THE DESIGN, DEVELOPMENT AND APPLICATION OF A DUAL-BEAM SPECTRAL–DOMAIN OPTICAL COHERENCE TOMOGRAPHY SYSTEM FOR THE NON-INVASIVE ASSESSMENT OF VASCULAR DYNAMICS BY STATISTICAL MEANS

A thesis presented to the University of Limerick in partial fulfillment of the requirements of the degree of Doctor of Philosophy

by

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Abstract

‘Where blood does not flow, life does not go.’

This assertion underscores the importance of haematological dynamic assessment, which can benefit greatly from non-invasive high-resolution imaging of blood flow especially at the microvascular level. This importance is further highlighted in oncological applications with recent emphasis on tumour vasculature, angiogenic processes and developments of anti-angiogenic therapies.

Optical coherence tomography (OCT) has established itself a firm foothold in the realm of non-invasive optical medical diagnostic imaging, enabling in vivo cross-sectional tomographic visualisation of the internal microstructure of biological systems, and is now considered an optical analogue to CT or MRI, but with microscopic resolution. The original concept of OCT was to enable non-invasive real-time in situ imaging of tissue microstructure with a resolution approaching that of histology, but without the need for tissue excision and processing; i.e. an optical biopsy. Doppler OCT has been the predominant force for the quantification of moving particles within media. However, despite the advancements of DOCT techniques, phase shift assessment of velocity values requires that the angle between the incident light source and the vessel in question be known a priori. Due to the extensive tortuosity of the microvasculature, the Doppler angular dependency may lead to incomplete vascular maps in vivo. In an effort to surmount the restrictions imposed by angular uncertainties, the in-house cross-correlation dual-beam SdOCT (db-SdOCT) system presented here operates by quasi-simultaneous illumination and measurement of two distinct planes; this forms a miniature time-gate. By analysis of light intensity fluctuations at two points a known distance apart, transit times may be deduced via temporal cross-correlation, thereby yielding velocity values irrespective of vessel tortuosity. This technique eliminates the need for phase sensitive detection and instead utilises the temporally evolving phase itself as a metric for quantifying velocity by statistical means.

Cross-correlation db-SdOCT analysis creates a variable velocity measurement range and can be set based on the flexibility of its parameters i.e. acquisition and beam separation distance. In general, the preliminary in vitro results obtained indicated a tentative first step in developing a robust tool for flow velocity quantification by means of cross-correlation. However, in order to investigate the applicability of the method for in vivo studies, a full characterisation of the system was performed and is outlined in terms of beam separation, functional extensions (axial profiling, discerning flow direction, turbulence investigations and pulsatility studies), and optimisation of the optics involved. Endoscopic OCT offers a means of obtaining high-resolution and high-speed depth-resolved visualisation of deep tissue structures in vivo. With a view to future potential applications of the db-SdOCT system and algorithm for intravital exploratory applications, a macro-model of a side-view endoscopic device was constructed to investigate the efficacy of this simplified approach. The capabilities of in vivo assessment were examined in the analysis of the nailfold plexus. The resulting velocity, directionality and axial profiling computation of the constituent capillaries have shown the capability of the db-SdOCT method in such environments.

This work chronicles the design, development and implementation of a db-SdOCT system for velocity assessment by cross-correlation analysis and its outlined functional extensions provide a multi-faceted modality for in vivo research. Adaptation of this method into existing OCT regimes is straightforward and cost-effective, providing a means of dynamic assessment, free from angularly induced artifacts.
I hereby declare that this submission is my own work and contains no material which has been submitted to any other University or higher education institution or for any academic award in this University. Where use has been made of the work of other people, it has been fully acknowledged and referenced accordingly.

Signed: ___________________________  Date: ______/_____/______

Susan McElligott-Daly
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"Great wits are sure to madness near allied,
And thin partitions do their bounds divide."

– from Absalom and Achitophel by John Dryden

The relationship one shares with their PhD and their emotional state in the pursuit of such is certainly a complex one. The study of which would be PhD worthy in itself. 'Love-hate', as one of my colleagues likes to put it. This seemingly unobtainable singular goal and all of its associated troubles and sleepless nights, coupled with momentary and fleeting instances of elation, makes one wonder why this endeavour is worthwhile. That is something that is learned at the very end. True passion, desire and determination: these are the fundamental requisites. Although the PhD process is certainly a solitary one, looking back it is humbling to think of all of those who contributed to its completion, but received no piece of the π(e):

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**Figure 90** In pulsatile FFT analysis, an evident error increase in the location of the FFT maximum occurred with decreasing velocity values. The above plots reveal the % error obtained for the maximal correlation frequency and the simulated applied pulse for (a) intensity and (b) phase -based correlation analysis. The errors obtained were more prominent for larger capillary sizes at lower flow rates. (A 2nd order polynomial fit is shown for clarity.)

**Figure 91:** In pulsatile FFT analysis, an increased error in the location of the FFT maximum occurred with decreasing velocity values. Data shows a 500 μm capillary with flowing 2% Intralipid solution at 9 mm/s at (top) 80 bpm and (bottom) 90 bpm, representing a respective deviation of 4.31% and 2.14% from the simulated pulse frequency (intensity-based cross-correlation velocity data).

**Figure 92:** Temporal cross-correlation maps resulting from analysis of a 50 μm capillary with flowing 2% Intralipid at 5 mm/s with simulated pulse at (left) 60 bpm and (right) 100 bpm. The increased pulse frequency resulted in a more turbulent correlation map.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aGM</td>
<td>angular control galvo mirror arm</td>
</tr>
<tr>
<td>AP</td>
<td>atherosclerotic plaque</td>
</tr>
<tr>
<td>AVA</td>
<td>arteriovenous anastomosis</td>
</tr>
<tr>
<td>cm-OCT</td>
<td>correlation mapping optical coherence tomography</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>db-SdOCT</td>
<td>dual-beam spectral-domain optical coherence tomography</td>
</tr>
<tr>
<td>DOCT</td>
<td>Doppler optical coherence tomography</td>
</tr>
<tr>
<td>DOF</td>
<td>depth of field</td>
</tr>
<tr>
<td>FCM</td>
<td>flow cytometry</td>
</tr>
<tr>
<td>FCS</td>
<td>fluorescence correlation spectroscopy</td>
</tr>
<tr>
<td>FdOCT</td>
<td>Fourier-domain optical coherence tomography</td>
</tr>
<tr>
<td>GRIN</td>
<td>gradient-index</td>
</tr>
<tr>
<td>IVUS</td>
<td>intravascular ultrasonography</td>
</tr>
<tr>
<td>LASCA</td>
<td>laser speckle contrast analysis</td>
</tr>
<tr>
<td>LDF</td>
<td>laser Doppler Flowmetry</td>
</tr>
<tr>
<td>LDPI</td>
<td>laser Doppler Perfusion Imaging</td>
</tr>
<tr>
<td>LDPM</td>
<td>laser Doppler Perfusion Monitoring</td>
</tr>
<tr>
<td>MRI(A)</td>
<td>magnetic resonance imaging (angiography)</td>
</tr>
<tr>
<td>MTF</td>
<td>modulation transfer function</td>
</tr>
<tr>
<td>NA</td>
<td>numerical aperture</td>
</tr>
<tr>
<td>OCT(M)</td>
<td>optical coherence tomography (microscopy)</td>
</tr>
<tr>
<td>OMAG</td>
<td>optical micro-angiography</td>
</tr>
<tr>
<td>OPT</td>
<td>optical projection tomography</td>
</tr>
<tr>
<td>PAT(M)</td>
<td>photoacoustic tomography (microscopy)</td>
</tr>
<tr>
<td>PCI</td>
<td>partial coherence interferometry</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>PIV</td>
<td>particle image velocimetry</td>
</tr>
<tr>
<td>PrDOCT</td>
<td>phase-resolved Doppler optical coherence tomography</td>
</tr>
<tr>
<td>PS</td>
<td>polarisation spectroscopy</td>
</tr>
<tr>
<td>PsOCT</td>
<td>polarisation-sensitive optical coherence tomography</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood corpuscle</td>
</tr>
<tr>
<td>RBC(_V)</td>
<td>red blood corpuscle velocity</td>
</tr>
<tr>
<td>Re</td>
<td>Reynolds number</td>
</tr>
<tr>
<td>SdOCT</td>
<td>spectral-domain optical coherence tomography</td>
</tr>
<tr>
<td>sGM</td>
<td>2-D raster scanning galvo mirror system</td>
</tr>
<tr>
<td>SLED</td>
<td>superluminescent diode light source</td>
</tr>
<tr>
<td>SLIC</td>
<td>scanning laser image correlation</td>
</tr>
<tr>
<td>SOCT</td>
<td>spectroscopic optical coherence tomography</td>
</tr>
<tr>
<td>SsOCT</td>
<td>swept-source optical coherence tomography</td>
</tr>
<tr>
<td>STED</td>
<td>stimulated emission depletion</td>
</tr>
<tr>
<td>StFT</td>
<td>short time Fourier transform</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>STICS</td>
<td>spatio-temporal image correlation spectroscopy</td>
</tr>
<tr>
<td>SV</td>
<td>speckle variance</td>
</tr>
<tr>
<td>TdOCT</td>
<td>time-domain optical coherence tomography</td>
</tr>
<tr>
<td>UHR OCT</td>
<td>ultrahigh resolution optical coherence tomography</td>
</tr>
<tr>
<td>UHS-OMAG</td>
<td>ultrahigh-sensitive optical microangiography</td>
</tr>
<tr>
<td>US</td>
<td>ultrasound</td>
</tr>
<tr>
<td>USn</td>
<td>ultrasonic</td>
</tr>
<tr>
<td>VDR</td>
<td>velocity dynamic range</td>
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1. Introduction

