Technology and Health Care 7 (1999) 143–162
IOS Press

Principles and practice of the laser-Doppler perfusion technique

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Abstract. This paper reviews the development and use of laser-Doppler perfusion monitors and imagers over the past two decades. The enormous interest in microvascular blood perfusion coupled with the 'ease of use' of the technique has led to 1500+ publications citing its use. However, useful results can only be achieved with an understanding of the basic principles of the instrumentation and its application in the various clinical disciplines. The basic theoretical background is explored and definitions of blood perfusion and laser-Doppler perfusion are established. The calibration method is then described together with potential routes to standardisation. A guide to the limitations in application of the technique gives the user a clear indication of what can be achieved in new studies as well as possible inadequacy in some published investigations.

Keywords: Blood supply, laser-Doppler flowmetry, microcirculation, Doppler shift, biomedical instrumentation

1. Introduction

The laser-Doppler perfusion monitor (LDPM) has been applied to measurements of blood perfusion in the undisturbed microcirculation for almost 20 years. The LDPM has emerged in the absence of any other method to fill the clinical requirements of a sensitive, continuous, non-invasive and real-time method for the measurement of microvascular blood flow. This paper attempts to track its development over the period and describe the definitions, the state of art in LD instrumentation and application, as well as its limitations and future perspectives.

1.1. Genesis

Measurement of the velocity of particles in solution by interpreting the Doppler frequency-shifted light backscattered therefrom was first described by Cummins et al. [1], only four years after the first working laser was demonstrated by Maiman [2]. Riva et al. [3] were the first to apply this technique to the measurement of red blood cell velocities in their glass tube flow model. However, the birth of laser-Doppler blood perfusion measurement in the undisturbed microcirculation must be attributed to an article entitled '*In vivo* evaluation of microcirculation by coherent light scattering' by M.D. Stern [4]. The author proposed that when coherent light impinges on the skin, some of it will be scattered by moving red blood cells and some by static tissue. If this light (which is spectrally broadened due to the Doppler-effect) is brought to the surface of a sensitive photodetector, optical beating will produce an

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audio-frequency photocurrent output. Spectral analysis of the photocurrent produced by backscattered He-Ne red laser light from the fingertip showed a clear difference between normal flow and occlusion of the brachial artery. The feasibility of real-time monitoring was demonstrated using the RMS (root-mean-square) of the photocurrent signal as a flow indicator. This method was shown to be sensitive to the microcirculatory response to temperature, posture, respiratory pattern and emotional stimulation. Furthermore, it was proposed that 'Because it provides information about the flow velocity distribution, it has the potential ability to analyse the distribution of flow in the different microvascular compartments'.

1.2. Technical development

Holloway and Watkins [5] redesigned this instrument to be of more practical use in the clinic, after some unpublished co-operation with Stern had demonstrated reasonable correlation with their established ^{133}Xe clearance technique. This system included fibre-optics for light delivery and collection, as well as a photodiode in place of the more cumbersome photomultiplier tube used by Stern. They again evaluated the instrument against the ^{133}Xe clearance technique and were able to measure the elevation in local blood perfusion due to injection trauma. This effect was suspected for some time but no other method was capable of measuring it. Further work by Stern [6] included an empirically based processor computing the square root of the second moment of the photocurrent spectrum. This approach was chosen because it could be implemented in simple analogue electronics in order to give a real-time index of blood flow, although it was realised that other algorithms could be as useful.

Watkins and Holloway [7], describing their instrument in more technical detail, identified the modal noise caused by the He-Ne laser as a major developmental problem, and found that measurement was limited to 50% of the observation time. The presence of more than one spectral line in the laser output may cause beat frequencies to occur on the photodiode, similar to those produced by Doppler-shifted light. This problem was overcome by Nilsson et al. [8] who proposed a dual photodetection system in which the noise common to both detectors could be rejected. The Doppler signal is not affected as the photons arriving at each detector are completely independent and random. Later that year Nilsson et al. [9] presented a further development incorporating the signal processor developed by Bonner and Nossal [10]. This remains the only signal processing algorithm with a predominantly theoretical base and has found favour amongst most researchers and manufacturers.

The instruments prototyped by Holloway and Watkins and also Nilsson et al. were soon commercially available. An early comparison by Fischer et al. [11] demonstrated a good correlation between the instruments as well as with microvascular blood flow. Clinical trials were undertaken in many areas and, while good results were often produced, widespread application has eluded the technique. One reason given by clinicians is that the lack of absolute units makes interpretation of the results difficult. However, the measurand (i.e., microvascular blood flow) is poorly predictable at any given tissue site and time. The assessment of sufficiency of perfusion must, therefore, be based on its variation in response to an appropriate physiological stimulus. Furthermore, the clinician is often unaware of limitations of the technique and clear guidance can only be given with reference to individual application areas. Diagnostic differences in microcirculatory response have been consistently reported where a ratiometric (% change) analysis has been employed. This invariably involves the comparison of a stimulated value to a resting level. However, even in this comparison there will be some dependence on spatial variation. This is best dealt with by moving the probe and repeating the experiment a number of times, taking the mean value.

Clinical trials of these improved instruments exposed the problem of motion artefact noise which occurs when the modes in which light passes through the fibre are disturbed by movement of the cable. This

phenomenon manifests itself in intensity fluctuations at the photodetector similar to the laser-Doppler beat frequency. One analytical study demonstrated that the range of frequencies produced is 0 to 3.5 kHz [12] and the magnitude can be much larger than the perfusion related Doppler signal. This problem was accentuated by the fact that the larger-core fibres translated the movement to the tissue, giving rise to tissue-related Doppler-shifting. Motion artefact noise is dealt with in greater detail in Section 4.

The wavelength of the diodes commonly used (c. 780 nm) has the further advantages of deeper penetration and reduced dependence on skin colour. Also, because the absorption of oxidised and deoxygenated haemoglobin are almost equal at this wavelength, any dependence on oxygen saturation is eliminated. The choice of laser wavelengths is confined (largely by commercial availability) to He-Ne red (632.8 nm) and near-infrared laser diodes (780 nm, 810 nm). More recently red (670 nm) laser diodes which are sufficiently stable have become more accessible. The He-Ne system is important for historical reasons as the first instruments were built using these lasers, however, laser diodes are now used in approximately 95% of instruments being sold. A great part of the literature refers to the He-Ne wavelength and consideration should be given to this fact when comparing results. A greater understanding of both the laser-Doppler signal and the associated noise has allowed us to develop instruments which can process the entire bandwidth of interest with excellent signal to noise performance.

