On the suitability of laser-Doppler flowmetry for capturing microvascular blood flow dynamics from darkly pigmented skin

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Abstract. Laser Doppler flowmetry (LDF) provides for the noninvasive monitoring of microvascular blood flow dynamics. It has been used extensively on light-skinned subjects, i.e. on skin with low melanin concentration, in both the healthy and pathological states. Because the optical properties of human skin could well affect the reliability of optically-based diagnostic equipment, the effectiveness of LDF needs to be checked and evaluated on dark skin, too, if this method is to be useful in global health care. Here we assess the performance of LDF in measuring blood perfusion from darkly-pigmented skin, i.e. skin with high melanin concentration. Thirteen dark-skinned subjects and ten light-skinned subjects were included in the study. Microvascular blood flow dynamics was measured on both the right and left ankles using LDF with a laser diode of wavelength 780 nm. The characteristics of time-varying blood flow oscillations were investigated by wavelet analysis, nonlinear mode decomposition and wavelet phase coherence. No significant differences were observed between the groups in the mean blood perfusion ($p > 0.1$), or wavelet power ($p > 0.6$). The instantaneous heart rate (IHR), extracted from the LDF at each of the recording sites, and from the electrocardiogram (ECG), did not differ significantly between the groups ($p > 0.8$). Nor did the wavelet power of the IHR differ ($p > 0.2$) between the groups. The only significant difference found between the groups lay in left/right ankle blood flow coherence near the cardiac frequency, attributable to known ethnic physiological differences. These results indicate that high melanin concentrations in skin exert no significant influence on the ability of LDF to monitor microvascular blood flow dynamics when using a laser diode of wavelength 780 nm. Hence LDF can help in the diagnosis and exploration of the pathogenesis of diseases such as diabetes, hypertension, or malaria in darkly pigmented patients across sub-Saharan Africa.

1. Introduction

Non-invasive optical techniques in biomedicine have made notable advances in recent decades (Peng et al. 2008, Vo-Dinh 2014, Tuchin 2016). One example is laser Doppler flowmetry (LDF). It has been shown to give results comparable to those from other methods of evaluating skin microvascular blood flow, and it possesses the particular

advantage of continuous detection of microvascular blood flow in a volume of tissue, as opposed to axial flow in a single vessel (Nitzan et al. 1988).

LDF provides a simple and non-invasive approach for assessing the dynamical properties of the skin microcirculation, and it can be applied in both the healthy and pathological states (Stefanovska et al. 1999). In combination with appropriate time-series analysis, it can yield valuable insights into the dynamics of microvascular blood flow. Its working principle depends on the Doppler shift in the frequency of light reflected from moving red blood corpuscles (erythrocytes). So it relies on the passage of incident light through the skin, twice. Some knowledge of the skin's optical properties is therefore required.

As illustrated in Fig. 1, human skin (Kanitakis 2002) is made up of several layers, of which the melanin chromophore responsible for skin pigmentation resides in the epidermal layer (Costin & Hearing 2007). Epidermal melanin consists of eumelanin (black-brown pigment) and pheomelanin (yellow-reddish pigment), whose main responsibility is skin photo-protection (Anderson & Parrish 1981, Costin & Hearing 2007).

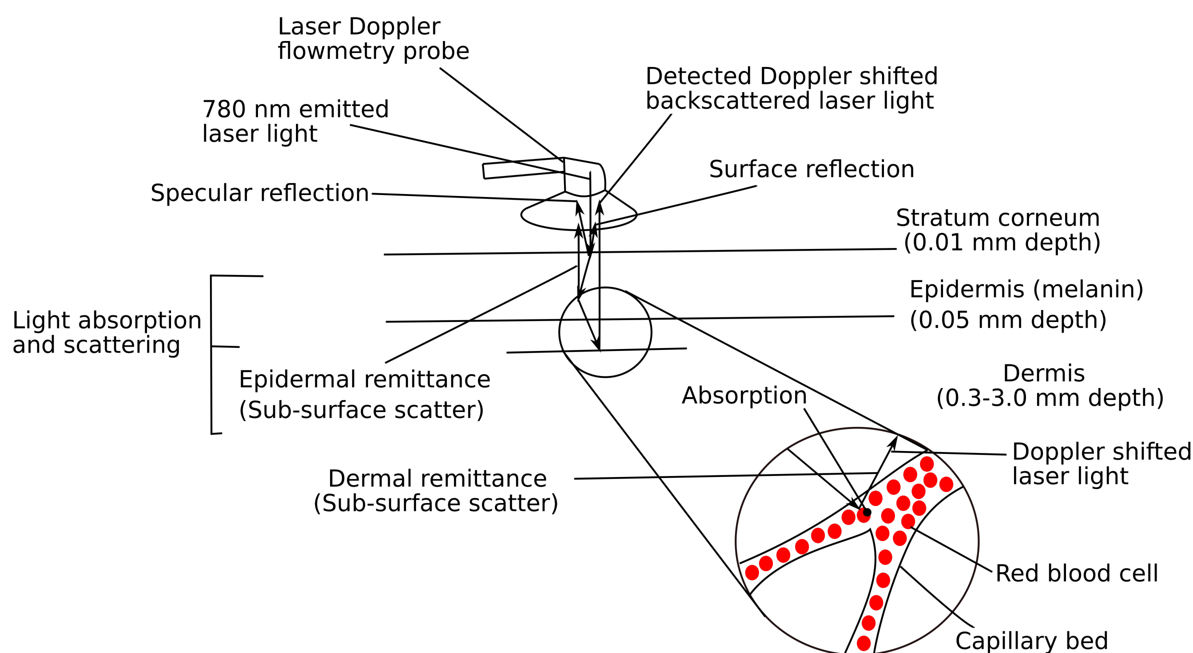

Figure 1. Schematic of the light-skin interaction. When laser light is delivered to the skin, there are three main contributions to the reflected light: specular reflection (which makes insignificant effect), back-scattering at the skin surface (i.e. the stratum corneum), and back-scattering from the epidermis and dermis levels. Scattering from moving erythrocytes (red blood cells) in the dermis results in a Doppler shift in the frequency of the light returned to the laser Doppler probe detector. This spectral broadening can then be related to the average speed and hence flux of the blood flow.

