself has richer microcirculation. Since the medial forearm and the finger under test belong to the same arm, the blood flow change on the medial forearm is basically the same as the change trend of the finger, but the amplitude is smaller.

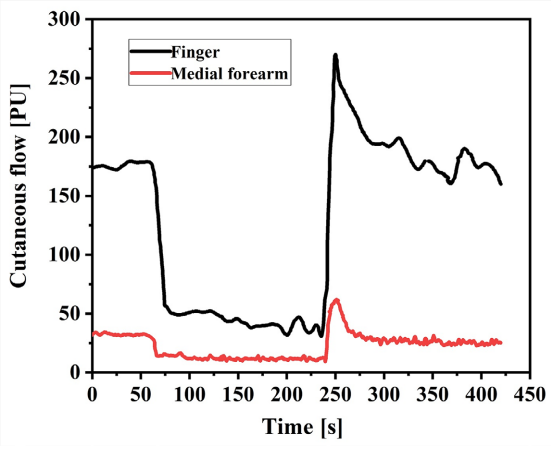

Figure 5. Recording of the blood flow of the medial forearm and finger by LSCI through PORH. PU: perfusion units.

3.2. Parameters of Quantity of NADH Fluorescence

Among a variety of non-invasive techniques for monitoring microvascular function in the skin, laser Doppler techniques and LSCI, when coupled with PORH, offer easy-to-perform and reliable tools to assess endothelial and neurovascular function in the cutaneous circulation [8] [9] [30]. Laser Doppler techniques provide an index of skin perfusion by measuring the Doppler shift induced by coherent monochromatic light scattering by moving red blood cells. Because it does not provide an exact measure of flow, it is often referred to as flux. A linear relationship between flux and actual flow has been demonstrated. LDF has very good temporal resolution at quantifying fast changes in skin blood flux. However, the inter-day reproducibility of LDF on the forearm is poor, most likely related to the regional heterogeneity of skin perfusion due to the skin anatomy leads to spatial variability [31].

Compared to laser Doppler techniques, full-field imaging techniques LSCI exhibits an excellent reproducibility when assessing microvascular reactivity. High-frame-rate LSCI is a recently marketed non-contact technique based on analysis of the reduction in speckle contrast, which has good temporal and spatial resolutions [32]. It allows assessment of perfusion over wide areas but with a high frequency. LSCI provides a perfusion index proportional to the concentration and mean velocity of red blood cells. These properties allow measurements of fast changes in skin blood flux over wide skin areas, with very good inter-day reproducibility [33]. However, LSCI also has its limitations. First, the skin penetration depth has been shown to be shallower for the LSCI (300 mm) than for laser Doppler (1 - 1.5 mm). This should be considered because the thickness of the skin is heterogeneous [34]. Second, laser Doppler has a better linearity with blood flow than LSCI. Direct comparison between LSCI and LDF has shown correlation over a wide range of human skin perfusion rates, despite a loss of correlation for low skin perfusion, which suggesting a non-linear relationship between LSCI signal and skin blood flow [35]. The third issue with LSCI is its sensitivity to movement artifacts, which is higher than that observed with laser Doppler. This is explained by the physical principle of the technique, even light movements decrease speckle contrast [9]. Despite these limitations, LSCI is a promising tool as well as laser Doppler that has been increasingly used in the past few years.

In contrast to LDF and LSCI, the newly-developed FMSF technique enables us to quantify skin fluorescence that theoretically may measure the biochemical redox equilibrium between NADH and NAD^+ in the tissue, in addition to microvascular perfusion. Recent evidence suggest that measurements of flow mediated metabolic changes are less variable and sensitive than direct recordings of perfusion [20]. Research suggests that FMSF measurements are feasible and reproducible [6]. The FMSF technique appears uniquely well-suited for diagnosing various pathologies and disorders (CAD, diabetes and its complications [25] [36] [37], SLE, chronic obstructive pulmonary disease (COPD) [38] [39]) for its abil-

ity, as well as for uses in sport physiology, prognostic observation means in COVID-19, enabling the early identification of potentially dysfunctional microcirculation and metabolic regulation.