Perhaps the most remarkable feature of the microcirculation is its enormous temporal and spatial heterogeneity. This has given rise to the introduction of a multichannel instrument and the laser-Doppler Perfusion Imager (LDPI). The multichannel array provides for the spatial assessment of blood flow without the loss of temporal resolution. This facilitates the continuous and real-time measurements of the effects of vaso-active stimuli in different compartments of the same organ or inter-organ comparisons. It can be particularly useful in disciplines where it is necessary to control the blood supply on a regional basis. One example is the use of pharmaceuticals to restrict the blood supply to the tumoural area without affecting local normal tissue. By simultaneously monitoring the perfusion at micro-regional sites within and in the locus of the tumour during infusion of such agents, immediate information is given as to the selectivity and severity of the drug's effect, e.g., [13]. Similar, but more graphic information may be obtained using the laser-Doppler Perfusion Imager [14] where the entire area of interest is visible and blood flow variations are expected to occur over a much longer time course.

Consensus in various application areas should promote the development of the full potential of the laser-Doppler technique. It is cost-effective and easy to use. It is possibly this advantage which has paradoxically become a stumbling block to more universal use of the instrument, because it is quite possible to use the instrument with little knowledge of what is being measured and how.

1.3. Future objectives

Given that absolute measurements of LDP are some way off, the possibility of a Standard Perfusion Unit (SPU) is a useful and attainable interim goal. As it was discussed during EU CA meetings, there are many reasons for desiring such a system, not least being the need for a practical definition laser of Doppler perfusion monitoring. If this unit is accepted then all instruments which can be satisfactorily calibrated to measure in this unit within their linear range belong to a new generation of laser-Doppler instruments. Such instruments might be distinguished by a new name, similar to the idea of 'Ultrasound B'. In practice all instruments with a listed probe and appropriate wavelength could be converted to operate according to the new specification. The specification might include additional requirements, such as the registration of the transfer function(s) of individual instrument models, so that these are verified laser-Doppler instruments.

Many recent attempts have been made to define blood perfusion (see Section 2) and what is measured by LDPMs. The results are inevitably different for earlier schemes because of the approach taken, as are the units one may derive. On the other hand, progress in any form of metrology requires the use of a generally accepted unit in which all such measurements can be expressed. All SI units are arbitrarily chosen or derived from such units. All measurements are subject to distortion by the measuring conditions, even time (e.g., travel on an inter-galactic scale). In the laser-Doppler perfusion technique it is difficult to define standard measurement conditions, due to the enormous variations of microvascular architecture, haematocrit and optical properties of the whole range of tissues which can be examined.

However, if one set of standard measurement conditions can be agreed by all parties, then great progress can be made on the comparability of instruments. All instruments calibrated in the same conditions, within the limits of velocities and concentrations over which the instruments are linear, would read the same value when probing exactly the same tissue site at the same time. In other words all calibrated laser-Doppler [B] instruments using the same probe configuration and wavelength will have the same response to signals within the linear portion of the instruments' transfer functions. For most purposes this can be deduced from their upper cut-off frequencies.

In the first instance such measurement conditions would have to include a low concentration of red blood cells (RBCs) moving with small velocities (say $< 1\,\mathrm{mm\,s^{-1}}$), ideally dispersed among a matrix of static-scatterers as described in Section 3. It should be possible to determine, both by calculation and direct measurement, the probed volume, the average speed and concentration within it. The laser-Doppler instruments should be calibrated to read a prescribed number of Standard Perfusion Units (SPU). To suggest that this would be equivalent to some SI derived unit would belie the complexity of the measuring environment. This unit would, however, allow users the confidence of knowing that another instrument would measure the same level in the same circumstances. Of course, it is desirable to check the linearity of response of the instruments at a third set-point, where a different concentration, for example, can be used.

Similar calibration of other probe types can be made by assessment of the effect of fibre separation and numerical aperture, from which an appropriate set-point can be established. This common reference is important from a practical point of view, but probes 'looking' at different volumes of tissue can never be directly compared. Clinical groups should agree on a probe type most suitable for their application areas.

Consensus in various application areas should promote the development of the full potential of the laser-Doppler technique. The EC-sponsored Concerted Action are working to provide a greater means of comparison of results between active groups, through the establishment of internationally standardised protocols. It is hoped that this will result in more published work which can be clearly reproduced by other workers. It can truly be said that 'We (still) know of no comparable method of demonstrating these microvascular reflexes' [4].

2. Theoretical background and definitions

2.1. Monochromatic light scattering in tissue

When laser light impinges on the tissue surface the individual photons migrate through the tissue in a random fashion, the exact statistical pattern of which is determined by the tissue optical properties. At interaction with one or more moving objects such as blood cells, the scattered photons will be shifted

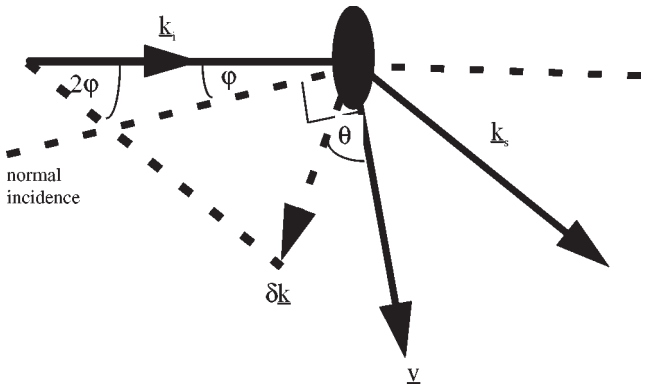

Fig. 1. Scattering of a photon by a moving RBC. Note that the angles are exaggerated. Scattering is strongly peaked in the forward direction, with a mean scattering angle of 5.4° [10].

in frequency by an amount determined by the scattering angle, the wavelength and the velocity of the scatterer. This frequency shift is generally named after the Austrian scientist Johan Christian Doppler [15,16] who studied and explained the phenomenon in the beginning of the nineteenth century.