LDF monitoring of blood flow is known to work efficiently in the case of light skin (Nilsson et al. 1980, Kvernmo et al. 1999, Stefanovska et al. 1999, Söderström et al. 2003), whose optical properties enable light to penetrate more easily, but the

question arises as to what happens in the case of darkly pigmented skin (Fredriksson et al. 2009, Karsten & Smit 2012)? The interaction of light with skin mostly happens just below the surface, i.e. in the stratum corneum. As shown in Fig. 1, some of the coherent laser light shone on the skin surface gets scattered from the stratum corneum, referred to as surface scattering. Some of the light propagates to deeper skin layers such as the dermis and epidermis. Absorption and scattering by the melanin and erythrocytes occur in the epidermis and dermis respectively. Unlike the epidermis and dermis, the stratum corneum layer is usually colourless and absorbs only a small portion of incident light.

Melanin absorbs light within the visible and near-infra-red (near-IR) parts of the optical spectrum. The absorption decreases with increasing wavelength, so that near-IR in the wavelength range 780–800 nm is best suited to non-invasive diagnosis and treatment (Peng et al. 2008). The fact that dark skins contain about twice the concentration of epidermal melanin, compared to lighter skins, inevitably means that less laser light reaches the deeper segments of dark skin (Peng et al. 2008).

Extensive investigations of the absorption and reflection of light in/from human skin began in 1911 with the spectrographic studies of Hasselbaich (Hasselbalch 1911). More recently, considerable effort has been devoted to quantifying the absorption spectrum of human skin, particularly darkly pigmented skins (Hardy et al. 1956, Kollias & Baqer 1985, Kollias & Baqer 1986, Kollias 1994, Lister et al. 2012). There has been significant disagreement in the results, perhaps due to challenges in conducting the experimental measurements. For example, the studies of both Pauli and Ivancevic (Pauli & Ivancevic 1927) and Cartwright (Cartwright 1930) reported that a substantial proportion of IR radiation penetrates deeply into the body through the skin, whereas Aldrich (Blaine 1932) and Hardy and Muschenheim (Hardy & Muschenheim 1934) reported a negligible proportion.

In an attempt to determine the effect of melanin on spectroscopic signal formation, Kollias and Baqer (Kollias & Baqer 1985) studied the absorption characteristics of melanin in the wavelength range 620–720 nm and observed that remittance spectra measured from vitiligo-involved skin and normal skin did not differ, from which they concluded that both skins have similar absorption. In a comparable study by Kollias and Baqer (Kollias & Baqer 1986), the remittance spectroscopy parameters measured from normal skin and amelanotic skin in the range 620–720 nm were found to be strongly correlated.

More recently, a model of fluorescence spectra from biological tissue based on the Monte Carlo approach has predicted the effect of melanin concentration on a spectroscopy signal (Dremin & Dunaev 2016). The use of near-IR diode lasers of relatively long wavelength (670, 780, and 810–850 nm) was shown to improve optical penetration (Murray et al. 2004).

Melanin and hemoglobin are known to dominate the absorption of light in skin within the visible range. About 4% to 7% of visible light is reflected from the skin surface, regardless of skin pigmentation and wavelength (Anderson & Parrish 1981,

Takiwaki 1998) (Fig. 1). Unlike hemoglobin which mainly absorbs light within the dermis, melanin present within the epidermis layer exhibits an absorption spectrum extending from the ultraviolet region to the IR (Lister et al. 2012). Several studies (Kollias & Baqer 1985, Kollias 1994) based on diffuse reflectance spectroscopy have investigated the absorption spectrum of melanin *in vivo*, with most of the data being analysed empirically. These studies have yielded a qualitative understanding of melanin absorption characteristics. Nonetheless, data analysis and the realisation (Karsten & Smit 2012, Dremin & Dunaev 2016) that a high epidermal melanin concentration attenuates laser light transmitted to the deeper skin layers, calls for a more extensive investigation.

Note that, because LDF computes the erythrocyte speed from the spectral broadening (see below) of the incident light, which is independent of the light's absolute intensity, the method might be expected to function effectively regardless of the degree of attenuation along the signal path. However, this conclusion can only hold true if two conditions are fulfilled. First, the signal/noise ratio of the light returned to the detector must be sufficient for the spectral broadening to be determined reliably by the measurement algorithm. Secondly, the incidence of frequency-dependent scattering process along the optical return path that would modify the shape of the spectrum must be insignificant. In practice, therefore, the only convincing way forward is empirical: to test LDF on dark-skinned subjects and look for significant differences in the results compared to those obtained from comparable light-skinned subjects.

