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Oxygen saturation, red blood cell tissue fraction and speed resolved perfusion during local heating and occlusion provocations - A new optical method for microcirculatory assessment

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Abstract

We have developed a new fiber-optic system that combines diffuse reflectance spectroscopy (DRS) and laser Doppler Flowmetry (LDF) for a multi-modal assessment of the microcirculation. Quantitative data is achieved with an inverse Monte Carlo algorithm based on an individually adaptive skin model. The output parameters are calculated from the model and given in absolute units: hemoglobin oxygen saturation (%), red blood cell (RBC) tissue fraction (%), and the speed resolved RBC perfusion separated into three speed regions; 0-1 mm/s, 1-10 mm/s and above 10 mm/s (% mm/s). The aim was to explore microcirculatory parameters using the new optical method, integrating DRS and LDF in a joint skin model, during local heating of the dorsal foot and venous and arterial occlusion of the forearm in 23 healthy subjects (age 20-28 years). There were differences in the three speed regions in regard to blood flow changes due to local heating, where perfusion for high speeds increased the most. There was also a high correlation between changes in oxygenation and changes in perfusion for higher speeds. Oxygen saturation at baseline was 44% on foot, increasing to 83% at plateau after heating. The larger increase in perfusion for higher speeds than for lower speeds together with the oxygenation increase during thermal provocation, shows a local thermoregulatory blood flow in presumably arteriolar dermal vessels. In conclusion, there are improved possibilities to assess microcirculation using integrated DRS and LDF in a joint skin model by enabling both oxygenation and speed resolved blood flow assessment simultaneously and in the same skin site. Output parameters in absolute units may also yield new insights about the microcirculatory system.

Key words: diffuse reflectance spectroscopy, laser Doppler flowmetry, microcirculation, skin blood flow, hemoglobin oxygen saturation.

Introduction

Recent studies show that there is a relationship between small vessel function and the heart and large vessel function [1]. There is increasing knowledge of the potential therapeutic/clinical value of the microcirculation as a model of vascular dysfunction on a systemic level [2, 3]. Assessing vascular function is very promising in that it reflects important vascular biology associated with disease burden and outcome, and response to interventions. For example, endothelial dysfunction in terms of vascular reactivity is shown to be an integral component of atherosclerotic vascular disease and that its presence is a risk factor for the development of clinical events [4-6].

Diffuse reflectance spectroscopy (DRS) and laser Doppler flowmetry (LDF) are two non-invasive methods to assess the microcirculation in skin. Hemoglobin oxygenation and concentration of red blood cells (RBCs) can be assessed with DRS by using white light in the visible to near-infrared range where hemoglobin has absorption spectra with distinct features. With light transport in tissue modeling, knowledge of absorption spectra for hemoglobin and calibration of DRS spectra, oxygenation and tissue fraction of red blood cells can be determined in absolute units [7-9]. Also by using a small source-detector distance, the sampling depth becomes small and mostly vessels from the microcirculation affects the determined values.

LDF measures perfusion (concentration of RBCs times their average speed) of the tissue by registering the frequency shift, i.e. Doppler shift, occurring when light is scattered by moving RBCs. Conventionally, a perfusion measure is obtained by calculating the first moment of the detected Doppler spectrum. However, due to optical properties and structure of the tissue, the perfusion measure is expressed in arbitrary units which can be seen as a limitation of the technique [10, 11] since it complicates between subjects and between sites comparison. One way to overcome this is by using a model-based approach together with Monte Carlo simulations of light propagation in the tissue to obtain perfusion in absolute units [12-14]. This is possible by performing an absolute calibration of the power in the measured spectra (see Data preprocessing below) whereas the frequencies are measured in absolute unit *per se*. By modelling the light transport with knowledge of the tissue optical properties in general (given from DRS measurements) and the scattering properties of RBC in specific (a priori knowledge), this model-based approach gives measured values in absolute units. In addition to the absolute values given from this approach and maybe even more important, the perfusion can also be separated into different speed regions of interest [12], which has also been shown by another group [15].