At present, a variety of parameters have been derived to quantitative measure the NADH fluorescence in the epidermis over time in response to brachial artery occlusion based on the FMSF device, which were presented in Table 1.

Table 1. Parameters derived to quantify NADH fluorescence based on FMSF device.

Parameter	Significance	Formula
B_{mean}	Mean NADH fluorescence at the wavelength of 460 nm, recorded at rest at the beginning of the measurement	——
FI_{max}	The maximal increase in NADH fluorescence that accumulated observed during forearm ischemia	——
FR_{min}	The maximal drop in NADH fluorescence below the baseline observed during reperfusion	——
I_{max}	A net increase in NADH in the skin during ischemia	$I_{max} = FI_{max} - B_{mean}$
R_{min}	A net reduction in NADH in the skin during the reperfusion	$R_{min} = B_{mean} - FR_{min}$
IR_{ampl}	Maximum range of fluorescence change during a single cycle	$IR_{ampl} = I_{max} + R_{min}$
IR_{max}	The ratio between the relative increase in NADH fluorescence and the baseline value	$IR_{max} = I_{max} / B_{mean} \times 100\%$
HR_{max}	The ratio between the relative decrease in NADH fluorescence and the baseline value	$HR_{max} = R_{min} / B_{mean} \times 100\%$
IR_{index}	The ratio of the area under the curve (AUC) of *IR* to the the curve of baseline	$IR_{index} = AUC_{IR} / AUC_{base} \times 100\%$
HR_{index}	The ratio of the area under the curve (AUC) of *HR* to the the curve of baseline	$HR_{index} = AUC_{HR} / AUC_{base} \times 100\%$
FM	A parameter characterizing basal flowmotion at rest	——
FM(R)	A parameter representing flowmotion during the reperfusion phase, which reflects the strong effect of hypoxia on flowmotion, mainly due to the increased activity of the vessels	——
HS	The fraction of the FM(R) value covering the intensity of flowmotion related to myogenic oscillations (0.052 - 0.15 Hz) during reperfusion	——
Log(HS)	As the values of the HS parameter can vary within a quite broad range it is more practical to use a normally distributed log(*HS*)	——
RHR	A parameter characterizes endothelial function related predominantly to the production of nitric oxide (NO) in the vasculature due to reactive hyperemia	$RHR = (FI_{max} - Frmin) / B_{mean} \times 100\%$

Initially, FMSF registers two principal parameters: *IR* and *HR*. The parameter IR_{max} is defined as the ratio of relative to maximal baseline increase in NADH fluorescence intensity observed over occlusion, whereas HR_{max} is expressed as the relative to maximal baseline decrease in NADH fluorescence intensity during the reperfusion phase. Subsequently, the parameter IR_{index} is calculated as the area under the curve (AUC) of *IR* in relation to the curve of baseline, while HR_{index} is defined as the AUC of the *HR* in relation to the curve of baseline [20] [26]. HR_{max} and HR_{index} are key parameters demonstrating the potential diagnostic power of the FMSF technique. HR_{max} refers to the efficacy of oxygen supply to the epidermis during hyperemia via the skin microcirculation and can be interpreted as an indirect measure of NO bioavailability in the microcirculation. HR_{index} corresponds to the recovery of metabolic status in the epidermis following hyperemia, caused by the release of pressure from the occlusion cuff. Parameters IR_{max} and IR_{index} are sensitive to deviation from the measurement procedure and carry auxiliary diagnostic significance [20]. **Figure 6** presented the four parameters on the NADH fluorescence trace recorded for a healthy volunteer (male, 29 years) in response to blockage and release of blood flow in the brachial artery.