The present chapter will provide a number of examples of imaging techniques aimed at providing both qualitative and quantitative metrics of morphological and functional information. The first section will focus on providing a framework of the structural and functional aspects of the (micro-) vasculature and will review the ailments associated with vascular irregularities and/or deformities. Furthermore, the inherent need for non-invasive imaging modalities in modern medicine is discussed, in addition to the advantageous aspects of their use in a clinical setting.

1.1 The Skin

1.1.1 Strata morphology

In the skin, the structure of vessels is arranged into superficial and deep horizontal plexuses [1] and serves a number of vital functions such as nutritional support for tissues and homeostasis [2, 3]. Biological tissues are absorbing media and are optically inhomogeneous [4]. Light microscopy and direct visual inspection of the skin is hampered by scattering, regular surficial reflections and absorption by superficial chromophores.

![Figure 1: Illustration of dermal and epidermal strata and the arrangement of blood vessels in the skin. Adapted from [5].](image)

Light interaction with a multilayer and multicomponent substance such as skin is a highly complicated process [4, 6].

Light propagation within tissues depends on the scattering and absorbing of its cells and the size, shape and density of these structures, relative refractive indices and polarisation states [7, 8]. For instance, the stratum corneum layer of the skin reflects 5-7% of incident
light and a collimated beam may be transformed into a diffuse beam by interfacial microscopic inhomogeneities. A collimated beam attenuated in a thin tissue layer of thickness $d$, may be described by the Bouguer-Beer-Lambert exponential law [9]:

$$I(d) = (1 - R_F)I_0 e^{-\mu d}$$  \hspace{1cm} (1)

where $I_0$ is the incident light intensity; $\mu = \mu_a + \mu_s$, extinction or total attenuation coefficient (see §1.3.1 and §1.3.2); and $R_F$, coefficient of Fresnel reflection, where at normal incidence:

$$R_F = \left[\frac{\eta - 1}{\eta + 1}\right]^2$$  \hspace{1cm} (2)

where $\eta$ is the relative mean refractive index of tissue and surrounding media.

At a microscopic scale, tissue components have no pronounced boundaries and appear to merge into a continuous structure with spatial variations in the refractive index. Quantitative models [10] assist in the comprehension of such complex morphologies by predicting the absolute magnitudes of optical coefficients, and of wavelength and angular dependencies. One such model is the discrete particle model of tissue [4, 11].

### 1.1.2 Vascular system

The *Ebers Papyrus*, an ancient Egyptian medical papyrus (16th BCE), outlined some of the earliest known acknowledgement of the connection between the heart to the surrounding arterial vessels. The Arabian physician, Ibn al-Nafis, provided the first accurate description of the process of pulmonary circulation [12], and did so more than 2700 years after the initial accounts of scientific thought on the subject by the Egyptians. It was not until the 17th CE that William Harvey deduced that the beating heart produced a continuous circulation through minute connections at the extremities of the body [13] (the Greek anatomist Erasistratus had previously postulated the existence of capillaries, 4th BCE). However, it is the Italian physician Marcello Malpighi who is credited with the discovery of the capillary interconnects between the arterial and venous vasculature, and ever since insights into the biophysical mechanisms that govern the haemodynamics of the microcirculation have been pursued.

The main constituents of blood are the liquid component ‘plasma’ and blood cells. White blood cells (or leukocytes) play an active part in the defence system of the body. Red blood corpuscles (RBCs) are specialised to transport respiratory gases. They are roughly 8
μm in diameter at their widest point with a volume of approximately 90 μm³ [4], are biconcave shaped and possess no nucleus [14, 15] (implying that the cell cannot repair itself and has limited lifespan). They produce one protein, the oxygen-carrying molecule haemoglobin and are one of the main chromophores (i.e. absorbers of light).

Microcirculation, the distal functional unit of the cardiovascular system, is the movement of blood or lymph through the vasculature via arterioles, venules, capillaries and arteriovenous anastomoses (AVAs) [2, 16]. AVAs serve as low resistance channels for large volumes of blood to pass directly from arterioles to venules (i.e. bypasses the capillaries) [2] and play an important role in the thermoregulation of the body [17]. Assessment of AVAs in the acral regions of the body has shown them to be implicated in diabetes, where the volume of blood transferred by AVAs increases and is thought to be the cause of neuropathic ulcerations [18] (see §1.4).

![Figure 2](https://via.placeholder.com/150)

**Figure 2**: (a) Illustration of the microcirculation including arterioles, capillaries and venules [15], displaying the extensive branching of capillaries; (b) Scanning electron microscopic image of the vasa vasorum of rat carotid sinus, showing the organised arrangement of vessels [19].

Capillaries and the smallest venules are formed from a single layer of endothelial cells supported externally by a collagen rich basal laminar layer [14]. Blood perfusion through the capillaries serves several key functions within the body including regulation of blood pressure, supplying oxygen and nutrients to tissue, and removal of carbon dioxide and waste metabolites [20]. The heart is unique among organs in the cardiovascular system in that coronary arterial flow is exclusively diastolic and venous outflow is systolic. Furthermore, coronary vessels function as a highly organised hierarchical system of microvessel flow regulators matching local blood flows with myocardial energy demands to support the viability of the heart [21]. The transport of blood is by no means trivial [2]; complex boundary conditions, pulsatile flow patterns, and deformable, elastic vessel walls all interact with this non-Newtonian fluid, thus increasing the complexity of analysis.
The scale of the cardiovascular system geometry spans several orders of magnitude (see Table 1), from the size of a minute capillary to the aorta (approximate diameter, 2 cm). In addition, these characteristic lengths are not consistent throughout the entire body; capillaries found in the skin have comparatively thicker walls (2-3 μm) [22] when compared with the rest of the body (0.1 μm) [15]. Furthermore, the type of flow experienced in these vessels varies greatly from strongly pulsatile (e.g. in arteries) to almost stationary.

Table 1: Reference values for mean velocity ($\bar{v}$), diameter ($d$), Reynolds number (Re) and Womersley number ($\alpha$); adapted from [23, 24].