To combine these two techniques into the same probe has been suggested by other groups [16, 17]. However, these systems analyze data from the two techniques separately, using conventional signal processing. This results in an uncertainty on the origin of the signal and the measured volume for the two systems, which is likely to be different [16]. By combining the two techniques into one joint model strengthens especially the robustness of the perfusion estimates, and the fact that one model can describe measured spectra from two different techniques increases the credibility of the method. Furthermore, with advanced light transport modelling, the output from the method can be obtained in absolute units, which makes it more compatible with other larger vessel measurement methods such as ultrasound.

With this newly developed state-of-the-art method for a more comprehensive characterization of the microcirculation, together with the new insight of the potential of microcirculatory assessments as a window to systemic pathophysiological conditions, suggest that this method could be an additional tool in clinical practice.

The aim of this study was to explore microcirculatory parameters using a new optical tool integrating DRS and LDF in a joint skin model. Standard provocations for determining the status of the microcirculation in the skin were performed; local heating of the foot and venous and arterial occlusion of the upper forearm. The output parameters oxygen saturation (SO_2), red blood cell tissue fraction (C_{RBC}) and speed resolved perfusion were quantified during these provocations in absolute units.

Material and methods

Subjects

Participants were non-smoking men and women with Caucasian skin type I-IV [18]. Exclusion criteria included disease or prescribed medication affecting the circulatory system, diabetes mellitus or hypertension. The participants were asked to refrain from coffee for 2 h prior to the measurements. Written informed consent was obtained from all participants. The study was approved by the Regional Ethical Review Board in Linköping (D. no M83-09).

Equipment

The DRS and LDF spectra were recorded using an integrated fiber optic probe (diameter: 200 μm ; numerical aperture: 0.37; material: fused silica) with two emitting optical fibers (white light: Avalight-HAL-S, Avantes BV, The Netherlands; laser light: 780 nm, modified Periflux 5000, Perimed AB, Järfälla, Sweden) and eight detecting fibers. DRS spectra were collected by two detector fibers at source-detector separation of 0.4 and 1.2 mm, respectively. Each detector fiber was connected to a separate channel in a multi-channel spectrometer (AvaSpec 2048-5-RM, Avantes BV, The Netherlands, grating: VB 600 lines/mm). The remaining six detector fibers were all placed 1.2 mm from the laser optical source fiber and connected to a single detector in a modified Periflux 5000 (Perimed AB, Järfälla, Sweden). The probe was placed in a thermostatic probe holder (PF 450, Perimed AB, Järfälla, Sweden) fixated to skin using double-adhesive tape, to ensure stable skin temperature and good tissue contact.

Measurement Protocols

Measurements were performed in a room with an ambient temperature of $24.6 \pm 0.7^\circ\text{C}$ (mean $\pm$ standard deviation). The volunteers were acclimatized to the room temperature for at least 15 min before the start of the protocols. During the measurements, the subjects were in a supine position with their arms and legs fully rested. Three different provocations were conducted: local heating of the foot and venous and arterial occlusion of the upper forearm. During the local heating of the skin the probe was placed on the dorsum of the foot (right or left foot chosen randomly). During the venous occlusion the probe was placed on the volar surface of the lower forearm (right or left chosen randomly). The probe was then moved to the other lower forearm for the arterial occlusion. Before attaching the probe, the skin was cleaned with 95% ethanol.

All protocols were preceded by a baseline of 5 min, where the thermostatic probe holder had a temperature of 32°C . During the heat provocation the temperature of the probe holder was increased to 44°C and held at that temperature for 25 min. For the two occlusion provocations, the probe holder was kept at 32°C . A blood-pressure cuff at a pressure of 60 mmHg was used to perform a venous occlusion of the arm for 5 min, followed by a 5 minute reperfusion phase. For the arterial occlusion, the cuff was inflated to 200 mmHg for 5 min followed by a 5 minute reperfusion phase.

Spatiotemporal variation in the measurement/ reproducibility was assessed at baseline on the foot and on the forearm. On the foot, this was done by performing additional measurements of one minute before the local heating at two randomly chosen sites, avoiding visible veins (resulting in three baseline measurements sites). On the forearm, the two baseline sites measured before the provocations were used.

Data preprocessing

A recording from a dark spectrum and an intensity reference was performed before and after each measurement. The intensity reference was used as a link to a white spectrum, recorded at a separate occasion, and also for assessing color drift in the spectrometer during the measurement.