The present study therefore tests the hypothesis that, in the case of dark skin, the attenuation of the incident laser light reaching (and scattering back from) the dermis is not sufficient to prevent LDF functioning effectively. A relatively long wavelength (780 nm) was chosen with the intention of minimising the attenuation. The paper describes a detailed experimental study comparing the LDF flux measured for both darkly and lightly pigmented skin. The time-varying oscillations in the microvascular blood flow dynamics were checked and compared for the two pigmentations. They are known (Kvernmo et al. 1999, Stefanovska et al. 1999, Söderström et al. 2003, Shiogai et al. 2010, Aalkjær et al. 2011) to include components ranging from the cardiac frequency at $\sim$1 Hz in healthy humans down to endothelium-related oscillations with frequencies of $\sim$0.01 Hz. Non-linear time series analysis (Stefanovska et al. 1999, Clemson & Stefanovska 2014, Iatsenko et al. 2015*b*, Clemson et al. 2016) was used to identify the oscillatory components in the signals. Specifically, the frequency interval from 0.0095 to 2 Hz was examined and categorized into 6 intervals: interval I (0.6–2 Hz) related to cardiac activity; interval II (0.145–0.6 Hz) related to respiratory activity; interval III (0.052–0.145 Hz) related to microvascular smooth muscle cell activity; interval IV (0.021–0.052 Hz) related to microvascular innervation; and intervals V & VI (0.0095–0.021 Hz and 0.005–0.0095 Hz, respectively) related to endothelial activity, both nitric oxide (NO) dependent and independent.

2. Methodology

2.1. Measurement of the skin blood perfusion

Laser Doppler flowmetry LDF provides a continuous measurement of microcirculation in the skin, thus reflecting perfusion in capillaries, arterioles, venules and dermal vascular plexus. The LDF (moorLAB, Moor Instruments Ltd, UK) used in the present study transmits a near-IR laser light from a temperature stabilized laser diodes operating at a wavelength of 780 nm and with a maximum power of 2.5 mW into the skin through an MP1-V2 probe (Moor Instruments Ltd, UK), which has two optical fibres. A time constant of 0.1 s was selected and the LDF processor bandwidth was between 18 Hz and 22.5 KHz. A flexible probe holder (PH1-V2, Moor Instruments Ltd., UK) was attached to the skin surface on the outer side of ankles (lateral malleolus) using double-sided adhesive discs. One fibre delivers light to the site under observation, while the backscattered (reflected) light is collected by the other fibre as shown in Fig. 1. According to the Doppler principle, the light reflected from moving red blood cells is shifted in frequency by an amount related to the blood flow in the illuminated volume of tissue – the frequency shift is proportional to red cell speed while the frequency of light reflected from stationary cells and tissue remains unchanged (Nilsson et al. 1980). The difference between incident light and the Doppler-shifted back-scattered light gives the LDF signal, known as the blood perfusion signal. The LDF output is semi-quantitative and is expressed in perfusion units (PU) of output voltage (typically 1 PU = 10 mV) (Nilsson et al. 1980). Skin perfusion on both left and right ankles was measured at a sampling frequency of 40 Hz. Throughout this paper we will therefore refer either to Doppler flow or blood perfusion.

Experimental protocol The protocol of this study was approved by the Faculty of Science and Technology Research Ethics Committee, Lancaster University UK. Thirteen healthy dark-skinned subjects, born in sub-Saharan Africa, without known ancestors of non-African origin, with a high melanin concentration in their skins, and ten light-skinned Caucasian subjects of European origin with low melanin concentration, between the ages of 18–27 years, were recruited. All subjects were male. Their anthropometric data are given in Table 1. Written informed consent was obtained from all participants. Volunteers were asked to abstain from food, coffee, and alcohol for 3 hours prior to the experimental measurements. LDF was used for non-invasive and simultaneous measurement of the skin blood flow at two different sites, on the outer sides of the left (LA) and right (RA) ankles (lateral malleolus). An electrocardiogram (ECG) was used to record the electrical activity of the heart with a sampling frequency of 1000 Hz. ECG was measured using a bipolar precordial lead. The electrodes were attached on both shoulders and the lowest left rib, as this maximizes the sharpness of the R-peak. Using an elastic belt fastened across the chest and fitted with a Biopac TSD201 Respiratory Effort Transducer (Biopac Systems Inc., CA, USA), the respiration was also measured. Skin temperature was monitored using two high sensitivity, low heat capacity thermistors –

YSI 709B Thermilinear sensors (YSI Inc, Yellow Springs, OH, USA) of 8.5 mm diameter, which were taped on the skin. The thermistors were positioned outside the left ankles, over the lateral malleolus, close to the LDF probes.

The time series were recorded simultaneously using a signal conditioning system (Cardiosignals, Institute Jožef Stefan, Slovenia) and recordings lasted for 30 minutes with the volunteers lying relaxed. The recorded data are publicly available (see Sec. 7).

Blood pressure was measured prior to the initiation of signal acquisition. A Digital Automatic Blood Pressure Monitor (Omron, M10-IT) was used, wrapping a cuff on the subjects upper right arm while the subject was seated. The subject then moved to a supine position on a comfortable bed, where the necessary sensors were installed. In this way, subjects were in a supine position for 15–20 min of acclimatisation, before the recordings started. The bed and the equipment were housed within a well-ventilated Faraday cage, with a controlled ambient temperature of 20-21°C and constant low illumination. The equipment was either battery supplied or plugged in to the electrical supply via a mains filter.