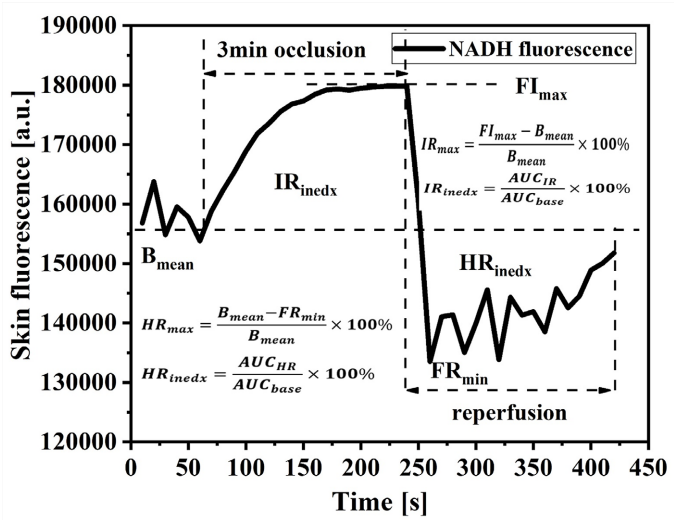

Figure 6. The typical NADH fluorescence trace recorded for a healthy volunteer (male, 29 years) in response to blockage and release of blood flow in the brachial artery. The ischemic response (IR_{max} and IR_{index}) is relative to baseline increase in NADH fluorescence intensity observed during cuff occlusion and the hyperemic response (HR_{max} and HR_{index}) is relative to baseline decrease in NADH fluorescence intensity after cuff release.

These four parameters have been used in numerous studies based on FMSF. In clinical study on patients with DM1, compared to healthy subjects, the low value of the HR_{index} parameter while the HR_{max} parameter is moderately disturbed indicates serious metabolic dysregulation with moderately dysfunctional microcirculatory status. And the majority of DM1 patients with $HR_{index} < 8\%$ showed signs of dysfunctional metabolic regulation [25]. A pilot study in notably

CAD patients found that IR_{max} and HR_{max} were significantly inversely correlated with endothelin-1 (ET-1) and asymmetric dimethylarginine (ADMA), respectively [7]. ET-1 is a potent vasoconstrictor and mitogen produced in response to hypoxia and vessel wall stress, high levels of ET-1 have been also been associated with microvascular dysfunction. It was shown that CAD patients with high ET-1 plasma levels presented low values of ischemic response suggesting their low sensitivity to intermittent hypoxia. The endogenous competitive inhibitor of NO synthase, ADMA, has been shown to decrease both the production and bioavailability of NO. Therefore, elevated plasma concentrations of ADMA have been considered to be an indicator of endothelial dysfunction and a risk factor for CAD.

While analyzing the measurements of hyperemic microvascular response, both HR_{max} and HR_{index} were found lower in patients with familial hypercholesterolemia (FH) compared to age- and sex-matched healthy controls [40]. Low-density lipoprotein cholesterol (LDL-C) levels and total cholesterol (TC) levels were inversely correlated to both HR_{max} and HR_{index}. Additionally, HR_{max} was significantly higher in FH patients examined on statins compared to those without any lipid-lowering treatment. SLE is a model autoimmune rheumatic disease, and although its pathogenesis is not fully understood [26]. Researcher adopted FMSF technique in patients with SLE to examine changes of NADH fluorescence from the epidermis of a forearm and to investigate whether they are associated with clinical manifestation of the disease. Results showed that IR_{max}, IR_{index}, HR_{max}, and HR_{index} were all lower in patients with SLE compared with controls. Such changes of NADH during reperfusion in patients with SLE could be associated with their possible lower sensitivity to hypoxia and possibly with endothelial dysfunction.