In the simple case of a source with frequency, f_s, reflected from an object moving with velocity, v, in the direction of propagation of the light, the detected frequency is different from that of the source by:

$$f_s - f_d = \delta f = \frac{2v}{\lambda}, \tag{2.1}$$

where f_d is the Doppler shifted frequency and λ is the wavelength of the light in the scattering medium.

In a more realistic situation the beam is incident at some angle, φ (see Fig. 1), to the normal to the direction of motion, in which case the frequency shift becomes:

$$\delta f = \frac{2v \sin \varphi}{\lambda}. \tag{2.2}$$

If the photon is detected without further scattering from moving objects, it can be considered detected in the direction k_s and the resultant Doppler-shifted frequency becomes:

$$\delta f = \frac{2v \sin \varphi}{\lambda} \cdot \cos \theta. \tag{2.3}$$

In a system of moving particles, such as RBCs in tissue the photon can undergo more than one Doppler-shift, in which case the detected frequency difference is the sum of the individual events thus:

$$\delta f = \sum_{i}^{n} \frac{2v_i \sin \varphi_i}{\lambda} \cdot \cos \theta_i. \tag{2.4}$$

Typical frequency shifts detected by a laser-Doppler perfusion monitor using a wavelength of 780 nm in the microcirculation range from 0–20 kHz. Paradoxically for 633 nm the range is smaller, typically 0–12 kHz (*ceteris paribus*). The apparent contradiction with Eq. (2.3) is caused by the deeper vessels probed by the longer wavelength which contain faster flowing blood. When one considers that it is necessary to

extract this shift from a frequency of 3.8×10^{14} Hz, it seems an impossible task. In fact it is impossible to resolve these frequencies directly as there is no detector capable of such a response time. If, however, the shifted and unshifted light are allowed to interfere on the surface of a sensitive photodetector, a beat frequency will be produced, the detectable component of which is equal to the difference frequency. It should be noted that although multiple Doppler-shifting can cause much higher difference frequencies to be detected, the individual shifts can be positive or negative.

As the angles are more or less random and a wide range of velocities are present in the microcirculation, a continuous Doppler frequency spectrum is produced. It is interesting to note that a similar result can be achieved by considering the scattering particle to be a reflector of light moving with velocity v. The reflected light will interfere with the reference beam and the resultant intensity will vary with the difference in optical path, opd, between the two beams. The number of interference fringes to pass the detector in time, t, will be given by OPD$/\lambda$ or $2d/\lambda$, where d is the actual distance moved by the particle. The number of fringes per second is then:

$$f = 2d/\lambda t = 2v/\lambda. \tag{2.5}$$

Thus, the relationship of the resultant frequency to velocity is the same in each case. Indeed, one group [17] have re-interpreted the technique in terms time-varying laser speckle. The speckle pattern is a granular variation of light intensity obtained from any surface, such as the skin, which is rough on a scale comparable with the wavelength of light. The scattering inhomogeneities can be considered as point sources and the small differences in the distances travelled to the detector (eye/photodiode) cause bright or dark speckles to be seen at all points in space. If some of the scatterers are moving a fluctuating speckle pattern will be observed, which fluctuations are related to the velocity of the motion.

Since the tissue volume occupied by moving blood cells is generally small, most photons will not be shifted in frequency before they are backscattered or absorbed. The backscattered and Doppler broadened light carries information about both the speed of the blood cells traversing the scattering volume (frequency broadening) and their concentration (fraction of total photons that is frequency shifted). If a portion of the backscattered light is brought to impinge on the surface of a photodetector, the frequency shifted and non-frequency shifted photons mix, and beat notes related to the Doppler shift is superimposed on the photocurrent [4].

2.2. Definition of perfusion

The quantity that is measured in laser-Doppler perfusion monitoring and imaging is generally referred to as perfusion. Although this quantity has been used extensively, a thorough definition is still lacking. The following definition of perfusion is therefore proposed:

Perfusion is the product of local speed and concentration of blood cells.

Comments: The word speed is preferred instead of velocity since velocity by definition is a vector implying information of both direction and magnitude. Speed refers only to the magnitude of the velocity vector. In many reports the laser-Doppler perfusion monitor is referred to as probing only the red blood cell movement. Practically it is substantiated by the fact that red blood cells present the majority (99%) of the blood-borne particles in the undisturbed microcirculation. However, since laser-Doppler perfusion monitors do not selectively measure the movement of the red blood cells, the word “red” should

preferably be omitted in the definition. It must be pointed out, however, that because of the different relation between scatterer dimension and wavelength, a different sensitivity must be expected for different scatterers (e.g., white and red cells) in the tissue perfusion.

2.3. What is recorded by laser-Doppler perfusion monitors?

The laser-Doppler perfusion monitor records the integrated perfusion within the sampling volume. Due to the non-uniform photon density within the sampling volume, the sensitivity is a function of the local co-ordinates within this volume. The spatial distribution of photons within the sampling volume is described by a weighing factor $H(r)$ dependent on the tissue optical properties at location r and properties of emitted light (wavelength, beam, intensity, diameter and profile). What is recorded by laser-Doppler perfusion monitors is thus proposed to be:

$$LDP \approx \int_{r^3} H(r)s(r)c(r)\,\mathrm{d}r^3, \tag{2.6}$$

where $H(r)$ is a sensitivity weighing function, $s(r)$ is the speed and $c(r)$ is the concentration of blood cells at location r. Integration limits depends on probe design, especially numerical aperture (NA) of detector, emitter-detector distance as well as the optical properties of the tissue under investigation.

Comments: By the introduction of the weighing function $H(r)$, the sampling depth distribution and the median sampling depth can be calculated by Monte Carlo simulations for a specific tissue – probe combination. Numerical values of the median sampling depth for some of the most generally used probes and for various tissues have been presented in the literature [18]. The weighing function $H(r)$ accounts for the local density of photons in tissue that eventually migrate to the photo detector surface and become available for detection but is virtually unrelated to the blood cell distribution. It is furthermore assumed that the density of the blood cells is such that successive Doppler shifts as well as homodyne detection can be neglected.