DRS spectra were calibrated in three steps: first the dark spectrum was subtracted, second the white reference spectrum was used to normalize DRS spectra, and third the two channels were normalized with a relative calibration using the average

intensity from a measurement where the detecting fibers at the face of the probe was evenly illuminated [19]. Only the relative intensity between the two DRS channels is considered in the data processing, not the absolute light intensity levels.

LDF calibration was performed by measuring in motility standard (Perimed AB) where all detected light can be assumed to be Doppler shifted. The LDF spectra were normalized with the total spectral power of that calibration measurement. In that way, the power in the measured LDF spectra can be directly compared to the power in the simulated LDF spectra [20]. The conventional perfusion measure was calculated as the sum of the frequency weighted LDF spectrum, i.e. the first order moment, and normalized to 250 PU (perfusion units) for the measurement in motility standard. The conventional CMBC (concentration of moving blood cells) measure was calculated as the total power of the LDF spectrum normalized to 400 CU (concentration units) for the measurement in motility spectrum.

Calculating microcirculatory parameters

An adaptive three layer skin model [13, 21], was used to analyze DRS and LDF spectra. Modelled DRS and LDF spectra were generated based on Monte Carlo simulations of the model. The model parameters were updated in an iterative non-linear optimization manner in order for the modelled spectra to fit measured spectra. This was first done for DRS spectra for all model parameters except perfusion parameters. In a second step, the remaining model parameters, i.e. the perfusion parameters, were updated for the modeled LDF spectra to fit the measured LDF spectra. The output parameters i.e. SO_2 in percent, C_{RBC} in percent and perfusion in percent RBCs times their speed in mm/s, separated into three speed regions (0-1 mm/s, 1-10 mm/s and above 10 mm/s) were then calculated in the sampling volume that was also achieved from the model. The mean hemoglobin concentration of red blood cells was assumed to be 345 g/l RBC.

Microcirculatory evaluation

DRS and LDF spectra were averaged over 1 s before analyzed using the adaptive individualized model. Baseline values for all provocations were calculated as the median value over the first three minutes of each measurement. First peak (median over one minute) and maximal thermal response (plateau, median over minute 22-25 after heat onset) were calculated from the heat provocation. The response after arterial occlusion for the perfusion measures was calculated as peak value and time to peak from release of the cuff pressure. SO_2 and C_{RBC} at time for total perfusion peak, together with maximal values (referred to as peak values) were also calculated. The response during occlusion was calculated as median value over the last minute, 10 s before release of the cuff pressure. The after occlusion measure was calculated as the median over minute 4-5 after release. The venous occlusion measures were calculated similar to the arterial occlusion, except for no peak value.

Statistics

Data are presented as mean $\pm$ standard deviation (SD). Since some of the parameters were not normally distributed, Wilcoxon signed rank test was used for pairwise comparisons. Correlation coefficients between changes in SO_2 , C_{RBC} , perfusion for all speed regions as well as total perfusion and conventional perfusion during local heating were analyzed using Pearson correlation. A p -value <0.05 was considered significant. Statistical analyses were performed using IBM SPSS Statistics v21.0 (IBM Corporation, New York, USA).

Reproducibility was expressed as within subject coefficient of variation (CV) [22].

Data exclusion

To assess spectrometer color drift during the measurements, the calibration recording from the intensity reference before the start of the protocol was compared to the one after. The ratio of normalized intensity (before/after) was evaluated and if the spectral difference between maximal and minimal ratio was $> 20\%$, the subject was excluded [21].

Output parameters obtained from spectra with poor fitting for the DRS spectra or the LDF spectra were not included in the median value calculations. The SO_2 level was considered uncertain if the hemoglobin spectral area (HemArea) in the 506 to 614 nm region for the DRS spectrum at 1.2 mm distance (Figure 1) was too small (less than 4% of the total spectral area, Total Area). If that was the case for more than 20% of the spectra during the measurement, SO_2 was not calculated. A small HemArea is generally the result of a low C_{RBC} .

Figure 1. Diffuse reflectance spectra from source-detector distance 1.2 mm. Definition of HemArea and Total Area for exclusion criteria.