<table>
<thead>
<tr>
<th>Vessel Type</th>
<th>$\bar{v}$ (m s⁻¹)</th>
<th>$d$ (mm)</th>
<th>Re (-)</th>
<th>$\alpha$ (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artery</td>
<td>0.45</td>
<td>4</td>
<td>450</td>
<td>0.4</td>
</tr>
<tr>
<td>Arteriole</td>
<td>0.05</td>
<td>0.05</td>
<td>0.5</td>
<td>0.05</td>
</tr>
<tr>
<td>Capillary</td>
<td>0.001</td>
<td>0.008</td>
<td>0.002</td>
<td>0.1</td>
</tr>
<tr>
<td>Venule</td>
<td>0.002</td>
<td>0.02</td>
<td>0.01</td>
<td>0.2</td>
</tr>
<tr>
<td>Vein</td>
<td>0.1</td>
<td>5</td>
<td>125</td>
<td>50</td>
</tr>
</tbody>
</table>

Physiological blood flow velocities range from $10^{-6}$ to $10^{-2}$ m s⁻¹ and can reach $> 1$ m s⁻¹ in the major vessels [25].

Flow is characterised by two dimensionless quantities: the Reynolds number ($Re$) and the Womersley number ($\alpha$), defined as:

$$Re = \frac{d\bar{v}\rho}{\theta} \quad \quad \alpha = d \sqrt{\frac{2\pi f\rho}{\theta}}$$

where $d$ is the vessel diameter; $\bar{v}$, bulk velocity; $\rho$, density; $\theta$, dynamic viscosity; and $f$ is the characteristic frequency. Generally, Reynolds numbers < 2300 imply laminar flow rates (turbulent flow patterns, $Re > 4000$), whereas for low Womersley values, pulsatile flow is dominated by viscous effects [23].

1.2 Motivation for optical imaging techniques

Direct visualisation of tissue anatomy and physiology provides physicians with important information for diagnosis and disease management. Non-invasive modalities capable of high spatial resolution imaging of structure and blood flow dynamics could have great value for biomedical research and clinical diagnostics.

The analysis of light interactions with biological tissue facilitates a non-invasive morphological and functional ‘optical biopsy’ [26], both of which are of outward importance diagnostically and prognostically. A biopsy of this form may be juxtaposed to its core or excisional biopsy precursor, wherein the procedure involves the surgical removal of a
sampling of cells or tissues for examination *via* microscopy or chemical analysis. The medical removal of tissue from a living subject to determine the presence or extent of a disease is a commonplace procedure in clinical practice.

In addition to gleaning structural data, vascular imaging makes it possible to quantify the number and spacing of blood vessels, assess blood flow and permeability [27, 28], analyse cellular and molecular abnormalities, and vessel wall shear stresses [23]. Structural and functional parameters may be elucidated by methods ranging from fluorescence to confocal and multiphoton microscopy. Magnetic resonance imaging, computed tomography, positron emission tomography, ultrasonography and optical imaging have been shown to provide non-invasive, functionally relevant images of angiogenesis* in animals and humans [19, 29-33]. The principles upon which such methods are based vary widely, as does the suitability (e.g. resolution, imaging depth, etc.) and the relative technology and costs involved [34].

Great attention has been paid in the past two decades to the measurement of flow velocity on the micrometer scale and many techniques for assessing the blood supply have been investigated. This is borne out of recognition of the vitally important role that blood flow plays in the health of the individual. Blood flow detection conveys diagnostic significance in many pathological conditions and potentially in the monitoring of therapeutic interventions [35]. There are a myriad of (biological) reasons for this importance; for example, the onset of atheroma, a degeneration of the arterial wall which leads to arteriosclerosis or ‘artery hardening’ [36, 37] manifests in disruption of localised flow patterns. In particular, there is growing clinical evidence that goal-directed intervention based on functional capillary density and heterogeneity of microcirculatory blood flow, in critically ill patients with septic and cardiogenic shock, led to improved outcomes [38]. However, the clinical applicability of optical methods is not solely confined to assessment of localised tissue regions. For instance, full-field skin perfusion techniques can assess the extent and aid in the diagnosis of burn depth [23, 39, 40] by visualising the velocity distribution as a scalar velocity map. Assessing flow pattern disturbances is not of interest uniquely to the biomedical community; similar methods have been used in the field of chemical analysis, for example, in efforts to improve fluidic system performance [41].

There appears to be an ever-increasing emphasis placed upon knowledge of precise distribution of flow velocities and the relative dynamics. To meet this need, a wide variety of methods aimed towards measuring values of flow velocity within media have come into

* Angiogenesis: the formation and development of blood vessels
existence. However, every technique contains limitations or restrictions which must be minimised or compensated for in order to utilise the technique in a practical manner (see Figure 3). In general, microscopic methods provide the highest resolution in terms of preserved tissue specimens, whereas clinical methods provide images of living tissues deep within the body, but at much lower resolution and specificity [19]. These constraints throw down the gauntlet of developing new imaging methods that can bridge this resolution dilemma.

![Figure 3](image)

**Figure 3:** Illustration of the relative domains occupied by various imaging modalities in terms of resolution and sampling depth. DOT: Diffuse Optical Tomography; CM: Confocal Microscopy; TiVi: Tissue Viability; LSPI: Laser Speckle Perfusion Imaging.

Non-invasive *in vivo* imaging modalities have obvious advantages for the clinical realm; noteworthy are their ability to provide clinically relevant information without disturbing the normal biological environment. Methods which would allow the routine clinical assessment and monitoring at the microcirculatory level could greatly improve early detection and improve patient recovery rate. Furthermore, *ex vivo* tissue is not representative of the natural biological environment. Thus, non-invasive optical imaging techniques have filled this niche, garnering a reputation for fulfilling patient comfort during clinical assessment. Nonetheless, difficulties remain in obtaining accurate data in an environment which is subject to such large biological variability.
1.3 The physics and origins of biological light-matter interactions

As light travels through different media, its speed can change. The refractive index ($\eta$) of a medium is a dimensionless constant which is a measure of the speed that light will travel in a medium of different optical density ($\nu$) compared with that of a vacuum ($c$):

$$\eta = \frac{c}{\nu}$$  \hspace{0.5cm} (4)

When a beam of light is incident on a boundary between two media of different refractive index, the direction of the incident beam changes; this is termed refraction. The optical path length ($\Omega$) travelled in a medium is therefore dependent on $\eta$:

$$\Omega = \eta d$$  \hspace{0.5cm} (5)

where $d$ is the geometrical length of the light path.

Biological tissues are optically inhomogeneous and absorbing media whose average refractive index is higher than that of air. As such, partial reflection of the radiation at the tissue/air interface (Fresnel reflection) occurs, while the remaining portion penetrates the tissue. Absorption (mechanical or chemical) of a photon can elevate a molecular electron from the ground state to an excited state, which is termed excitation. Relaxation of the excited electron may impart luminescence (i.e. another photon – fluorescence/phosphorescence) or heat; otherwise it is referred to as non-radiative relaxation.

Light propagation within tissue is dependent on the scattering and absorption properties of its components; the size, shape, density, refractive index and polarising abilities of these structures all play important in the function of light in tissues [42]. The diffusion-like behaviour of light in biological tissue presents a key challenge for optical imaging. Most tissues are composed of structures on a wide range of sizes and can be described as a random continuum of inhomogeneities of the refractive index with a varying spatial scale [4]. The contribution of spatial variations in the refractive index of cells and other tissue components to the scattering properties of tissue can be estimated theoretically and experimentally. Quantitative models that relate the microscopic properties of cells and other tissue components should be able to predict the absolute magnitudes of the optical scattering coefficients as well as their wavelengths and angular dependencies [11]. At the microscopic scale, tissue components have no pronounced boundaries and appear to merge into a continuous structure with spatial variations in refractive index. Such a complex arrangement requires description via a statistical model. Another approach considers tissue as a discrete
system of scattering particles. The first theoretical models were based on single-scattering theory [43], but were restricted to the superficial layers of the skin. At larger probing depths, however, light is subject to multiple scattering. The effects of multiple scattering have been investigated on the basis of solving the radiative transfer equation in small-angle (diffusion) approximations [44], on models based on the extended Huygens-Fresnel principle [45] and for Monte Carlo simulations [46].