Statistical analysis Application of the Lilliefors test for normality (Lilliefors 1967) showed that there were no consistent normal distributions of data among the groups being compared. Non-parametric statistical tests were therefore used, meaning that no assumptions were made about any underlying distributions, thus allowing robust conclusions to be drawn. The Kruskal-Wallis ANOVA test (Kruskal & Wallis 1952) was used when all IHRs (derived from the LA and RA blood flows and from the ECG) were compared. The Kruskal-Wallis test checks whether two or more independent sets of data originate from the same distribution, and it does not assume normal distributions. Where significance is found, pairs of groups are tested either by the Wilcoxon signed rank test for paired data, or by the Wilcoxon rank sum test for unpaired data (Wilcoxon 1945). The latter was used to test for possibly significant differences between blood flow measured from dark-skinned and light-skinned subjects, respectively, as the corresponding time-series do not match. The Wilcoxon rank sum test is used to determine whether two unmatched samples come from similar distributions, whilst the sign rank test requires that the samples are matched. In all cases, $p < 0.05$ was considered as being statistically significant.

3. Time series analysis

Recorded signals can be analysed to extract statistical properties of the data, or to investigate potential oscillatory characteristics, by quantifying the amplitude and power of oscillations and their phase characteristics. In this study, we particularly focus on characterization of time-varying oscillatory properties, resolving the dynamics with an optimal time localization and frequency resolution.

3.1. Wavelet analysis

The effective visualisation of the oscillations present in LDF generated signals greatly depends on the method used for their analysis.

Traditionally, representations of time series in the frequency domain are obtained with the fast Fourier transform, which constitutes a periodic function in terms of sines and cosines. This makes it suitable for analysing time series whose components are strictly periodic in nature, but it is unsuitable for LDF signals whose components are inherently non-periodic. The limitations of the Fourier transform can partly be addressed by use of the short-time Fourier transform through windowing, i.e. by dividing the time series into shorter time-windows within which there is not much time variation so that the fast Fourier transform (FFT) can usefully be calculated; in practice, this is usually done by sliding a window across the whole signal.

In the short-time Fourier transform, however, the spectral resolution depends strongly on the length of the window, with short windows leading to poor frequency resolution but good time localisation, and *vice versa*. This method may therefore fail when dealing with non-stationary physiological signals with varying frequencies, such as the LDF time series considered in this study. Its limitations can be overcome by use of wavelet analysis (Stefanovska et al. 1999) which, by using an adaptive window length that simultaneously analyses time series at each moment in time, provides both optimal frequency resolution and good time localisation (Iatsenko et al. 2015*a*, Clemson et al. 2016). These features are particularly important for the analysis of slowly-changing oscillatory dynamics over a broad frequency interval, of the kind giving rise to an LDF signal.

Wavelet analysis is a scale-independent method comprising an adaptive window length allowing low frequencies to be analysed using longer wavelets, and higher frequencies with shorter wavelets. The continuous wavelet transform $W_s(s,t)$ of a signal $f(t)$ is defined as

$$W_s(s,t) = |s|^{-1/2} \int_{-\infty}^{\infty} \psi\Big(\frac{u-t}{s}\Big) f(u) du, \quad (1)$$

where s is a scaling factor, t is the temporal position on the signal, and the wavelet function is built by scaling and translating a chosen mother wavelet ψ. In this study the complex Morlet wavelet:

$$\psi(u) = \frac{1}{\sqrt{\pi}} (e^{-i\omega_0 u} - e^{-{\omega_0}^2/2}) e^{-u^2/2} \quad (2)$$

was chosen because it maximizes joint time-frequency resolution (Stefanovska et al. 1999).

3.2. Extracting the instantaneous heart frequency

The instantaneous heart rate (IHR) was extracted using both time-frequency and time domain analysis techniques. The methods used to estimate heart frequency

include nonlinear mode decomposition (NMD) (Iatsenko et al. 2015*b*), a technique that decomposes a signal into set of components, or modes. Using NMD, the instantaneous frequency of the heart beat was extracted from the wavelet transform of the ECG, thus yielding the IHR. Similarly, the IHR was also derived from the LDF signal using the same technique. Note that in the literature (Malik et al. 1996, Iatsenko et al. 2013) IHR is mostly referred to as HRV or, occasionally, as IHF.

3.3. Wavelet phase coherence

The coherence $WPC_{s_1,s_2}(f)$ between the two signals $s_{1,2}(t)$ is determined through their WTs $W_{s_{1,2}}(t,f)$ as

$$WPC_{s_1,s_2}(f) = \left| \frac{1}{T} \int_0^T e^{i \arg[W_{s_1}(t,f) W^*_{s_2}(t,f)]} dt \right| \tag{3}$$

and it reflects the extent to which the phases (and thus the underlying activities) of these signals at frequency f are correlated (Bandrivskyy et al. 2004, Sheppard et al. 2012). Unlike the usual coherence measures, wavelet phase coherence does not take into account the amplitude dynamics of the signals. This is appropriate because (i) the amplitudes of most physiological signals are subject to artefacts and noise, and (ii) the relationships between the amplitudes of common physiological oscillations in different signals can be complicated and nonlinear, but in all cases the relationship between their phases remains the same (up to a constant phase shift).

3.4. Effective (or significant) coherence

The wavelet phase coherence between the oscillations in the two blood flow signals is computed by evaluating the difference between the wavelet transform phases of the signals at each frequency, and at each moment in time, as given by Eqn. (3). The oscillations are considered to be coherent at any given frequency if their phase shifts remain unchanged (with a coherence value ranging between 0 and 1); otherwise they are said to be incoherent. Coherence does not provide information about synchronization between oscillations, as this would require them to be coupled, which is not necessary for coherence to exist. However, information on possible synchronization between oscillations, particularly at the smaller ratios, e.g. 1:1 synchronization, can be obtained by estimating the wavelet phase coherence. Note, however, that the coherence computed in the first instance does not necessarily reflect a genuine phase relationship and requires careful evaluation. The problem arises because some of the coherence values obtained can be less than zero (although formally coherence values range between 0 and 1). These negative coherence values are then subtracted. Following this procedure, the very low frequency oscillations may appear to have a coherence values close to 1, because of bias resulting from the use of recordings that are too short to encompass the content at low frequencies.