Results

Measurements were performed on 23 subjects. Three subjects were excluded due to spectrometer color drift and three additional subjects were excluded due to missing before-and-after measurements on the intensity reference. One subject was excluded from the venous occlusion protocol due to the wrong pressure in the cuff. In total, measurements from 17 subjects were included in the local heating and arterial occlusion protocol, and 16 subjects during the venous occlusion protocol. The included subjects (10 male; 7 female) had a mean age 25 ± 2 years and BMI $23.8 \pm 3.0 \text{ kg/m}^2$.

The sampling depth, calculated as described in [13], was on average 0.67 mm.

Reproducibility of baseline measurements

Variability was less than 40% for all parameters on the forearm as well as on the foot (Table 1). Highest variability (above 35%) was seen in perfusion for higher speeds on the foot. The variability was lowest for SO_2 and low speed ($< 1 \text{ mm/s}$) perfusion on both forearm and foot.

Table 1. Reproducibility at baseline on the forearm and on the foot.

	CV [%]	
	Forearm	Foot
SO_2	10	14
C_{RBC}	17	24
Total perfusion	22	26
Perfusion 0-1 mm/s	10	17
Perfusion 1-10 mm/s	22	36
Perfusion $>10 \text{ mm/s}$	23	36

Local heating

A representative example of the parameters during the local heating of the foot is presented in Figure 2. Total perfusion increased from 0.15 %RBC x mm/s at baseline to 1.10 %RBC x mm/s at first peak and 1.49 %RBC x mm/s at plateau, while SO₂ increased from 28% at baseline to 79% at first peak and 84% at plateau. There are small differences between the levels of the different speed regions during baseline, while upon heating the perfusion for high speeds (> 10 mm/s) increased the most and perfusion for low speed (0-1 mm/s) remained almost at baseline level.

Figure 2. (a) Oxygen saturation (SO₂) and RBC tissue fraction (C_{RBC}) during local heating of the foot. (b) Perfusion in percent RBC times speed divided into three speed regions (0-1 mm/s, 1-10 mm/s and above 10 mm/s) together with total perfusion during local heating.

Mean values at baseline, first peak and plateau during local heating are presented in Table 2. SO₂ for one subject during baseline was excluded due to small HemArea. The first peak, which mostly depends on local sensory nerves, was seen for all perfusion estimates but not for all subjects in the lowest speed region. It was not possible to distinguish a first peak for all subjects in SO₂ (4 subjects), C_{RBC} (3 subjects) or perfusion for the lowest speed region (4 subjects). There was a significant increase from baseline to first peak (in cases where first peak was identifiable) for all parameters ($p < 0.01$). The increase from baseline to plateau was also significant for all parameters ($p < 0.05$ or $p < 0.01$).

Table 2. Tissue model parameters during baseline and local heating of the foot; oxygen saturation (SO₂), RBC tissue fraction (C_{RBC}) and perfusion in percent RBC times their speed (divided into speed regions 0-1 mm/s, 1-10 mm/s, above 10 mm/s and total perfusion). Conventional perfusion in perfusion units (PU) and concentration of moving blood cells (CMBC) in concentration units (CU) are also given. N=17 if not other specified.

	Baseline	First peak	Plateau
SO ₂ [%]	44 (14) ¹	79 (7) ^{4†}	83 (8) [†]
C _{RBC} [%]	0.17 (0.088)	0.25 (0.099) ^{3†}	0.30 (0.11) [†]
Total perfusion [%RBC x mm/s]	0.13 (0.069)	0.91 (0.67) [†]	1.26 (0.77) [†]
Perfusion 0-1 mm/s [%RBC x mm/s]	0.039 (0.014)	0.049 (0.016) ^{4†}	0.047 (0.018) [*]
Perfusion 1-10 mm/s [%RBC x mm/s]	0.051 (0.029)	0.29 (0.17) [†]	0.37 (0.17) [†]
Perfusion >10 mm/s [%RBC x mm/s]	0.041 (0.033)	0.58 (0.52) [†]	0.84 (0.64) [†]
Conventional perfusion [PU]	42 (17)	269 (130) [†]	356 (129) [†]
CMBC [CU]	287 (31)	-	318 (28) [†]

Data expressed as mean (SD)

ⁿnumber of excluded values

Statistical comparison with baseline * $p < 0.05$; $†p < 0.01$.