The refractive indices of tissue structure elements can be derived using the law of Gladstone and Dale [47], which states that the resulting value represents an average of the refractive indices of the components related to their volume fractions:

$$\bar{n} = \sum_{i=1}^{N} \eta_i f_i$$  \hspace{1cm} (6)

where $\eta_i$ is the refractive index and $f_i$ is the volume fraction of the individual components; $\sum f_i = 1$; and $N$ is the number of components. The refractive index of a particle can be defined as the sum of the background index and the mean index variation:

$$\bar{n}_s = \bar{n}_0 + \langle \Delta \eta \rangle$$  \hspace{1cm} (7)

The spatial organisation of particles forming tissue plays a substantial role in the propagation of light; for example, in very small packing densities situations, incoherent scattering by independent particles occurs. Light scattering of particles composing tissue (or a phantom) are modelled exactly by Mie theory, which reduces to the simpler Rayleigh theory if the spherical particle is much smaller than the impinging light wavelength [48, 49].

### 1.3.1 Scattering

For a single scatterer, the scattering cross section ($\sigma_s$) is related to its geometric cross-sectional area ($\sigma_g$) through its scattering efficiency ($Q_s$), i.e. $\sigma_s = Q_s \sigma_g$. In a medium containing a density $N_s$ of scatterers, the scattering coefficient can be considered as the total cross-sectional area for scattering per unit volume:

$$\mu_s = N_s \sigma_s$$  \hspace{1cm} (8)

where $\mu_s$, the scattering coefficient, is defined as the probability of photon scattering per unit length in a medium; 100 cm$^{-1}$ is a representative value in biological tissue [4].

Most biological tissues are characterised by strong optical scattering and are therefore referred to as either scattering or turbid media. Optical scattering originates from light interaction with biological structures, which range from cell membranes to whole cells.
Photons are scattered most strongly by a structure whose size matches the optical wavelength and whose refractive index mismatches the surrounding medium. The extinction coefficient ($\mu_t$), is given by:

$$\mu_t = \mu_a + \mu_s$$  \hspace{1cm} (9)

where $(\mu_t)^{-1}$ is the mean free path between interaction events; and $\mu_a$ is the absorption coefficient (see §1.3.2). The probability that a photon incident on a small volume element will survive is equal to the ratio of the scattering and extinction cross-sections; it is referred to as the *albedo* ($\Lambda$) for single scattering:

$$\Lambda = \frac{\sigma_s}{\sigma_t} = \frac{\mu_s}{\mu_t}$$  \hspace{1cm} (10)

which ranges from zero for a completely absorbing medium, to unity for a completely scattering medium.

The phase function $p(\vec{s}, \vec{s}' )$ describes the scattering properties of a medium and is the probability density function for photon scattering in the direction $\vec{s}'$, originally travelling in the direction $\vec{s}$. If scattering is symmetric relative to the direction of the incident wave, then the phase function is dependent on the scattering angle, $\theta$, only:

$$p(\vec{s}, \vec{s}' ) = p(\theta)$$  \hspace{1cm} (11)

The phase is usually approximated with the aid of the Henyey-Greenstein function [50]:

$$p(\theta) = \frac{1}{4\pi} \frac{1-g^2}{(1+g^2-2g\cos(\theta))^{3/2}}$$  \hspace{1cm} (12)

where $g$ is the scattering anisotropy parameter:

$$g \equiv \langle \cos(\theta) \rangle = \int_0^\pi p(\theta) \cos(\theta) 2\pi \sin(\theta) d\theta$$  \hspace{1cm} (13)

g = 0 corresponds to isotropic (Rayleigh) scattering; $g = 1$ corresponds to total forward (Mie) scattering; and $g = -1$ corresponds to total backward scattering.

### 1.3.2 Absorption

For a single absorber, the absorption cross section ($\sigma_a$) is related to its geometric cross-sectional area ($\sigma_g$) through its absorption efficiency ($Q_a$), i.e. $\sigma_a = Q_a \sigma_g$. In a medium containing a density $N_a$ of absorbers, the absorption coefficient can be considered as the total cross-sectional area for absorption per unit volume:

$$\mu_a = N_a \sigma_a$$  \hspace{1cm} (14)
where \( \mu_a \), the absorption coefficient, is defined as the probability of photon absorption per unit length in a medium; 0.1 cm\(^{-1} \) is a representative value in biological tissue [4]. Light attenuates as it propagates in an absorbing-only medium according to the following expression:

\[
\frac{dl}{l} = -\mu_a \, dx \tag{15}
\]

where \( l \) denotes the light intensity and \( x \) denotes the distance along the light propagation direction; see also Eq. 1. Transmittance is defined by:

\[
T(x) = \frac{I(x)}{I_0} \tag{16}
\]

which represents the probability of survival after propagation \( x \). Optical absorption in biological tissue originates primarily from the presence of haemoglobin, melanin and water.

1.4 The clinical implications of blood flow assessment

Aberrant microcirculatory morphology and haemodynamics are signatures of many human diseases such as cancer, macular degeneration, and many others. The skin is easily accessible and any compromise in nutritional support [51] and/or impairment in tissue viability can lead to disease manifestation [52]. Skin blood flow measurements are used to study clinical conditions which either intrinsically affect skin blood [22] or where measurement of skin blood flow is impeded by the underlying pathology. Assessment of skin structure and function can provide evidence of a disorder, the dysfunction extent, serve as an early indication of disease onset, or as a means of post-operative assessment, e.g. skin flap viability following plastic surgery [51].

A brief list of ailments and the respective techniques used in their detection and identification are outlined on Table 2. Although the quoted imaging techniques do not proffer a solution to such ailments, their clinical value and the justification for their use may be understood from their potential to characterise disease progression and determine response to therapy, enabling timely modifications to therapeutic strategies. This is particularly pertinent from the perspective of preventative medicine and patient management, as the manifestation of vascular aberrations can occur initially in the peripheral areas of the body prior to clinical suspicion. Such early diagnoses and subsequent early intervention may offer the patient a decreased degree of suffering, a welcome measure for those who suffer habitually from such ailments.
Table 2: Compilation of ailments which manifest in the acral regions and the respective technique used in their detection. OPS, Orthogonal Polarisation Spectral; SDF, Sidestream Dark Field; LDF, Laser Doppler Flowmetry; PAOD, Peripheral Arterial Obliterative Disease [34, 53-70].

<table>
<thead>
<tr>
<th>Ailment</th>
<th>Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systemic Lupus Erythematosus</td>
<td>Wide-field microscopy</td>
</tr>
<tr>
<td>Osteoarthritis</td>
<td>Capillaroscopy</td>
</tr>
<tr>
<td>Scleroderma</td>
<td>OPS imaging</td>
</tr>
<tr>
<td>Reflex Sympathetic Dystrophy</td>
<td>SDF Illumination</td>
</tr>
<tr>
<td>Acrocyanosis</td>
<td>Fluorescence videomicroscopy</td>
</tr>
<tr>
<td>Polymyositis</td>
<td>LDF</td>
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<tr>
<td>Rheumatoid Arthritis</td>
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<tr>
<td>Diabetes mellitus</td>
<td></td>
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<tr>
<td>Migraine</td>
<td></td>
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<tr>
<td>Antiphospholipid Syndrome</td>
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<tr>
<td>Hypertension</td>
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<tr>
<td>Acromegaly</td>
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<tr>
<td>Pre-eclampsia</td>
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<tr>
<td>Menopause</td>
<td></td>
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<tr>
<td>General microcirculation</td>
<td></td>
</tr>
<tr>
<td>Mixed Cryoglobulinaemia</td>
<td></td>
</tr>
<tr>
<td>Psoriasis &amp; Psoriatic Arthritis</td>
<td></td>
</tr>
<tr>
<td>Raynaud's Phenomenon</td>
<td></td>
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<tr>
<td>Dermatomyositis</td>
<td></td>
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<tr>
<td>(mixed) Connective tissue disease</td>
<td></td>
</tr>
<tr>
<td>Sjögren's syndrome</td>
<td></td>
</tr>
<tr>
<td>PAOD</td>
<td></td>
</tr>
<tr>
<td>Systemic Sclerosis</td>
<td></td>
</tr>
<tr>
<td>Rheumatoid vasculitis</td>
<td></td>
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<tr>
<td>Paediatric rheumatism</td>
<td></td>
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2 A review of pioneering methods towards dynamic data

The present chapter will serve as an historical review of pioneering methods of imaging, utilising both exogenous and endogenous contrast mechanisms. A summary of up-to-date high resolution optical sectioning techniques is provided and the differing means by which each yields dynamic data. In addition, an examination of current correlation based mechanisms, capable of yielding information pertaining to both the static and dynamic aspects of the vasculature through statistical means is presented.