2.4. What about flow and flux?

The terms ‘flow’ and ‘flux’ are often used in the context of laser-Doppler measurements, but their meanings can be ambiguous. The term (blood) flow is used in a very general way to describe the passage of a quantity (usually unspecified) of blood through the region of interest – but is – thankfully – rarely now used to describe what is measured by a LDPM. The term flux is often used in place of perfusion (particularly in clinical papers) and can be loosely defined as the product of mean blood cell number and speed. From a pure physical point of view these terms are too broad to allow a proper definition. Flow can be particulate, volume or mass and in each case refers to the quantity which has passed the specified point per unit time. It will have units of *number* (*e.g., of blood cells*)/s, l/s, or kg/s. Flux usually indicates particulate flow, but is often used as the shortened form of flux density and in this context has the stricter definition; *the rate of flow of a physical quantity through a reference surface* (*i.e., an area*). As such it will have units (e.g., cells/m^2/s).

2.5. Modelling

Analytical models for dynamic light scattering in tissue have earlier been published in the scientific literature [10,19]. These models, however, do not account for a varying local density of blood cells

in the tissue or for the three-dimensional and limited sampling volume. In order to be able to further study more realistic situations, new models for dynamic light scattering in tissue therefore need to be developed. These models can probably not be analytical in nature, but Monte Carlo simulation of photon migration in tissue may be useful to further understand the basic operating principles of laser-Doppler perfusion monitors and imagers. By the use of such Monte Carlo simulation models it is possible to simulate the influence of, e.g., single small vessels located at different depths in tissue on the laser perfusion monitor output signal. Software packages for simulation of photon migration in tissue have been developed by two groups within the concerted action [20,21]. By the further use of these models it should be possible to calculate for instance the degree of scattering with multiple Doppler shifts for a given microvascular architecture and thereby the amount of non-linearities in the output signal. It is further possible to compare the depth sensitivity for different probe – tissue combinations. Such activities aim at developing a look-up table for selection of the correct probe for a specific measurement problem.

3. Standardisation and calibration

3.1. Standard measurement conditions

Laser-Doppler perfusion measurements are easy to perform in many clinical situations. However, to exploit this method it is necessary to know and to take into consideration various environmental and individual-related variables which effects microcirculation. A review on this problem was presented by Bircher et al. [22]. It is obvious that the temperature has very significant effect on microcirculation (thermoregulation) but it was also experimentally confirmed that position and motion, anatomical site, physical and mental activity as well as food and drugs have considerable effect. Thus, these environmental factors should be stabilised by recordings of LD perfusion signal in standard measurement conditions. Ideally the temperature controlled room should be used where temperature could be stabilised to $\pm 1^{\circ}$C. A period of about 15 min should be allowed for the investigated subject to acclimatise to the measurement site surroundings before starting LD recordings. During the measurements all movement, like change of position, and speaking should be very restricted and possible mental stress should be avoided. Smoking, abundant food and drinks, especially with caffeine or alcohol, should be avoided some hours before the measurements. The standard measurement conditions should be specified in a detailed measurement protocol for selected clinical investigation. The stimulation (provocation) tests applied should also be specified in detail in the measurement protocol. This concerns frequently used tests, e.g., large blood vessels occlusion, thermal test and orthostatic manoeuvres, as well as procedure of drugs administration, especially if applied during the LD measurements. The above-mentioned measurement conditions should be reproducible and have to be exactly followed during LD recordings especially in comparative or follow-up studies.

3.2. Instrumentation parameters

A typical laser-Doppler Perfusion (LDP) signal is derived from the detector signal by calculating the first moment of the power spectrum of the frequency spectrum of the detector signal. In most cases a normalisation is performed, based on the total amount of light reflected from the tissue or the zeroth moment of the power spectrum. In normal cases the resulting signal is presented in relative numbers, called Perfusion Units (PU). Since these numbers are relative, it is necessary that a proper Doppler scattering standard is available. Therefore, all manufacturers should provide a standardisation method

and equipment for calibrating the instrument, enabling the user to check that the instruments are correctly calibrated.

The LDP signal is from definition dependent on the speed and concentration of blood cells. There are, however, a number of factors that additionally influence the reading of the LD instrument:

- the optical properties of the tissue and of the blood content,
- the wavelength of the laser light,
- the physical and optical properties of the fibres, if present for the transport of the light to and from the tissue,
- the detection efficiency and bandwidth of the detector and signal processing electronics,
- the software for calculation of frequency moments, if present.

Some of these factors are tissue dependent and cannot be known *a priori*, but the others are generally instrument dependent and should be specified in the measurement protocols. There are mainly following parameters:

- wavelength of applied laser,
- LD probe configuration, fibres diameters and separation distance,
- signal processing algorithm and frequency bandwidth,
- data acquisition parameters, e.g., time constant, sampling frequency,
- calibration parameters.

The leading European manufacturers (Perimed, Moor Instrument, Oxford Optronix; all represented in the Biomed-I Concerted Action) provide instruments with differences in probe design and dimensions, wavelength, signal processing and perfusion index calculation procedures. For optimum results in physical and clinical research it is important to have a possibility to change the various parameters in the instruments. However, a comparison of results between the various instruments is desirable, especially for clinical purposes. Therefore a certain restriction and standardisation is required by the users of the instruments.

A common motility standard which could be used for all instruments as a Doppler calibration standard is the first step to meet such a requirement. Since all readings on tissue, obtained with the different instruments will render different results, depending on the specification of the type of instrument, a "European" laser-Doppler calibration device and protocol will require a common design.

3.3. Calibration methods

The calibration of the measurement instruments is usually performed by using universal standard of measured quantity. However, in the case of microcirculation there is no such standard of microperfusion, because of heterogeneity in tissue requirements and function. Thus, the absolute calibration, e.g., in ml/min/g, is possible only for particular measurement site in a specific preparation.

The real absolute calibration can be obtained by recording on isolated organs or tissue perfused with blood at a well-controlled rate. There are many studies of this type demonstrating experimentally a correlation between LD measurements and some other technique of blood flow measurements like isotope washout, radioactive labelled microspheres, photo-plethysmography, hydrogen clearance and capillaroscopy. However, the difference in measured volume (much smaller sampling volume in the case of laser-Doppler methods) make these comparisons difficult and often questionable. Nevertheless, the most valuable experiments performed on very fine preparation of feline small intestine and the human bowel well [23,24] showed excellent correlation between the LD signal and venous outflow.