The mean relative increase from baseline to plateau was 89% for SO₂, 76% for C_{RBC}, 869% for the total perfusion and 748% for conventional perfusion. The increase for the different speed regions were 21%, 625% and 1950% (low, medium and high speeds). The conventional LDF measure concentration of moving blood cells (CMBC) increased 11%.

There was positive correlation between absolute changes (plateau-baseline) in SO_2 and relative changes (plateau/baseline-1) in total perfusion ($r=0.75$, $p=0.001$, Figure 3.a), all speed regions (low: $r=0.61$, $p=0.012$; medium: $r=0.64$, $p=0.008$; high: $r=0.64$, $p=0.008$) and conventional perfusion ($r=0.71$, $p=0.002$). There were no correlation between absolute changes in C_{RBC} and absolute changes in SO_2 , relative changes in total perfusion, perfusion for speed region 1-10 mm/s or perfusion above 10 mm/s. However, there was a positive correlation between absolute changes in C_{RBC} and relative changes in perfusion for the lowest speed region ($r=0.59$, $p=0.013$). The relative changes in total perfusion were strongly correlated to those in conventional perfusion ($r=0.92$, $p=0.000$, Figure 3.b).

Figure 3. (a) Relative changes in total perfusion (RelCh TotPerf) and absolute changes in oxygen saturation (AbsCh SO_2 in %) (b) Relative changes in total perfusion (RelCh TotPerf) and conventional perfusion (RelCh ConvPerf).

Venous occlusion

A representative example of SO_2 , C_{RBC} and perfusion parameters during venous occlusion is presented in Figure 4. SO_2 was 58% at baseline and decreased slowly during the occlusion to 18%. After release of the cuff, SO_2 returned immediately to baseline values (52%). RBC tissue fraction increased constantly during occlusion, from 0.12% baseline to 0.47% during occlusion and returned to 0.10% after release. Total perfusion was at baseline 0.14 %RBC x mm/s, decreased to 0.038 %RBC x mm/s during occlusion and returned to 0.083 %RBC x mm/s 4 minutes after release. Perfusion for higher speeds (>1 mm/s) were also lower than baseline values 4 minutes after release, while perfusion for the lowest speeds had returned to baseline values.

Figure 4. (a) Oxygen saturation (SO_2) and RBC tissue fraction (C_{RBC}) during venous occlusion of the arm (b) Perfusion in percent RBC times speed divided into three speed regions (0-1 mm/s, 1-10 mm/s and above 10 mm/s) together with total perfusion during venous occlusion.

Mean values at baseline, during and after venous occlusion are presented in Table 3. SO_2 for one subject during baseline and after occlusion was excluded due to small HemArea. SO_2 was significantly lower than the baseline value during occlusion, while C_{RBC} was significantly higher ($p<0.01$). All perfusion parameters were significantly lower during occlusion, including conventional perfusion ($p<0.01$). After the release of the cuff SO_2 , C_{RBC} and perfusion for lower speeds returned to baseline values after four minutes, but the total perfusion, conventional perfusion and perfusion for higher speeds were still significantly lower than baseline ($p<0.05$ or $p<0.01$).

Table 3. Tissue model parameters during baseline and venous occlusion of the arm; oxygen saturation (SO_2), RBC tissue fraction (C_{RBC}) and perfusion in percent RBC times their speed (divided into speed regions 0-1 mm/s, 1-10 mm/s, above 10 mm/s and total perfusion). Conventional perfusion in perfusion units (PU) and concentration of moving blood cells (CMBC) in concentration units [CU] are also given. N=16 if not other specified.

	Baseline	During venous occlusion	After occlusion
SO_2 [%]	49 (12) ¹	16 (5) [†]	49 (13) ¹
C_{RBC} [%]	0.15 (0.067)	0.56 (0.29) [†]	0.15 (0.073)
Total perfusion [%RBC x mm/s]	0.14 (0.094)	0.048 (0.027) [†]	0.11 (0.098)*
Perfusion 0-1 mm/s [%RBC x mm/s]	0.031 (0.012)	0.015 (0.0077) [†]	0.029 (0.014)
Perfusion 1-10 mm/s [%RBC x mm/s]	0.052 (0.045)	0.013 (0.0093) [†]	0.039 (0.034)*
Perfusion >10 mm/s [%RBC x mm/s]	0.054 (0.073)	0.020 (0.017) [†]	0.042 (0.088) [†]
Conventional perfusion [PU]	48 (29)	19 (9) [†]	38 (24)*
CMBC [CU]	279 (31)	277 (27)	278 (31)

Data expressed as mean (SD)

¹number of excluded values

Statistical comparison with baseline * $p<0.05$; [†] $p<0.01$.