To minimise random effects giving rise to apparent (but spurious) coherence, whether at low or high frequency, we checked/tested the significance of the computed coherence using the method of surrogates (Schreiber & Schmitz 2000, Lancaster et al. 2018) – by setting as a null hypothesis that, for all frequencies, the phases in the signals are independent. We used iterative amplitude-adjusted Fourier transform (IAAFT) surrogates to estimate the significance level of the apparent coherence, thereby removing the bias associated with the power spectrum of the more commonly used amplitude-adjusted Fourier transform (AAFT) surrogates. First, the IAAFT surrogates are constructed by randomizing all the properties of the signals in question, whilst keeping only the phases unshuffled. Subsequently, this is accomplished in an iterative fashion, simply by using the appropriate value and re-scaling the distribution to substitute Fourier amplitudes, which allows us to obtain resemblance between the distributions and power spectra of the surrogates and the original signals. At each frequency we took the coherence threshold to be 95% of the highest value of 100 random realisations of IAAFT surrogates.

Finally, the effective/significant coherence was estimated by subtracting the 95th percentile of the 100 surrogate values, thus giving the extent to which the phases of the two signals at each frequency are correlated.

4. Results

The anthropometric data of the subjects are shown in Tables 1, while the median and interquartile ranges of the instantaneous respiratory rate and IHR for both dark-skinned and light-skinned groups of subjects are presented in Table 2. There are no statistically significant differences between the groups.

Table 1. Anthropometric data of subjects measured, median values, ranges [25th and 75th percentiles] and significance

	Dark-skinned subjects ($n = 13$)	Light-skinned subjects ($n = 10$)	p
Age (years)	21.0 [20.0 24.0]	22.0 [19.0 25.0]	0.66
Body mass index (kg/m^2)	23.15 [20.2 24.2]	23.15 [21.6 24.7]	0.34
Skin temperature (^{o}C)	29.9 [25.9 31.1]	30.1 [28.9 30.4]	0.90
Instantaneous respiratory rate (Hz)	0.28 [0.27 0.3]	0.26 [0.24 0.27]	0.06
Systolic BP (mm Hg)	115.5 [112 125]	118.5 [109 126]	0.85
Diastolic BP (mm Hg)	71.5 [68.0 75.0]	76.5 [67.0 77.0]	0.64

Typical recordings of LDF blood flow time series simultaneously recorded from the right and left ankles of volunteers in both groups, together with their respective time-frequency representations, are presented in Fig. 2. No differences in skin perfusion or fluctuations between the groups were evident. Table 2 presents the median and interquartile ranges of the blood flow of dark and light-skinned groups. Although the

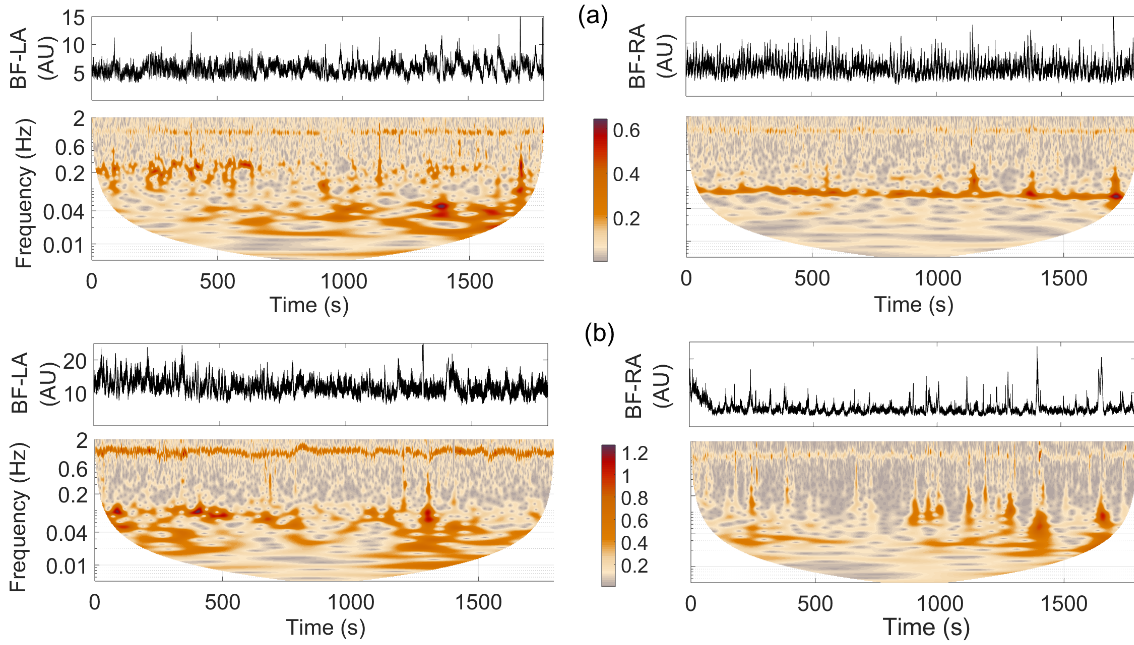

Figure 2. Typical LDF blood flow (BF) signals recorded from the left (LA) and right (RA) ankles together with their continuous wavelet representations for: (a) a dark-skinned volunteer with high melanin concentration; and (b) a light-skinned volunteer with low melanin concentration. The wavelets provide time-resolved frequency content of the blood flow signals.

values for dark-skinned group are slightly lower, no statistically significant differences were found in mean blood perfusion between the two groups ($p > 0.1$).