2.1 Exogenous contrast mechanisms

2.1.1 Radionuclide techniques

Historically, the first means of measuring flow and perfusion were performed through invasive procedures. Examples of such procedures include direct measurements of capillary blood pressure [71], transcutaneous oxygen measurements [22, 72] and radionuclide techniques. Xenon clearance is the ‘gold standard’ choice of radionuclide and subcutaneous injections of such are used to evaluate skin blood flow [73, 74] by measurement of the rate of isotope clearance (activity) from the injected area by the local blood supply [75]. Tissue blood flow \( (Q) \) may be calculated from the half-life \( (t_{1/2}) \) of the isotopic tracer involved [22]:

\[
    Q = 100 \ln(2) \frac{\lambda}{t_{1/2}}
\]

(17)

Although many other tracers have been used for these purposes (e.g. \(^{22}\)Na, \(^{24}\)Na, \(^{125}\)I), difficulties arise with regards to the choice of tracer as each has a different degree of diffusibility upon injection. The choice of an appropriate tracer and use of a suitable detector is crucial for measurement success, in addition to assessment of skin type for biological variation. The measurements of blood flow by these methods have been proven to be disadvantageous, however, by being very much a point-by-point measurement modality whose results may not bear any relationship to regional blood flow metrics. A poor choice in measurement site would therefore lead to inaccurate results.
Indocyanine (ICG) fluorescence method is a widely used infrared optical imaging technique superior to fluorescein angiography [76] which has been used to monitor blood flow in various clinical imaging applications. The ICG dye is intravenously introduced to the patient and upon accumulation in the location of interest, it is laser excited and the resulting fluorescence is detected by a CCD camera. ICG fluorescence analysis has been employed in numerous environments, for example, in the assessment of burn depth [77], intraoperative skin flap viability [76] and the patency of coronary artery bypass grafts [78]. The feasibility of the detection of exogenous chromophores in the adult human brain has been demonstrated. ICG contrast agents have been used in various oncological studies, i.e. the detection of carcinogenic cells with a sensitivity of $1:10^7$ in the background of normal cells [79] and in the analysis of lymph flow in breast cancer patients [80].

2.1.2 Thromodilution

Cardiac output (CO) is regarded one of the most important haemodynamic variables for the assessment of cardiac function [81] and therapeutic guidance in the intensive care setting. Thromodilution is a method of measuring blood flow based on the premise that when an indicator substance is added to circulating blood, the rate of blood flow is inversely proportional to the indicator concentration change over time. A fluid miscible with blood (e.g. a dextrose or saline solution) and at a lower temperature is injected into the coronary sinus at a constant rate and the resulting change in temperature is detected a short distance downstream [82]. The cold fluid mixes with blood in the right heart chambers, is ejected into the pulmonary artery and flows past a thermistor positioned on the distal end of the catheter which records temporal changes in blood temperature. CO is determined from the following equation [83]:

$$CO = \frac{V_i(T_b-T_i)K_1K_2}{\int_0^\infty \Delta T_b(t)dt}$$

where $V_i$ is injectate volume; $T_b$ and $T_i$ are the blood and injectate temperatures, respectively; $K_1$ is a density factor; and $K_2$ is a computation constant.

The method of thromodilution is gainful from the perspective that it permits measurements to be repeated in short intervals and allows rapid changes in coronary circulation to be studied. Detrimental and possibly harmful aspects of the method include the requirement of catheterisation of the coronary sinus and the introduction of relatively large amounts of fluid. Additionally, CO determination by thromodilution may be unreliable or
impossible in patients with low CO states [84], and studies have shown that the thermodilution method overestimates true cardiac output in the low output range [85].

2.1.3 Iontophoresis

Iontophoresis is defined as the facilitatory motion of ions across a membrane, permitting the transdermal delivery of vasoactive drugs [2, 86]. The technique examines the relationship between pharmacological provocation and physiological responses of the vascular bed. Active agents capable of holding an electric charge are applied topically to the skin in a gel form. An electrode, oppositely charged with respect to the active agent, repels the ions down towards an anode/cathode via an electrical gradient, thus penetrating the skin. As the skin generally possesses a net negative charge, positively charged drugs are generally easier to deliver [18]. Although the iontophoretic method does not cause localised pain or swelling, it is a time consuming exercise and must be repeated at regular intervals.

2.2 Endogenous contrast mechanisms

2.2.1 Thermography

In thermography, two-dimensional (2-D) maps of radiation differences are constructed of regional temperature distributions across the skin and are used as a direct measure of blood perfusion [22, 87]. There are contact and non-contact thermographic devices; the former utilises liquid crystal technology directly on the skin surface, and the latter employs the infra-red (IR) portion of the electromagnetic spectrum. The energy flux emitted by the skin is given by Stefan-Boltzmann’s law:

\[ R = \theta_s T_s^4 \]  

where \( \theta_s \) is the Stefan-Boltzmann constant and \( T_s \) is the temperature at the skin surface. Although thermography has good spatial resolution and is relatively easy to use [88], it provides details of surface temperature only and has poor responsiveness. However, microwave radiometry can penetrate to subcutaneous tissue depth (1-2 cm) and has been used in studies to assess peripheral vascular disease [89].
Figure 4: Thermograms of the right hand of a healthy control (left) and a patient with systemic sclerosis (right). A temperature gradient persists irrespective of ambient temperature, suggesting an underlying vascular disease [90].

2.2.2 Laser Doppler imaging

A system composed of both stationary and mobile scatterers is illuminated with coherent light; some photons undergo a frequency change. This phenomenon is known as the Doppler Effect, named for its discoverer, Austrian physicist Christian Johann Doppler. The effect occurs when photons are scattered from particles which are moving and the change in frequency is determined by the frequency of the emitting source and the velocity relative to the observer. As the reflected portion of the incident beam is found to be spectrally broadened [91, 92], this frequency shift can be used to determine the velocity of the moving object. Laser Doppler Flowmetry (LDF) refers to a general class of non-invasive techniques which utilise the optical Doppler effect to measure changes in microcirculatory blood perfusion for both in vitro [93] and in vivo [94] purposes. Perfusion is defined as the product of the local speed and concentration of blood cells [8, 95]

Within the vasculature, dynamic components (such as RBCs) will undergo a frequency shift whereas the static constituents will not. These frequency shifts are allowed to impinge on the surface of a photodetector, producing beat frequencies from which it is possible to determine the perfusion of the moving components within the tissue volume in question. The scattering angle, the wavelength of the light in tissue ($\lambda_o$) and the velocity of the moving scattering object ($\vec{v}_s$) determine the frequency shift produced by Doppler scattering; see Figure 5.