CMBC did not change during the venous occlusion. The RBC tissue fraction on the other hand had a mean increase of 273% during the occlusion. SO_2 decreased 65%, the total perfusion 66% and conventional perfusion 60%. The different speed regions decreased 52%, 75% and 63% (low, medium and high speeds).

Arterial occlusion

A representative example of SO_2 , C_{RBC} and perfusion measures during arterial occlusion is presented in Figure 5. SO_2 was 42% at baseline and decreased during the occlusion to -2%. Maximum value at release was 90% and four minutes after release, SO_2 was 65% which was higher than baseline. RBC tissue fraction increased during occlusion, from 0.16% baseline to a maximum of 0.32% during occlusion and returned slowly to 0.14% after release. Total perfusion was at baseline 0.089 %RBC x mm/s, decreased to 0.044 %RBC x mm/s during occlusion, peaked at 0.69 %RBC x mm/s and returned to 0.12 %RBC x mm/s 4 minutes after release.

Figure 5. (a) Oxygen saturation (SO_2) and RBC tissue fraction (C_{RBC}) during arterial occlusion of the arm (b) Perfusion in percent RBC times speed divided into three speed regions (0-1 mm/s, 1-10 mm/s and above 10 mm/s) together with total perfusion during arterial occlusion.

Mean values at baseline, during occlusion, at peak and after arterial occlusion are presented in Table 4. SO_2 for one subject during baseline, during occlusion and after occlusion was excluded due to small HemArea. It was not possible to distinguish a post occlusive peak for two subjects in the lowest speed region. All perfusion parameters and SO_2 were significantly lower than baseline values during occlusion ($p < 0.01$), while C_{RBC} was significantly higher ($p < 0.01$). All parameters were significantly higher than baseline at peak ($p < 0.01$). Both SO_2 and C_{RBC} were at 91% of maximal value at the time for total perfusion peak. SO_2 reaches maximal value after total perfusion peak for all subjects and C_{RBC} reaches maximal value both before and after time for total perfusion peak. SO_2 was still significantly higher than baseline value 4 min after the release of the cuff ($p < 0.01$). Note the difference between total perfusion and conventional perfusion after occlusion, where total perfusion was at baseline level while conventional perfusion was still significantly higher than baseline ($p < 0.01$).

Table 4. Tissue model parameters during baseline and arterial occlusion of the arm; oxygen saturation (SO_2), RBC tissue fraction (C_{RBC}) and perfusion in percent RBC times their speed (divided into speed regions 0-1 mm/s, 1-10 mm/s, above 10 mm/s and total perfusion). Conventional perfusion in perfusion units (PU) and concentration of moving blood cells (CMBC) in concentration units [CU] are also given. For SO_2 and C_{RBC} maximal values are referred to as peak values. $N=17$ if not other specified.

	Baseline	During occlusion	Peak	After occlusion
SO_2 [%]	47 (13) ¹	-2 (3) ^{1†}	84 (6) [†]	63 (12) ^{1†}
C_{RBC} [%]	0.16 (0.051)	0.28 (0.12) [†]	0.35 (0.12) [†]	0.15 (0.043)
Total perfusion [%RBC x mm/s]	0.10 (0.039)	0.029 (0.0088) [†]	0.70 (0.33) [†]	0.11 (0.058)
Perfusion 0-1 mm/s [%RBC x mm/s]	0.031 (0.013)	0.012 (0.0059) [†]	0.070 (0.023) ^{2†}	0.032 (0.013)
Perfusion 1-10 mm/s [%RBC x mm/s]	0.042 (0.019)	0.0044 (0.0023) [†]	0.28 (0.15) [†]	0.051 (0.031)
Perfusion >10 mm/s [%RBC x mm/s]	0.027 (0.013)	0.012 (0.0033) [†]	0.38 (0.19) [†]	0.031 (0.023)
Conventional perfusion [PU]	35 (11)	11 (2) [†]	215 (62) [†]	43 (24) [*]
CMBC [CU]	279 (29)	226 (39) [†]	-	286 (35) [*]

Data expressed as mean (SD)

ⁿnumber of excluded values

Statistical comparison with baseline ^{*} $p < 0.05$; [†] $p < 0.01$.