Table 2. LDF skin perfusion and IHR (derived from LDF) of measured subjects, median values and ranges [25th and 75th percentiles]

	Dark-skinned	Light-skinned	p
Blood flow (RA) (AU)	6.12 [5.0 6.9]	9.3 [5.4 12.5]	0.11
Blood flow (LA) (AU)	7.54 [5.5 11.11]	9.06 [7.01 11.75]	0.37
IHR (from ECG) (Hz)	0.99 [0.91 1.05]	0.99 [0.83 1.17]	0.98
IHR (from RA) (Hz)	1.00 [0.95 1.10]	1.01 [0.83 1.18]	0.73
IHR (from LA) (Hz)	0.99 [0.93 1.11]	0.99 [0.84 1.17]	0.78

The IHR values calculated from the LDF time series recordings measured on the ankles did not differ between groups, as shown in table 2. Figure 3(a)(c) illustrates how the IHR was extracted from the ECG and (b),(d) from LDF blood flow signals; and the intra-group comparison between the IHRs (Figures 3(e) and (f)). Comparisons made between mean values of the IHR signals derived from both the ECG and LDF data (measured from both LA and RA) (table 2) revealed no statistically significant differences ($p > 0.7$).

Figure 4 summarises the quantitative analyses of the oscillations in LDF blood flow

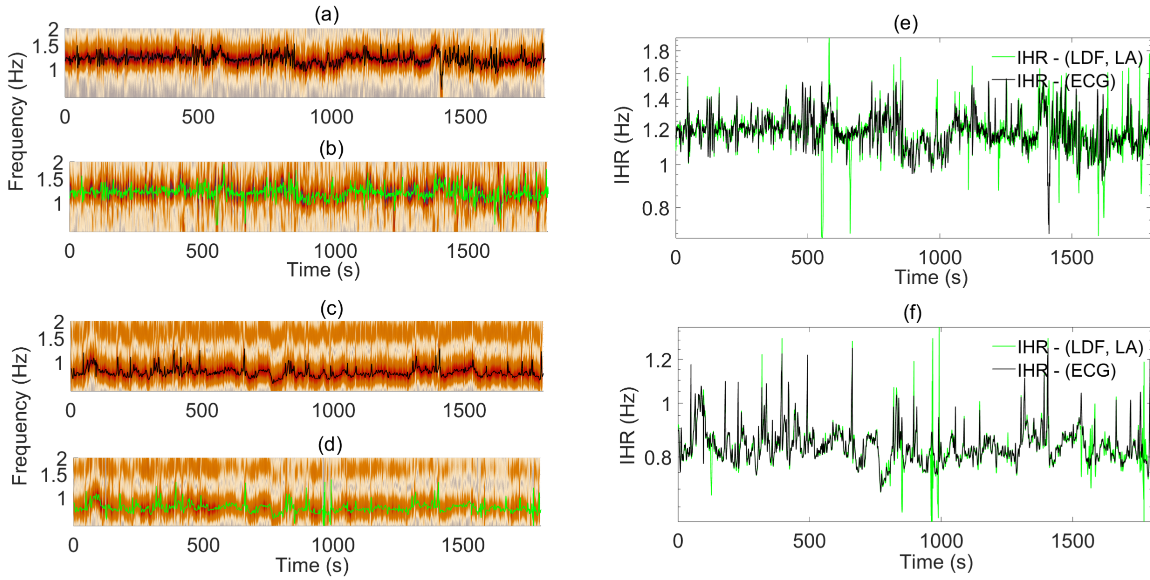

Figure 3. Time-frequency representations illustrating the extraction of IHRs from the heart beat detected in the wavelet transform of the ECG (a),(c), with their variation in time traced with black curves for dark (a) and light (c) skin; (b),(d) wavelet transforms of the LDF blood flow and their variation in time traced with green curves, for both dark (b) and light (d) skin. The frequency variation over time shown in (a)-(d) gives the IHR. (e),(f) comparisons between the IHR obtained from ECG and LDF for dark (e) and light (f) skin.

and in the IHRs derived from both ECG and LDF. No statistically significant differences in time-averaged wavelet power across the frequency intervals were observed in the LA and RA (Fig. 4(a),(b)) blood flows between the dark- and light-skinned groups ($p = 0.95$ and $p = 0.62$ respectively). The 25th and 75th percentiles (indicated in dark green for dark skinned and light green for light skinned subjects) of both LA and RA LDF blood flow spectrum between groups are presented in Fig. 4(a)(i) and (b)(ii). There is no obvious difference in inter-subject variations between the two groups.

Intra-group comparison of the time-averaged power of the IHR derived from ECG, LA and RA LDF blood flows revealed no significant differences between light-skinned (Fig. 4(c)) and dark-skinned (Fig. 4(d)) groups: $p = 0.99$ and $p = 0.21$ respectively as obtained by the Kruskal-Wallis test. Fig. 4(e) shows no significant difference in the inter-group comparison of the time averaged power for IHR derived from (i) ECG, (ii) LA LDF and (iii) RA LDF, $p = 0.64$, $p = 0.37$, and $p = 0.20$, respectively.