Figure 5: Illustration of single Doppler shift event; $\theta$, scattering angle; $\phi$, azimuthal angle.
To derive this frequency shift, if an incident photon is described by the propagation vector $\vec{k}_i$ and is scattered by a moving particle in the direction $\vec{k}_s$, the scattering vector $\vec{q}$ (invariable with elastic scattering) is given by:

$$\vec{q} = \vec{k}_i - \vec{k}_s = 2k \sin(\frac{\theta}{2})$$  \hspace{1cm} (20)

The Doppler frequency shift ($f_{DS}$) for a single scattering event may be given by [4]:

$$f_{DS} = \frac{2v_s n \cos\theta}{\lambda_0}$$  \hspace{1cm} (21)

In a system of moving particles such as RBCs in tissue, a more realistic scenario would involve a single photon undergoing multiple Doppler-shifts. In this case $f_{DS}$ would be the sum of the individual scattering events. The velocity of RBCs in cutaneous circulation is of the order of mm s$^{-1}$ and correspondingly produces a Doppler shift of several kHz. Since this is very small, optical heterodyning techniques are utilised to obtain Doppler shifts [22]. By analysing the spectral content change of scattered light, LDF can detect flow velocities from 0.06 to over 1 mm s$^{-1}$ [25, 96]. However, multiple scattering of photons in tissue makes it extremely difficult to determine the scattering angle of each photon-RBC interaction and thus cannot provide an absolute measurement of blood cell velocity. In addition, strong scattering coupled with an inability to discriminate depth severely limits the spatial resolution obtained in non-transparent biological tissues, often preventing flow analysis in specific small vessels of interest [96].

**Figure 6**: Perfusion images of the finger obtained before, during, and after occlusion of the upper arm using LDPI (64 × 64 pixels) [97].

Laser Doppler Perfusion Monitoring (LDPM) and Laser Doppler Perfusion Imaging (LDPI) were developed for point-wise monitoring and imaging of skin perfusion, respectively. LDPI techniques have good spatial resolution due to their wide field of vision and ability to record fluctuation patterns across a large area of the skin [1]. The first portable clinical instrument was developed by Holloway and Watkins although it had some practical
limitations due to poor signal to noise ratio [22, 98]. Although Laser Doppler technology has many potential applications, it has yet to become fully integrated into a clinical setting [99]. Angular limitations, the stochastic nature of the Doppler signal from a highly scattering medium [100, 101], phase wrapping, and interferometric fringe washout effects, prevent accurate assessment of the detected Doppler signal [102] causing measurement ambiguities as well as loss of information. Additionally, disturbances of the fluid refractive index resulting from large temperature fluctuations have caused the incident laser beam to wander and defocus [103]. In spite of this, LDPI technology has been demonstrated and used in the assessment of a plethora of clinical situations; for instance, assessment of burn extent [39, 40] and relative perfusion [8, 104, 105], post-operative malignant skin tumour assessment [106], and in the study of arthritis and joint inflammation [107].

2.2.3 Laser Speckle imaging

Image speckle refers to the pattern observed on a surface as a result of random coherent light interference [108]. As outlined in §2.2.2, analysis of the Doppler shift of the photon or the distribution of Doppler shifts from a distribution of photons, enables the discernment of the dynamics of scattering particles. In vivo experiments have shown a strong correlation between Doppler and speckle modalities [109, 110]. A speckle pattern is formed either by the reflection of coherent light from a rough surface, or by transmitting the light through a transparent medium having a randomly fluctuating refractive index distribution and by subsequent 3-D multiple beam interference [111]. The speckle pattern is a granular variation of light intensity (see Figure 7) obtained from any rough surface such as skin and is characterised by a random intensity distribution that may be described by statistical means [34, 112].

Scattering inhomogeneities within the skin can be considered as point sources and small differences in distances traversed, to the photodiode detector used, result in dark and bright speckles dispersed at all points in space. This motion results in a speckle pattern that appears decorrelated or ‘blurred’ [113]. The amount of decorrelation depends on the speed and volume of the scatterers in the tissue. To quantify the blurring of the speckles, the speckle contrast is computed as the ratio [114, 115]:

\[ K_s = \frac{\sigma_s}{\langle I \rangle} \leq 1 \quad (22) \]

where \( \sigma_s \) is the standard deviation; and \( \langle I \rangle \) is the mean intensity. A fluctuation in light intensity is recorded each time a scatterer moves through a distance of 0.5\( \lambda \). Determinations
of velocity may be obtained by temporally assessing the statistical behaviour of speckles. The time-varying component of speckle may be quantified by comparisons of like pixel intensities recorded during successive scans of the subject, thus forming 2-D maps of flow across the region of interest. A successful approach was developed using laser speckle contrast analysis (LASCA), which assumes that (considering a Lorentzian flow profile) the speckle contrast could be related to correlation time $\tau_c$ (and hence RBC$_V$) [114, 116]:

$$K = \beta^{0.5} \left\{ \frac{\tau_c}{T} + \frac{\tau_c^2}{2T^2} \left[ \exp\left( -\frac{2\tau_c}{T} \right) - 1 \right] \right\}^{0.5}$$

(23)

where $\beta^\dagger$ accounts for loss of correlation related to the ratio of the detector size to the speckle size and polarisation; and $T$ is the exposure time of the camera.

In full-field measurements, tissue is illuminated by an expanded laser beam and the resulting image speckle is recorded by a CCD camera.

Speckle techniques have the advantage of being able to acquire images in real-time, making it possible to measure blood flow response to occlusion and hyperaemia, for instance. Although specular reflection effects may be disregarded with the addition of polarising filters, static tissue reduces the signal-to-noise (SNR) ratio [118-120]. These factors contribute to the confinement of the speckle technique to superficial measurements, with deeper measurements requiring the necessity of being performed on surgically exposed tissue.

Progress has been made in establishing a flow decorrelation time using laser speckle contrast techniques [121]; however, issues still remain of how to relate this time constant to the flow velocity [122]. Although accurate measurements of absolute RBC$_V$ cannot be

If the source is polarized and the detector is not, then $\beta = 0.5$.
calculated, perfusion changes can be successively monitored and tracked. Laser speckle imaging has been demonstrated on the rodent skin fold model [123, 124], assessed the vasodilation responses of intestinal tissues [125], analysed retinal blood flow and cerebral perfusion [126-129], and stroke [114].

![Figure 8: (Left) Photograph of a free flap on the face after surgery; (Right) Laser Doppler image of the same area, showing much lower perfusion in the flap than in the surrounding tissue. Adapted from [108].](image)

2.2.4 Polarisation spectroscopy

If polyvinyl alcohol (PVA) chains are stretched, they form an array of aligned, linear molecules known as a polariser. This device is capable of changing the exact form that light, which passes through, may take, i.e. its *state of polarization*. Polarisers come in many different configurations, capable of circular or elliptical polarisation. However, if an iodine dopant is attached to the PVA molecules, this makes these chains conducting along their length and thus light polarised parallel to these chains is absorbed whilst perpendicularly polarised light is transmitted (a linear polariser). The technique of polarisation spectroscopy (PS) makes use of this phenomenon for imaging purposes by gating photons returning from different skin tissue compartments [130].

Light from the superficial skin layers can be differentiated from light backscattered from the dermal tissue matrix by use of simple polarisation filters. When monochromatic or white incoherent light is linearly polarised by a filter and is subsequently incident on the skin surface, 5% of the light is specularly reflected by Fresnel reflection as surface glare and a further 2% is reflected from the superficial *stratum corneum* layers. These two light fractions retain their original polarisation state, dictated by the orientation of the first polariser (see Figure 9). The remaining portion penetrates through the epidermal layers, where it is absorbed or backreflected. The backscattered portion is exponentially depolarised by scattering [131] and tissue birefringence [132]; it has been suggested that more than 10 scattering events are required to sufficiently depolarise light [133, 134]. Light remerging from tissue structure is now diffusely reflected, consisting of equal (approximately 22%) divisions of parallel and perpendicular polarisations with respect to the original filter
direction. By placing another polarising filter over the detector, surficial [135] and subdermal [136] light fractions (i.e. co-polarised (CO) and cross-polarised (CR)) may be discriminated.

Figure 9: Illustration of the operating principle of PS using polarisation gating method. Polarisation filters (1,2) are arranged so that their pass-directions are perpendicular, thus creating a cross-polarised image [137].