For calibration of the LD instrument as a flowmeter the various mechanical flow models were used. The simple flow models consisted of polythene tubes filled with latex microspheres suspension or out of date blood. The flow rate was controlled by precise syringe pumps. The mechanical models allow tests of linearity and scaling in the wide range of input values (velocity and concentration) and in various experimental conditions.

Linearity and stability of LD instruments can be also tested by using a matrix with moving scatterers, usually in form of rotating discs. The typical example is the thin disc made of fluorocarbon simulating moving scatterers, which was commonly used in the earlier studies.

All above-mentioned calibration and standardisation methods are too complicated to be used in clinical conditions. Therefore a more simple method was proposed for frequent and easy checking of the LD instrumentation. This method is based on two-point calibration with use of motility standard being the water suspension of latex microspheres in Brownian motion.

3.4. Motility standard

Each of the European manufacturers provide a motility standard (MS) to facilitate calibration. Basically this standard consists of a small jar of water (or another suitable liquid) containing polystyrene microspheres in suspension. These spheres act as light-scattering particles. The motility of the spheres is sustained by Brownian motion. Either the calibration facility is provided to the customer by the manufacturer (Perimed, Moor) or the calibration is performed at the factory. This method is, however, inadequate for a number of reasons:

- Light scattered in solution has very little or no unshifted component and the signal is self-beating or homodyne in origin. High concentrations must be employed to increase and stabilise backscatter level and ensure that the signal is derived from within the solution.
- The presence of only moving scatterers within the solution promotes multiple Doppler-shift scattering, manifesting in a broader spectrum of beat frequencies.
- The calibration signal is likely to be far above the physiological range of interest and usually beyond the linear range of the instrument (see Fig. 2). This hypothesis needs to be investigated further.
- Spheres used are much smaller than red blood cells so as to reduce the sedimentation rate and increase velocity components.
- While most theoretical work approximates the erythrocyte to a sphere with a volume of approximately 90 μm^3, e.g., [25], the reality of the flexible disk shape (see Section 2.3) has a somewhat unknown effect on its scattering properties [26].

Thus, the presently used two point calibration method allows one to compare the response of two identical instruments with the same probe configuration. However, as the second calibration point is beyond the linear range of the processor and first point is instrumental zero (see Fig. 2), different instrument-probe combinations calibrated to the same sensitivity in the standard solution may have quite different responses *in vivo*. A new type of calibration standard has to be developed, in order to overcome some of these problems.

3.5. Zeroing of LDP measurements

All manufacturers provide a zeroing disk, usually a white static scatterer of a plastic material. This zeroing procedure works well when the total scatterer is concerned, but cannot distinguish between Doppler and non-Doppler scattering, and thus Doppler and non-Doppler zero levels. Research on this topic must continue.

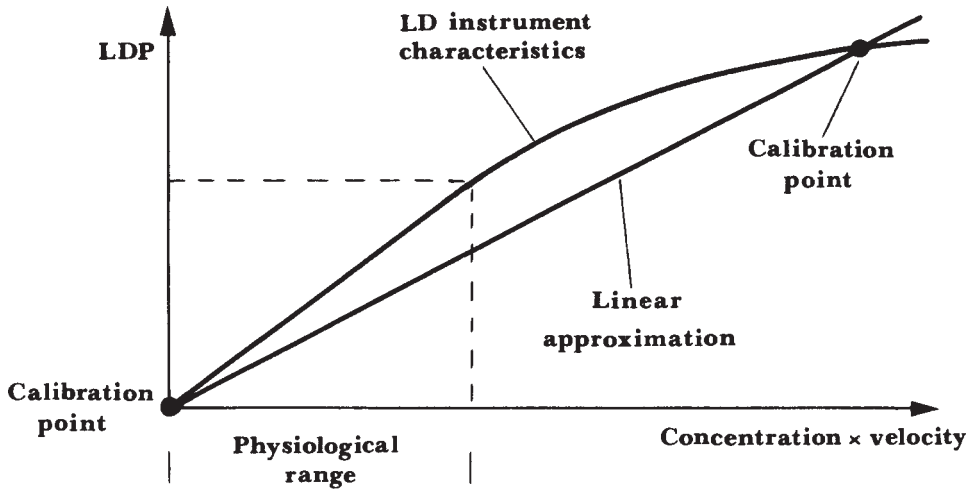

Fig. 2. Representation of the two point calibration procedure presently used.

3.6. New calibration standard

The ideal calibration standard, which will exactly simulate the tissue, does not exist and cannot be simulated either. A good substitute for tissue, to be used as a calibration device should meet following criteria:

- Easy to be used by the instrument operator, who normally will have a clinical background;
- Durable over years and completely sealed;
- Accurate, depending upon the field of application of the instrument;
- Inexpensive, i.e., much cheaper than the instrument itself;
- Having the opportunity to check linearity;
- Stable, no drift, over at least 24 hours.

All manufacturers are interested in such a common standard becoming available within the next two or three years. Recently, connected to the Biomed-I project, some attempts to improve the calibration procedure have been started:

- A new calibration standard for laser-Doppler perfusion has been developed using commercially available materials by Liebert et al. [27]. This standard consists of a porous material (Vyon) containing a suspension of polystyrene microspheres. The mixture of static and dynamic scatterers is expected to produce a Doppler spectrum similar to that of tissue. It was concluded that adding a matrix of static scattering particles to the motility standard does improve the applicability of the standard.
- Another approach was undertaken by de Mul et al. [28]. They developed a standard consisting of a set of layers containing scattering and/or absorbing particles, and either at rest or moving with respect to each other. This approach has the advantage of absolute reproducibility and of predictability using Monte Carlo simulations of the Doppler light scattering process.
- An optoelectronic calibration procedure was proposed by Boggett (Moor Instruments Ltd.) and also tested by Liebert and Maniewski in Warsaw. The method is based on light impulses of various frequencies and amplitudes transmitted into the detector of LD instrument by the receiving fibre.