Wavelet phase coherence between LA and RA blood flows from each groups are shown in Fig. 4(f). Compared to the dark-skinned cohort, the light-skinned group exhibits a significantly higher coherence in the $0.6 - 2$ Hz frequency interval, corresponding to cardiac activity. The coherence was significantly lower in the dark-skinned group also near 0.1 Hz.

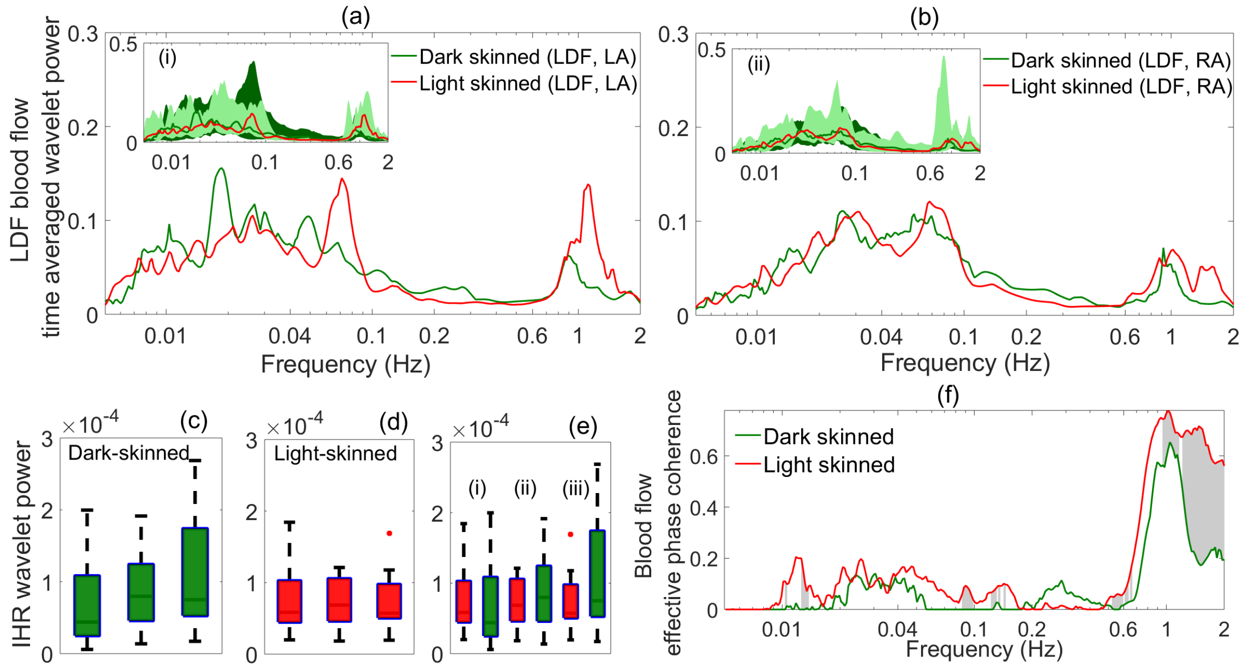

Figure 4. Group median time-averaged spectral power calculated from the wavelet transforms of LDF signals recorded for 30 minutes in dark-skinned (green) and light-skinned (red) groups for (a) the left ankle (LA) and (b) the right ankle (RA). In neither case was any statistically significant difference seen at any frequency. The insets (a-(i)) and (b-(ii)) are provided to give an idea of inter-subject variability, which was similar in both groups. They show the 25th and 75th percentiles of the individual spectra from dark-skinned (dark green) and light-skinned (light green) groups. (c) and (d) show IHR time-averaged wavelet power for dark-skinned (green) and light-skinned groups (red). The box-plots represent the time-averaged wavelet power for IHR derived from ECG (first), LA LDF (second) and RA LDF (third box). The Kruskal-Wallis test revealed no significant differences among the three IHR powers ($p = 0.21$ for the light-skinned group and $p = 0.99$ for the dark-skinned group). (e) Comparison between groups: (i) IHR derived from ECG, (ii) IHR derived from LA LDF and (iii) IHR derived from RA LDF. No significant differences are obtained for any of the three comparisons. (f) Median wavelet phase coherence between left and right ankle blood flow for dark-skinned (green) and light-skinned (red) groups. Light-gray indicates frequencies at which there are significant differences ($p < 0.05$) as determined by the Wilcoxon rank-sum test.

5. Discussion

Analysis of the spectrally broadened signal due to backscattering of the incident 780 nm coherent laser light from darkly- and lightly-pigmented skins has provided a measure of the flux, which is proportional to the product of the average speed of the moving erythrocytes and their concentration. It has enabled the hypothesis underlying this study – that dark skin colour (melanin concentration) does not attenuate the light sufficiently to vitiate LDF as a method for measuring blood flow – to be tested.

Our study sheds additional light on the long-running debate about the significance for LDF of the optical difference between Caucasian and non-Caucasian skin, a question

that has been under discussion ever since the early days of the technique (Leahy et al. 1999). Dark skin colour significantly attenuates the incident laser light that reaches the deeper skin tissue (Goldman et al. 1963). Fredriksson and co-authors (Fredriksson et al. 2009) used Monte Carlo simulations of light propagation in tissue, for wavelengths between 543 and 780 nm, to show that skin pigmentation is expected to have a negligible effect on the measurement depth. The wavelength of the light certainly influences the optical penetration, irrespectively of the skin colour. Zhoa and Fairchild (Zhao & Fairchild 1998) showed that, in the wavelength interval 532–1064 nm, laser light of longer wavelength penetrates more deeply into skin. Also using Monte Carlo simulations (Ash et al. 2017) it was recently confirmed that an increase in the wavelength of incident light increases its penetration, consistent with the fact that melanin absorption decreases monotonically with increasing wavelength (Keilhauer & Delori 2006). This arises because the scattering and absorption coefficients of melanin and other chromophores inside the skin are wavelength-dependent. In this study we tested one of the commonest wavelengths used for LDF measurements.