Image processing of PS techniques employ an algorithm which generates a normalised difference between CO and CR images [34]:

\[ P = \frac{CO-CR}{CO+CR} \]  (24)

where \( P \) represents a composite image observing surface histology. The denominator cancels any non-uniform illumination. Using polarisation imaging and Kubelka-Munk theories, an algorithm was developed [138] to produce an output variable (\( TiVi_{index} \)) capable of relaying information regarding RBC concentration:

\[ TiVi_{index} = \frac{M_{red}-M_{green}}{M_{red}} \exp \left[ -p \frac{M_{red}-M_{green}}{M_{red}} \right] \]  (25)

where \( M_{red} \) and \( M_{green} \) represent the red and green image colour planes; and \( p \) is an empirical factor which produces the best linear fit between \( TiVi_{index} \) and RBC concentration. The technique has high temporal and spatial aspects, with lateral resolution estimated at 50 \( \mu \)m [137].

\( P \) images have been shown to be comparable to histopathology examination, the invasive ‘gold standard’ of skin examination [34]. Polarisation filtering has been applied in specular reflection elimination in LSI [113] and in LDPI, which otherwise would have resulted in an overestimation of readings [139]; to provide chemical signatures to generate Raman spectra of the superficial layers [140]; and it has been applied to studies of burn depth [34, 39, 40, 141, 142]. The PS method has some limitations, however. A prominent error relates to the
measurement inaccuracies obtained on persons with darker skin types due to the high absorption of melanin present. Nonetheless, owing to its low cost, ease-of-use and portability, PS imaging systems are an attractive alternative for imaging purposes.

2.2.5 Side-stream dark-field illumination

The use of green light ensures optimal physiological optical absorption by the haemoglobin in the RBCs (independent of oxygenation state) with respect to the lack of absorption by the tissue embedding the microcirculation [61]. The use of green light illumination thus acts as a contrast mechanism, where RBCs are visualised as dark moving globules and tissue is visualised as white/gray. This was previously discussed in § 2.2.4 where computation of a $TiVi_{index}$ value relays information regarding RBC concentration. Side-stream dark field illumination imaging was introduced as the successor of Polarisation Spectroscopy [143].

![Figure 10](image)

**Figure 10:** (a) Hand-held SDF imaging device, imaging the tissue-embedded microcirculation [61]; (b) SDF image of the sublingual microcirculation (area = $0.98 \times 0.73 \text{ mm}^2$) [143].

The SDF imaging device consists of a central light guide, surrounded by concentrically placed light emitting diodes that emit green light. As in the case of PS imaging, backscattered (and thus depolarised) light is projected onto a CCD camera after it passes through an analyzer. Polarised light reflected by the tissue surface is not detected. Several shortcomings of SDF imaging have been reported [144]; the most prominent of which is the suboptimal imaging of the capillaries due to motion-induced image blurring. In order to overcome this motion-induced artifact, intravital stroboscopic illumination of the green light emitting diodes [61], i.e., providing pulsed illumination in synchrony with the CCD frame rate, (partially) prevents smearing of moving features and motion-induced blurring of capillaries due to the short illumination intervals.
OPS imaging has had an important clinical impact by observation of the microcirculation during sepsis, shock, and resuscitation [144-147]. In fact, results have shown that OPS observation of sublingual microcirculatory alterations provided more sensitive information about patient outcome from sepsis and shock than conventional clinical parameters. OPS imaging as well as SDF imaging are both validated techniques to investigate the microcirculation; reported investigations include that of the hamster dorsal nailfold [148], the human nailfold plexus [61, 149] (see §6.1), the liver [142] and the sublingual tissues [61].

2.2.6 Ultrasound

Ultrasound (US) imaging systems rely on the propagation of mechanical (ultrasonic) waves from a transducer into tissue and the subsequent interactions of these waves with mechanical discontinuities in the tissue. Upon interaction with these discontinuities, a small reflected ‘echo’ wave (scatter) is created and is detectable by the transmitting transducer. Payne et al. were the first to report on the topic of Doppler US and the potential the technique held for user defined preselection of measurement depths [150, 151]. The range, \( z \), of the scattering structure is determined by the time of flight of the US to and from the target,

\[
z = \frac{ct}{2}
\]  

where \( c \) is the speed of sound; and \( t \) is the time taken from initial emission to subsequent scatter remission. The frequency dependent attenuation of US within biological media limits the maximum depth to 300 wavelengths, suggesting that minimum wavelengths of 33 \( \mu \)m (45 MHz) to 100 \( \mu \)m (15 MHz) imaging 10 to 30 mm tissue depths, respectively, is appropriate for small animal studies.

US is a well established means of measuring blood velocity using the Doppler principle (see §2.2.2). Analogous to its optical counterpart, scattering of US waves from moving RBCs leads to a shift in the frequency relative to the initial sonification pulse. The expression for velocity of blood flow, \( \nu \), is given by:

\[
\nu = \frac{f_{DS} c}{2 f_0 \cos \theta}
\]  

where \( f_{DS} \) is the Doppler frequency shift, \( f_0 \), the sonification frequency, \( c \), the local speed of sound, and \( \theta \), the angle between the axis of beam propagation and the flow velocity vector.

Recent work has been undertaken to examine the potential of US Doppler findings of tumour stage and/or patient prognosis [152-154]. Doppler US using frequencies ranging
from 2-15 MHz is the most widely used clinical imaging modality for assessing the vasculature, typically targeting larger vessels such as the carotid. In practice, it remains difficult to use Doppler US at clinical frequencies (~10 MHz) to detect blood flow slower than a few cm s\(^{-1}\) [25]. It is, however, clear that these methods are sensitive only to large vessel flow, i.e. arterioles and venules larger than about 50 µm. Utilising shorter wavelengths in the development of high frequency micro-US [155, 156] has enabled the measurement of flow velocities as slow as 2 µm s\(^{-1}\). Additionally, these wavelengths may be focused tighter than US, facilitating the non-invasive imaging of microscopic structure and blood flow with improved spatial resolution.

![Figure 11: 3-D image of an experimental tumour growing in a mouse [157, 158].](image)

The intensity of scatter echoes depends on the reflective property of the tracers [23]. The introduction of US microbubble contrast agents (diameter less than 1-5 µm) has created a significant opportunity for visualisation of the microcirculation [159, 160] by enabling anatomical and functional aspects to be visualised and quantified. Due to these improvements in US imaging, the modality is now ripe to be exploited in a plethora of clinical applications. Efforts are underway to use the US technology in prostate imaging [161], neonatal imaging [162], skin and ocular imaging [163] and a wide range of intraluminal imaging applications [164]. The extensive applicability of US in the clinical domain may be seen from its qualitative and prognostic assessment of cardiovascular disease [165], developmental biology [166], musculoskeletal disease [167], arteriosclerosis [165] and a wide variety of other manifestations of human disease.

2.2.7 Magnetic resonance imaging

Magnetic Resonance Imaging (MRI) was first suggested by Lauterbur [168] and, independently, by Mansfield and Grannell [169]. Nuclei with an odd number of protons, neutrons or both, have an intrinsic nuclear spin [170, 171]. Ordinarily, nuclei spin with their axes randomly aligned. The potential energy of the magnetic momentum in a homogeneous external magnetic field depends on the direction of momentum. The magnetic momentum of
a proton (e.g. hydrogen atom) of low potential energy (i.e. parallel momentum and field vectors) can be flipped to a higher energy state (anti parallel vectors) by absorption of a photon of exactly the missing energy difference between these two states [23] – this is referred to as nuclear magnetic resonance. The nucleus reverts/realigns to its normal state via emission of a photon, whose frequency is dependent on the energy difference between the normal and excited spin states. It is this motion that is used to construct a 3-D image composed of information from the strata of the object; this depth information is obtained by transmitting multiple radio waves at differing frequencies in space [170].