Table 1
Comparison of the manufacturers instruments

Manufacturer	Instrument model	Wavelength	Filtering	Motility standard: Particle size
Moor Instrument	MBF3, DRT4, MULTI4, FLOLAB	780 nm	Dig. 20 Hz–3.1 kHz; 14.9–22.5 kHz	0.5 μm
		780 nm	Dig. 20 Hz–14.9 kHz	
Oxford Optronix	Oxford Array	780 nm	Anal. 10 Hz–20 kHz	0.78 μm
Perimed	PF4001	780 nm	Anal. 20 Hz–12 kHz	0.3 μm
	PF1, PF2, PF2B, PF3	632 nm	20 Hz–4 kHz–12 kHz	

The procedure can be useful for simple testing and adjusting of the instrument (signal processing unit) neglecting the laser source effects.

– Steenbergen and de Mul have prepared a system with thin plastic-like layers containing scatterers. This system of scatterers moving independently at different depths should elucidate the response of the LDPM to cell motion at various depths.

These approaches were started within the framework of the Biomed-I CA project and are interrelated. The work in this field will continue.

3.7. Temporary solutions

While waiting for the new standards to be developed, in the mean time some temporary solutions have to be found:

3.7.1. Conversion factors

A comparison between the different manufacturers' instruments will at present be only approximate. Although the various instruments are calibrated using the same motility standard, they will show differences when measuring the same tissue. This is caused by the fact that the present calibration is a one-point calibration only. Besides that, the signal processing is different (Table 1).

3.7.2. The standard test probe: "Europrobe"

Standardisation of probe geometry is an essential prerequisite for comparison of results. To enable comparison of clinical protocols it was agreed to develop a common probe design. This probe is not intended to be superior in any sense to other existing designs, but its use will remove the influence of one of the variables which complicates a sound interpretation and inter-comparison of clinical results and signal analysis.

The probe to be developed has been designated: "Europrobe". Further research to determine the optimum probe design characteristics for particular applications is necessary.

The Europrobe will be available from all manufacturers, with the following specification (preliminary):

– the probe will be comprised of only two optical fibres, one emitting and one receiving, or equivalent,
– the diameter of the emitter and receiver fibre will be 125 ± 5 μm, including cladding $140(+2-5)$ μm and buffer diameter 0.25 mm,
– the centre-to-centre distance between the fibres will be 0.250 mm ($\pm$10%),
– fibre type: Ensign Bickford code HCP-M0125 T-12 or equivalent.

The perfusion units as read by the Moor instruments and the Perimed PF4001 instrument are similar. Using the Europrobe, similar results will be obtained, provided the instruments are properly calibrated (according to the Instructions). An analogue similarity holds for the other Perimed instruments, except for the different wavelength.

4. Limitations and perspectives

In order to be widely clinically accepted the laser-Doppler method of perfusion measurement must be proven to have diagnostic value.

This can be achieved as long as the normal action and functions of the microcirculation are not ignored. There are very many parameters which affect both the actual and measured blood perfusion, which must be borne in mind when using a LDPM. The effects of such parameters must be limited or at least accounted for before the results become useful. It is interesting to note that many of the 'limitations' of the technique described in the literature relate to the physiology and environment of the measuring site rather than the instrument. From this point of view there are two separate issues. Firstly, it is impossible to say what is the 'correct' blood flow for a particular piece of tissue, especially in the skin. Secondly, we do not know the optical properties or microvascular architecture (geometry) in advance. There are, however, a number of specifically technical limitations which are most appropriate to this paper. These include:

- motion artefact noise;
- multiple Doppler shifting;
- variations in instrument specifications;
- lack of quantitative units and knowledge of depth of measurement;
- instrument zero/Biological zero.

The measurand (i.e., microvascular blood flow) is poorly predictable at any given tissue site and time. The assessment of sufficiency of perfusion must, therefore, be based on its variation in response to an appropriate physiological stimulus. Furthermore, the clinician is often unaware of limitations of the technique and clear guidance can only be given with reference to individual application areas (cf. report of the clinical groups). Diagnostic differences in microcirculatory response have been consistently reported where a ratiometric (% change) analysis has been employed. This invariably involves the comparison of a stimulated value to a resting level. However, even in this comparison there will be some dependence on spatial variation. This is best dealt with by moving the probe and repeating the experiment a number of times, taking the mean value.

4.1. Motion artefact

Clinical trials exposed the problem of motion artefact noise which occurs when the modes in which light passes through the fibre are disturbed by movement of the cable, resulting in small losses of light from the core into the cladding. It can also occur when the probe head moves relative to the tissue. This phenomenon manifests itself in intensity fluctuations at the photodetector similar to the laser-Doppler beat frequency. One analytical study demonstrated that the range of frequencies produced is 0 to 3.5 kHz [29] and the magnitude can be much larger than the perfusion related Doppler signal. This problem was accentuated by the fact that the larger-core fibres translated the movement to the tissue, giving rise to tissue-related Doppler-shifting.

Technological improvements have facilitated the use smaller fibre-optic cables, a wider range of probes encompassing new application areas and less noisy semiconductor laser diodes. This led to an increased number of instruments being made available throughout the ’80s. Smaller diameter fibres reduce sensitivity to motion artefact in three ways.

– Firstly the smaller core produces fewer modes.
– Secondly, the optical beating will be more efficient if there is a reduced spread of paths travelled by the light collected.
– Finally, less of the movement will be transmitted to the probe head.

However, it is still desirable to avoid unnecessary movement. Where motion is likely, any averaging of the output such as response time settings should be kept at or below 0.2 s. Longer times would make it difficult to identify artefacts which may manifest as apparent increases in flow. The use of laser diodes in LDPMs was reported by de Mul et al. [30] with the diode laser source and detection integrated in the probe head. This eliminated the need for fibre-optic cables and is insensitive to noise contributions from voluntary motion.