As mentioned earlier, an important and unique feature of the FMSF technique concerns the oscillations in the microcirculation that can be observed on the baseline and the hyperemic (reperfusion) part of the FMSF trace. Oscillations in the microcirculation, known as flowmotion, are a well-recognized characteristic of cutaneous blood flow [36]. As there is very low noise in the recorded FMSF traces, flowmotion can be observed very distinctly and precisely. Flowmotion analysis based on the FMSF trace at rest could expand the scope of the application of FMSF. Based on the flowmotion, three parameters *FM*, *FM*(*R*) and *HS* shown in **Table 1** are derived to quantitative measures of oscillations. Since myogenic oscillations are predominantly stimulated on the reperfusion line following transient ischemia, the *HS* parameter seems particularly well-suited for quantitative characterization of the microcirculatory response to hypoxia. Another clinical test concluded that the *HS* parameter representing the microcirculatory response to hypoxia induced by transient ischemia can be used to identify diabetic foot ulceration (DFU) with low prognosis for healing. A very low value for the *HS* parameter signifies serious microcirculation disorders. Since changes in microcirculation occur sooner, the FMSF method allows for the detection of

disorders at an early stage, allowing for prophylactic or therapeutic interventions or more accurate and complex diagnostic tests. The FMSF technique can be recommended to both monitor the health status diabetic patients and to predict vascular complications including DFU.

Recently, two parameters were derived from FMSF measurements: reactive hyperemia response (*RHR*) and hypoxia sensitivity [log(*HS*)]. These parameters can be used for efficient characterization of vascular circulation based on the response to transient ischemia. *RHR,* based on the combined response from both the ischemic and hyperemic parts of the measured FMSF trace, which characterizes endothelial function related predominantly to the production of nitric oxide (NO) in the vasculature due to reactive hyperemia [41] [42]. As the values of the *HS* parameter can vary within a quite broad range it is more practical to use a normally distributed log(*HS*). For example, very low values for the log(*HS*) parameter can effectively predict a limited chance for healing in patients with DFU. Diagnostic use of the *RHR* parameter can be greater in CVD, where dysfunction in microcirculation prevails. Characterization of vascular circulation based on the RHR and log(*HS*) parameters can be effective across a broad segment of the population, from physically active healthy individuals to individuals suffering from serious health problems related to vascular dysfunctions. This simple two-parametric approach based on distinguishable macro- and microcirculatory responses to hypoxia will be recognized as a powerful diagnostic tool for characterization of vascular circulation in the future.

This study has several limitations. First, the study group was relatively small, further research with larger sample sizes is needed. Secondly, further mechanistic studies are needed to explain the regulation mechanisms of FMSF signal, which represents the complex effects of the cellular NADH metabolism changing microvascular circulation. While we use LDF and LSCI to quantify blood flow, results should be confirmed by other technique measuring metabolic changes or microvascular endothelial function, for example, flow mediated dilation (FMD).

To enhance the generalizability and reliability of our findings, we plan to include more cases in subsequent studies by increasing the sample size and diversity. This includes recruiting a larger number of participants to improve the statistical significance and representativeness of the data; covering individuals of different ages and genders to ensure broader applicability of the results; and particularly focusing on including individuals with relevant health conditions, such as metabolic diseases or diabetes, to explore the applicability of the findings across different health backgrounds.

4. Conclusion

In conclusion, noninvasive, real-time, in vivo measurement of NADH fluorescence were performed through a homemade FMSF system as a function of time in response to blocking and releasing blood flow in the forearm. The dynamical changes of NADH fluorescence were analyzed with the comparison of skin

blood flow and blood physiological index measured by LDF and LSCI experiments simultaneously with FMSF measurements. The physiological significance of the existing evaluation parameters derived from the FMSF method is analyzed, which lays the foundation for further research on the clinical application of the FMSF method. As the first method to measure metabolic changes which directly depend on local perfusion, FMSF could be of great interest for assessing microvascular function in various diseases, may provide new avenues for in vivo physiological, clinical and pharmacological research on mitochondrial metabolism.

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Conflicts of Interest

The authors declare that there are no conflicts of interest related to this article.

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