Here we would like to emphasise that there are two different approaches to the evaluation of the efficiency of LDF. One of them is based on the assumption of equilibrium and is concerned with temporal and spatial reproducibility, treating the tissue as a static and unchanging system. Under this approach readings are usually taken over a short time, or at an instant of time. The other approach, which is what we use here, is based on evaluation of dynamical changes over time, where the interval over which the recording is made is of particular importance. To ensure sufficient information about the dynamical changes, one needs to record over sufficient time to resolve them, but also time-resolved methods of analysis are needed to cope with time-varying nature of the changes. These are the characteristics on which this study was based. Our focus is on whether the dynamics is influenced by pigmentation.

Our results show that LDF can provide reliable information about the dynamical properties of blood flow in darkly pigmented skin: a significant fraction of the laser light is able to penetrate, interact with the moving erythrocytes, and be back-scattered to reach the detector. By quantifying the power of oscillations and their phase characteristics, our findings show that the oscillatory characteristics of LDF time-series recordings from darkly pigmented skin did not differ significantly from those measured from light skins. This indicates that the intensity of 780 nm light penetrating to 1.15 mm below the epidermis is sufficient for LDF to gather information about the blood flow dynamics (Braverman 1997, Braverman 2000). Our findings differ from some of those reported earlier (Goldman et al. 1963, Karsten & Smit 2012, Dremin & Dunaev 2016), because the wavelength of the laser diode in the present study differed, although the exact depth to which the LDF method can be effective remains unknown (Braverman et al. 1992). One expects differences in the amplitude of the flux measured from different skins, due to possible difference in the density of erythrocytes between individuals, given that the flux (the Doppler-shifted signal) depends on the erythrocyte concentration. In this study, however, the average values of Doppler perfusion did not differ to a

statistically significant extent between the darkly- and lightly-pigmented skins.

Wavelet analyses of LDF blood flow measured from dark and light skins produced closely similar results. The cardiac activity could clearly be observed in each case. Oscillations throughout the full 0.0095-2 Hz frequency interval investigated were the same, suggesting that the fluctuations in laser Doppler perfusion are being properly captured from dark-skin. This is contrary to earlier reports that reproducible estimation of laser Doppler perfusion in darkly pigmented skin is difficult (Bonner & Nossal 1990).

Two methodological issues deserve comment. First, the exact melanin concentrations were not measured directly. However, we have considered the two extreme cases of a) darkly-skinned subjects that were born and raised in sub-Saharan Africa, without known ancestors of non-African origin, and b) white-skinned Caucasians of European origin. It is well known that of all skins, the sub-Saharan black Africans skin possesses the highest concentration of melanin. It is packaged into larger singly-dispersed melanosomes of $\sim 1\ \mu$m, whereas in lighter skin types the melanosomes is known to be smaller with a size of $\sim 0.5\ \mu$m (Alaluf et al. 2002). Secondly, the group sizes are relatively small. We made every possible effort to make the groups homogenous, including healthy, non-obese males in a very narrow age-range (see table 1). Additionally, we would comment that a large number of studies with similar numbers of participants have been conducted with clinically and physiologically relevant results, e.g. (Kvernmo et al. 1999, Stefanovska et al. 1999, Söderström et al. 2003, Sheppard et al. 2011, Ticcinelli et al. 2017).

The significantly higher coherence around the cardiac frequency interval in the light-skinned group, compared to the dark-skinned one, may result from the known ethnic disparity in cardiac autonomic modulation between white and black people (Esco et al. 2010, Urbina et al. 1998, Guzzetti et al. 2000). It could also be associated with the ethnic differences in left ventricular wall thickness (Hinderliter et al. 1996). Also, it is known that nitric oxide (NO) and endothelium-derived hyperpolarizing factor are of crucial importance in resting blood flow dynamics and that blacks exhibit reduced NO compared to Caucasians (Cardillo et al. 1998, Kalinowski et al. 2004, Ozkor et al. 2014, Kim et al. 2018). So the significant difference in blood flow coherence observed here may reasonably be attributed to the physiological differences underlying the ethnic disparities (Berardesca et al. 1991, Hill et al. 2015) between the two groups of subjects, and we will provide a fuller analysis and discussion of these differences elsewhere.

The IHR values from the laser Doppler signals exhibited no significant differences between dark-skinned and light-skinned groups. Similarly, we obtained no significant difference in the spectral power over the 0.005-0.6 Hz frequency interval. The IHRs derived from ECG were considered as the reference signal in these comparisons. Although the curve of IHR does not match perfectly between groups, the observations still prove that the fluctuations in Doppler perfusion measured in high melanin skin are physiologically meaningful. The fact that the observed microcirculatory dynamics is similar for both groups shows that frequency-dependent scattering processes along the

optical return path are insignificant.

6. Conclusion

Our investigation has shown that, with illumination derived from a laser diode of wavelength 780 nm, LDF provides an effective method of studying blood flow dynamics, even in darkly pigmented skin. We found no evidence that the greater light attenuation in the latter case has an adverse effect on measurements, and we were able to obtain the same information about the microcirculatory dynamics regardless of skin pigmentation.

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