4.2. Multiple Doppler shifting

The majority of applications of the laser-Doppler perfusion technique have been in the skin where the blood volume fraction is of the order of 1%. There are many applications, e.g., [31] in which the technique is used to probe internal organs (e.g., liver and kidney) with much higher blood volume fractions and this leads to an increased likelihood that the photons backscattered from these tissues will have been scattered from more than one moving blood cell. In this case some part of the light will have multiple Doppler shifts in frequency. Larger blood volumes may also lead to homodyne mixing because a large proportion of the light will have undergone frequency shifts. As the processor is designed to interpret heterodyne mixing and single Doppler shifting, it may produce an inaccurate result, since its response to such flow will be non-linear. This issue was addressed by Bonner and Nossal [10]. Some processors (e.g., Vasamedics, USA) have attempted to detect and correct for such cases, although the effectiveness of such attempts is not well-tried. In general a degree of scepticism is required in the interpretation of results from such organs until further research is done in this area.

4.3. Lack of quantitative units and knowledge of depth of measurement

Currently available LDPMs cannot express the measurement in absolute units because of the enormous inter- and intra-individual variation in microvascular architecture, physiology, and optical properties. We expect the maximum sampling depth to be the radius of the hemisphere describing the tissue volume from which returned light is captured. It is difficult, however, to define the measuring volume and the locus of flowing blood within this volume, both of which are specific to the site. As we saw in Section 2 there is a dependence between the sensitivity and the position of the moving blood cell. In the present system neither the measuring depth/volume nor the sampling depth/volume are known on-line. At least one of these is a prerequisite for a quantitative perfusion output. It is, of course, possible to calibrate any of the instruments for a particular site on a particular organ, but such a calibration is only valid if the optical properties remain constant – i.e., without moving the probe. In general then, it is best to interpret the results in terms of a ratiometric (% change) analysis – cf. contribution of the clinical group.

One of the major limitations of the laser-Doppler perfusion technique is the lack of knowledge of and control over the mean sampling depth (and hence volume) in skin tissue. To measure changes in nutritional blood supply we ideally wish to confine our measurement to the papillary loops, lying underneath the epidermis [32]. The epidermis varies in depth at different areas of skin from 17–485 μm [33]. Recent attempts to vary the sampling depth have included:

(a) 'twin wavelength laser systems', e.g., [20,34–36],
(b) varying the distance between the source and the detector on the skin surface [18].

In the first case variation of the measurand (i.e., RBC flux, and hence volume changes) will vary the sampling depth. Also it relies on short-wavelength lasers which are bulky, expensive and difficult to stabilise [34]. Sampling depth in the second method is also dependent on the scattering properties of the site. Neither of these methods have potential to provide the user with continuous adjustment/assessment of the sampling/measuring depth. Further work is required to address these issues.

Although the depth of measurement is of fundamental importance to any interpretation of results, it is often overlooked because of the difficulty in defining the locus of measurement. In clinical papers it is generally dealt with by saying that the 'laser-Doppler perfusion measurements are made within a hemisphere of approximately 1 mm radius'. Although this hand-waving statement is in general true, there are two fundamental problems with it. Firstly, the actual depth measured is dependant on the source wavelength and probe used as well as the optical and anatomical properties of the site. Secondly, it is not easy to determine whether the measured perfusion relates to nutritional, thermoregulatory (largely associated with shunting of blood through the arterio-venous anastomoses) or both types of flow.

4.4. Instrument zero/biological zero

When occluding the blood flow by for instance inflating a pressure cuff positioned around the upper arm while measuring the tissue perfusion in, e.g., the forearm skin, the laser-Doppler perfusion monitor output signal does not reach the absolute zero level but stabilises at the so called biological zero level. By convention, this biological zero level has generally been taken as the zero perfusion level from which the actual perfusion is calculated. Studies of the origin of this biological zero level, point to the fact that the residual movement of the arrested blood cells constitutes a major component of the biological zero [37] but movements of other aggregates in the tissue matrix may also contribute to a varying degree, depending on the circumstances under which the recordings are performed. Further studies of the origin of the biological zero level recorded under different conditions are needed in order to ascertain its origin. Only when the origin of the biological zero level has been identified, the issue as to whether this biological zero level should or should not be subtracted from the recorded perfusion value in order to give an accurate measure of the tissue perfusion, can be resolved. Since this is a most important practical issue, scientific studies of the biological zero under healthy and pathological conditions are urgently needed.

Comment: A better understanding of the origin of the biological zero is of special importance at recordings of low and ischaemic perfusion levels, in which cases the biological zero constitutes a significant fraction of the total laser-Doppler perfusion monitor output signal. In these cases the magnitude of the recorded perfusion level and the following therapy-related decision making may be dependent on whether this biological zero level is subtracted or not from the laser-Doppler perfusion monitor output signal. Inflating the pressure cuff as quickly as practicable is recommended to ensure that arterial and venous occlusion occur at approximately the same time so that the blood volume in the tissue is similar to that during normal conditions.

4.5. Basic nature of the microcirculation

When using the laser-Doppler perfusion monitor, repetitive recordings of skin perfusion generally do not result in identical perfusion values. The technology is therefore often said to be non-reproducible. Recording the mobility of scatterers from a calibration standard or from a physical model with a carefully controlled flow, however, yields highly reproducible results. The non-reproducibility therefore cannot be explained by instrumentation related factors. The tissue perfusion, however, possesses both a temporal and a spatial variation [38,39] implying that the recorded perfusion values cannot be expected to be constant either in time or space.

4.6. Temporal variations

The temporal variation is composed partly of a deterministic signal (vasomotion and heart synchronous variation), partly of a non-deterministic signal (slow drifts and stochastic changes). This is the common nature of any tissue perfusion. There is recent evidence that the absence or presence of specific waves in the perfusion signal relates to the influence of a specific disease on the microvascular perfusion [40, 41]. The perfusion signal is, however, non-stationary and spectral analysis must be carried out with this in mind.

4.7. Tissue perfusion heterogeneity

The spatial variation is explained by the fact that if the probe is positioned over an area with an underlying small artery, a high perfusion value is generally recorded [42]. If the probe is positioned over an area with only capillary vessels, the signal is normally much smaller. By using the laser-Doppler perfusion imager [43], the spatial perfusion pattern has been proved to be consistent and the spatial variation from a high to a low perfusion area occurs over a scale of typically a few millimetres. In order to be able to record reproducible values at repetitive measurements with the laser-Doppler perfusion monitor, the use of an integrating probe [44] is recommended when information about the average perfusion value over a certain tissue area is more important than the very local readings attained by most standard probes.