CMBC decreased 19% during occlusion while C_{RBC} increased 75%. SO_2 decreased 104%, total perfusion 71% and conventional perfusion 69%. The different speed regions decreased 61%, 91% and 56% (low, medium and high speeds, respectively).

Mean time to peak values are given in Table 5. Time to peak for perfusion for speeds 1-10 mm/s was significant longer than time to peak for the total perfusion ($p<0.05$).

Table 5. Time to peak for total perfusion, perfusion for speed regions 0-1 mm/s, 1-10 mm/s, above 10 mm/s and conventional perfusion. N=17 if not other specified.

	Total perfusion	Perfusion 0-1 mm/s	Perfusion 1-10 mm/s	Perfusion >10 mm/s	Conventional perfusion
Time to peak [s]	15 (6)	10 (5) ²	19 (9)*	14 (5)	16 (8)

Data expressed as mean (SD)
ⁿnumber of excluded values
Statistical comparison with total perfusion * $p<0.05$

Discussion

We assessed microcirculatory parameters for healthy subjects using a new method, integrating diffuse reflectance spectroscopy and laser Doppler flowmetry in a combined skin model. The oxygen saturation, RBC tissue fraction and speed resolved perfusion, in absolute units, were evaluated during local heating of the foot and venous and arterial occlusion of the arm. 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.

Spatio-temporal variability at baseline/ reproducibility, was acceptable (< 35%; [23]) on the forearm for all parameters and this indicates that the method has a better reproducibility compared to other studies regarding reproducibility of LDF on forearm [23]. When applying the method on the foot the variability was above 35% for perfusion for speeds above 1 mm/s. It is logical to assume that the spatial heterogeneity is higher for larger vessels since they are less densely distributed than smaller vessels. That could possible explain the larger CV for higher speeds.

SO₂ during baseline, at peak after arterial occlusion and after heating were comparable to other studies [16, 17, 24], although the sampling volume differs between the studies. SO₂ and C_{RBC} on forearm at baseline and during occlusion were comparable to our previous findings [21], using DRS modeling. The higher SO₂ peak after release in this study was probably due to the elevated cuff pressure from 30 mmHg above systolic pressure to 200 mmHg, which was seen as a limitation in the previous study [21]. We have previously presented perfusion values in absolute units and in separate speed regions in healthy controls [25]. The achieved values are on a similar level, but they cannot be directly compared since the LDF part of the presented method differs in multiple ways from the previous LDF-only method. In the new integrated DRS and LDF method the sampling depth is different (different source-detector fiber separation and different way to weight layers in adapted model), a compensation for inhomogeneously distributed blood is implemented and the perfusion estimates benefit from model parameters obtained from the DRS system which improves the robustness. The conventional perfusion estimates in this study originates from a source-detector distance of 1.2 mm which is different from the mostly often used standard Perimed probe, and cannot therefore be compared to previously presented values.

The average sampling depth for the method, 0.67 mm, is coherent with previous findings [26]. This sampling depth includes the dermal layers where capillaries, arterioles and venules are found. Larger vessels are normally associated with higher blood flow speeds than smaller vessels. Therefore, the speed resolved perfusion measures may aid in interpreting which vessels types that take part in the physiological response.

During local heating there was a difference in the different speed regions regarding the response. The increase from baseline to plateau was 869% for the total perfusion and 21%, 625% and 1950% (low, medium and high speeds, respectively) for the

different speed regions. The higher relative change in perfusion for higher speeds is most likely due to the different physiology in the different vessel structures they represent. More smooth muscle cells are involved in larger vessels resulting in a larger vasodilation compared to smaller vessel structures which consist primarily of capillaries that have smaller vasoactive function. Well known mediators of smooth muscle cells are prostaglandins, nitric oxide and endothelium-derived hyperpolarizing factor, factors shown to be involved in thermal provocations as well [27]. The higher relative change in perfusion for higher speeds is also according to our previous findings in healthy controls [25]. The SO_2 response supports the assumption of a non-metabolic flow, since there was a strong correlation between absolute changes in SO_2 and relative changes in perfusion for higher speeds. The correlation between SO_2 and the lowest speed region was weaker.