5. Instrumentation

5.1. Laser-Doppler Perfusion Monitor (LDPM)

5.1.1. Basic configuration

A laser-Doppler perfusion monitor typically comprises a laser (He-Ne or solid state) which delivers the light to the tissue (directly or via a fibre optic probe), a single or a pair of photodetector(s) and an analyzing circuit which provides an electrical output signal that scales linearly with the perfusion. This signal is presented on an analogue or digital display or fed to a pen recorder. The probe of the LDPM device is generally attached to the skin by a double adhesive tape. Most units are portable and can be brought to the patient for bed-side monitoring of tissue perfusion.

5.1.2. Processor and transfer function

Laser-Doppler signal processors in commercial instruments all have a linear relationship to blood flow in *in vitro* models. Where the red He-Ne/diode laser is used the bandwidth is generally chosen to be approx. 12 kHz, whereas for the near infrared diode lasers a larger bandwidth is preferred (see Section 3.4). This is to account for the higher shift frequencies from faster moving RBCs encountered at the larger depths penetrated by NIR light, even though the Doppler equation predicts an inverse relationship between frequency shift and wavelength. The filter characteristics can be verified by sweeping the post-preamplifier input with a frequency generator, from 0–30 kHz. The angle of incidence can be assumed random in a highly scattering medium with RBC's moving in all directions. The transfer function of the processor in general use is described in a rigorous theoretical analysis of Doppler shift scattering from RBC's in tissue by Bonner and Nossal [10] and given as:

$$LDP = CF\left(\int_{\omega_1}^{\omega_2} \omega P(\omega)\,\mathrm{d}\omega - \int_{\omega_1}^{\omega_2} \omega N(\omega)\,\mathrm{d}\omega\right)\Big/ I^2, \tag{5.1}$$

where *CF* is a calibration factor, $P(\omega)$ is the power spectral density of the Doppler shift frequencies ($\omega = 2\pi f$) and $N(\omega)$ is the power spectral density of the shot noise at intensity I. The frequency (ω)-weighted filter can be implemented in analogue or digital electronics. The noise correction and normalisation is usually performed after the slope and offset of the noise/intensity relationship are found by varying the light intensity backscattered from static objects. The photodetector shot noise has a linear variation with intensity as expected and the correction can be made on-line at all light levels.

Bonner and Nossal showed that it should give an output independently linear with RMS velocity and number of red cells in the measuring volume. This algorithm is preferred by most researchers and manufacturers, partly because of its strong theoretical foundation, but also for its improved sensitivity at low flow rates. The model can be intuitively understood by neglecting noise and normalisation and rewriting the first moment of the power spectral density thus:

$$\int_{\omega_1}^{\omega_2} \omega P(\omega)\,\mathrm{d}\omega = \langle\omega\rangle \int_{\omega_1}^{\omega_2} P(\omega)\,\mathrm{d}\omega, \tag{5.2}$$

where $\langle\omega\rangle$, the mean Doppler shift frequency, is, from the Doppler Eq. (2.3) proportional to the mean velocity and the integral is the sum of the Doppler shifted photons (proportional to the total number of RBC's). Therefore the product is proportional to flow of blood cells. Of course the validity of the latter component is dependent on the assumption that each detected photon has undergone at most one Doppler shift. De Mul et al. [28] and Nilsson et al. [19] have argued that calculation of both the weighted and unweighted moments of the power spectrum can yield the three related parameters; speed, concentration and perfusion. However, it must be kept in mind that the estimation of concentration based on the unweighted first moment is only valid at low concentrations where heterodyne detection and single Doppler-shifting predominate.

Other methods of normalisation may be at least as useful. For example, some researchers (e.g., de Mul and co-workers) advocate the use of the unweighted moment, i.e., $\int_{\omega_1}^{\omega_2} P(\omega)\,\mathrm{d}\omega$. Such a method has the advantage of limiting the normalisation to the Doppler-shifted component of the light reaching the detector and hence predominantly from blood cells.

5.1.3. State of the art

The rapid advance in recent years in the area of microelectronics and fibre optic technology have had a big impact in the field of non-invasive monitoring. In many cases it has brought clinical acceptability to instruments which might otherwise be confined to research. The application of digital technology can enhance the reproducibility and user-friendliness of the instrument. Apart from the inherent flexibility, the main advantage of this technology is the elimination of voltage offsets which plague analogue processing circuitry especially for multiply and differentiate-type operations. Similarly, laser-Doppler instruments based on digital technology are available (e.g., Vasamedics, USA) and many analogue based instruments now use PCs and specific software for convenient presentation of results.

5.2. Laser-Doppler perfusion imaging

5.2.1. Principle

The laser-Doppler perfusion imager [45] makes possible the creation of a perfusion map of a predetermined tissue area by successively scanning the laser beam from one measurement point to the next while the backscattered Doppler broadened light is detected by a remote photo detector. When a scanning procedure is completed, typically several thousand perfusion values have been collected. These perfusion values together constitute a two-dimensional map of the tissue perfusion and is displayed on a computer monitor. The laser-Doppler perfusion imager is a new device and it is currently being tested in many laboratories all over the world. One result of these test procedures points to the fact that more powerful image processing software packages for analysis of the images are required in some applications. In addition, a patient administrative data base that handles the images and makes editing as well as merging of the images with other clinical parameters of interest possible is under development. In order to make these image processing software packages and patient administrative data base more universally adaptable, specifications and conceptual ideas from users of the system in various countries throughout Europe are required.

5.2.2. Applications

Although in its infancy, laser-Doppler perfusion imaging has been used to map and evaluate the spatial distribution of tissue blood perfusion in association with the axon reflex [46], during application of microdialysis [47] and in evaluation of port wine stain perfusion [48]. Other investigators have used the laser-Doppler imager to study skin reactions induced by tuberculin [49] as well as hand perfusion in normal subjects and patients suffering from scleroderma [50]. Lam and Ferrell [51] examined the spatial blood perfusion in the rat knee joint capsule whereas Hermansson et al. [52] used laser-Doppler perfusion imaging to receive an immediate indication of the perfusion status in the myocardium during by-pass surgery.

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