A major difference between CMBC and C_{RBC} is that CMBC only involves red blood cells in motion and this causes differences in response between CMBC and C_{RBC} during the provocations. In addition, for the fiber separation used in this study almost all detected photons that penetrates the non-perfused top epidermal layer will be Doppler shifted already at low levels of moving RBC. This result in a CMBC estimate that already at baseline is close to saturated with a low degree of dynamics. These differences are clearly displayed in the response to local heating where the CMBC only increased 11% while the C_{RBC} increased 76%. Similarly, during venous occlusion CMBC remained practically non-affected of the occlusion while C_{RBC} increased 273%. During the arterial occlusion CMBC decreased 19% while C_{RBC} increased 75%.

It should be emphasized that the presented technique measure the fraction of RBC in the sampling volume, not the blood amount. The relationship between C_{RBC} and blood tissue fraction is directly dependent on the (local) hematocrit. For normal hematocrit values of 40-50%, the blood tissue fraction is thus slightly more than the double C_{RBC} . Note, however, that the hematocrit can vary within the vessel tree and can be significantly lower in the capillaries [28].

At release after arterial occlusion the SO_2 peak was slower than the total perfusion since at the time for perfusion peak, SO_2 was on average at 91% of maximal value and reached maximal value after the total perfusion peak. C_{RBC} reached maximal value both before and after release and was at 91% of maximal value at time for perfusion peak on average. After occlusion, the return to baseline values was slower for SO_2 than for C_{RBC} and the perfusion estimates. This could possibly be caused by a rapid inflow of oxygenated blood at release where the oxygen supply exceeds the demand of the tissue. Even after blood flow has returned to baseline values, SO_2 is higher than baseline. However, the detailed mechanism is still unknown.

Time to peak upon release after arterial occlusion was similar for conventional perfusion and total perfusion, and agrees with findings by Yvonne-Tee et al [29] for conventional perfusion on forearm. The inflation and deflation time of the cuff were similar in our study, since it could have an effect on time to peak [29]. The distance between the cuff and measurement site has also an effect on time to peak [30]. There was a difference in time to peak for the perfusion speed regions, where perfusion for speeds between 0-1 mm/s has the shortest time to peak and, interestingly enough, perfusion for the middle speed region (1-10 mm/s) has the longest.

SO_2 reached slightly below zero during the arterial occlusion for 10 out of 16 cases. This is an effect of the restrictions on the oxygen parameter, which allows for modeled oxygenation values both below 0 and above 100%. By allowing for oxygenation values outside the physically correct range i.e. 0 to 100%, the oxygen output parameter is allowed to fluctuate around the borders and therefore can attain a mean value of 0 or 100. The criteria on the HemArea takes care of the cases where the amount of blood in the sampling area is too low to accurately estimate the oxygen saturation. SO_2 from one subject was excluded during local heating and arterial occlusion, and from another subject during venous occlusion because of low HemArea. This could be an effect of the heterogeneity of the skin where a different site could produce a different estimate on the HemArea, or an individual difference in skin blood flow since the same subject was excluded from two provocations and also had the lowest SO_2 among all subjects in the third.

In conclusion, by integrating diffuse reflectance spectroscopy and laser Doppler flowmetry in a joint skin model, and by presenting oxygen saturation, RBC tissue fraction and speed resolved perfusion in absolute units, it is possible to achieve new insight into the microcirculatory system. Oxygen saturation, RBC tissue fraction and perfusion have different responses and different time to response during the three provocations which can be quantified by the method. With speed resolved perfusion it is possible to see the different responses for different vascular beds and with absolute units a comparison between individuals and sites can easier be obtained. This new optical method may provide further insight into the microcirculatory status and therefore also aid in the assessment of vascular dysfunction on a systemic level by using microcirculation as a model.

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