![Figure 12: Maximum intensity projection of the whole data-set showing normal appearance of the head and neck arteries using contrast-enhanced MR-Angiography [172].](image)

Contrast agents [173] are frequently used in MRI studies as a means of improving the visibility of internal body structures by contrast enhancement, the most common of which are gadolinium-based [174]. As tissue is approximately diamagnetic, a paramagnetic vascular contrast agent would change the environment and alter the relaxation times of tissues and body cavities in which they are present. Barium sulphate is commonly used as a means of signal enhancement in the gastrointestinal tract, but natural products high in manganese (e.g. green tea) serve the same purpose [175].

In the infancy of the MRI modality, signals from dynamic tissue constituents (e.g. RBCs) were considered an insidious nuisance as it generated frequent artifacts which detracted from image quality. In due course, it was realised that these apparently errant signals could be exploited to generate diagnostic quality images of blood vessels. For example, phase contrast velocity MRI [176] estimates the phase shift of hydrogen nuclei as they pass through a magnetic field and this phase change is proportional to velocity [177]. Other methods of time-resolved contrast-enhanced functional assessment have since been introduced, for example, time-of-flight MR angiography (TOF MRA) [178] and contrast-enhanced MR-
angiography (CEMRA) [179]. Contrast-enhanced techniques such as MRI offer unrivalled spatial and temporal resolution, in addition to having good soft tissue contrast and depth of penetration without any loss in sensitivity or resolution [2, 180]. However, it is an expensive research tool in terms of initial purchase and subsequent maintenance, requiring experienced operators. Image rendering is very time consuming, implying the technique is more suited to recordings of steady state conditions rather than transient phenomena. Moreover, MRI has not been well validated in terms of the microcirculation [181]. Nevertheless, functional MRI has become a most important tool for cognitive research since the latter part of the 20th century, with some of its most important applications being found in oncological studies [182, 183], cardiac perfusion and monitoring [184, 185] and neurological imaging [186, 187].

2.2.8 Positron emission tomography

Functional imaging with positron-emitting isotopes was first proposed as an imaging technique that could offer greater sensitivity with single photon-emitting isotopes than conventional nuclear medicine techniques [188]. It was recognised that high-energy photons produced by the annihilation of positron-emitting isotopes could be used to describe the physiological distribution of ‘tagged’ chemical compounds [189]. Positron emission tomography (PET) is a nuclear medicine imaging technique which utilises exogenous and endogenous contrast mechanisms (i.e. ‘tracers’) to yield a 3-D image of intrinsic functional processes and/or changes of regional blood flow in various anatomic structures.

The PET process is multifaceted. Imaging begins with a biological molecule that carries a positron-emitting isotope (a ‘tracer’) injected into the patient. $^{15}$O, $^{13}$N, $^{11}$C and $^{18}$F are considered among the most important radionuclides for medical applications of PET [190]. Over time, this isotope accumulates in an area of the body for which the attached molecule has an affinity; e.g. $^{11}$C accumulates in the brain or tumours where glucose is the primary source of energy [189]. The radioactive nuclei decay by positron emission and the emitted positron undergoes annihilation with an electron from the surroundings. The energy associated with this process divides equally between two photons (i.e. high energy $\gamma$-rays) which fly from each other at a 180° angle. An array of detectors surrounding the patient senses the $\gamma$-ray radiation (provided the line of coincidence is correct) and distribution of the positron-emitting tracer is computed via tomographic reconstruction procedures from the recorded projection data.

PET images qualitatively assess sites of unusual tracer accumulations (tumour sites) and quantitatively measure the tracer uptake for an assessment of the staging of a disease [189].
However, the use of radionuclides is not only invasive, as outlined in §2.1.1, but the use of such may induce patient-specific complications. Furthermore, PET utilises ionising radiation in its operation, and resulting images tend to be of poor resolution (in the order of mm [191]) in comparison to other methods. In spite of this, the ability of PET to analyse disease progression is well documented, for example, in Alzheimer’s disease [192, 193] (see Figure 13); Parkinson’s disease [194, 195]; epilepsy [196-198]; visualisation of the heart [199] and afflictions such as coronary artery disease [200]. PET has also been shown to be effective in locating metastatic disease in the breast [191], lung [201] and lower gastrointestinal tract [202], in addition to distinguishing between both benign and malignant cancer [203, 204].

![Figure 13: PET image of the progressive reduction in glucose metabolism indicative of Alzheimer’s disease. Adapted from [192, 205].](image)

Micro positron emission tomography (micro-PET) is an advanced form of current PET technology [206]. It provides information pertaining to the function of living organisms with very high sensitivity, high signal-to-noise ratio, and requires minimal use of radioisotopes (e.g. nanoparticles) for detecting physiological characteristics. Nowadays, PET is always combined with CT to put the function in a structural context.

2.3 Optical sectioning techniques

2.3.1 Histological staining

An essential tool of biology and medicine, histology is the study of the anatomy of cells and tissues via examination by microscopy, subsequent to sectioning and appropriate staining. As biological tissue has little inherent contrast in either light or electron microscopy, staining is employed to provide contrast and to highlight particular features of interest. Micro-Computed Tomography is one of the most common microscopy methods which employs histology (see §2.3.2.3).

Tissue processing initially involves ethanol dehydration to remove water, addition of an infiltration agent (e.g. paraffin wax or a resin) which solidifies the medium and then
embedding in an external medium (e.g. agar, gelatine) to harden. The hardened blocks containing the tissue samples are then ready to be sectioned. Freezing a tissue section or applying chemical fixatives (e.g. formalin) can also preserve samples from degradation [207]. Depending on the section thicknesses involved, a steel or diamond knife mounted in a microtome is used to cut tissue into sections. Post-sectioning, specimens are mounted on a slide and treated with an appropriate stain. Haematoxylin and eosin is the most commonly used microscopy stain in histology and histopathology (see Figure 14). Different tissue classifications such as blood cells (connective tissue) and the endothelial lining of blood and lymphatic vessels are subject to selective staining [15].

![Figure 14: Haematoxylin and eosin stained sections (× 100) of a rodent dorsal skin flap [124].](image)

Processing of tissue samples in this manner can cause shrinkage and alterations in tissue pigments and structure, rendering it unrepresentative of in vivo morphology. Information on functional (perfused) capillary density is lost along with any dynamic data. Additionally, naturally occurring melanin within the skin can alter tissue appearance and obscure structures in histopathological examination [208]. However, live tissues may be stored indefinitely at room temperature, and nucleic acids may be recovered decades after fixation.

Given the extensive clinical reliance on the value of histopathological findings, the application of its use is very broad. Reported studies have shown it to be used in the quantitative analysis of cancer metastatic growth [209, 210] and in the detection of hypoxic cells [211]; it has provided contrast in ocular surgery [212]; qualitative assessment of its use in esophageal lesions has been reported [213]; and analysis of irregular pigmentation, for example, port wine stains [214-216]. Histology has also been employed for flow purposes, for example, in the observation of novel threadlike structures on organ surfaces [217] and has been used in conjunction with LSI (see §2.2.4) to visualise blood vessel density [124].
2.3.2 High-resolution optical sectioning methods

2.3.2.1 Optical projection tomography

Previously, the standard technique for mapping 3-D gene and protein expression patterns involved serial sectioning [218]. However, reconstruction of images by these means was not only time consuming but also induced distortion. The wide range of optical methods which exist at present are optimised for the differing levels of scattering of various sized specimens within tissue. Small, non-scattering samples (through which photons will travel with a ‘ballistic trajectory’) can be imaged with Optical Projection Tomography (OPT). OPT provides molecular specificity, resolution on the order of microns to tens of microns, and is suitable for imaging of transparent specimens whose thicknesses in the imaging direction lie in the 1-15 mm range [219]. With reference to Figure 3, OPT occupies the same imaging domain as that of Optical Coherence Tomography.

Figure 15: (a) Schematic of OPT microscopy setup [220]; (b) False-colour image of a mouse embryo, stained with alcian blue and then scanned by OPT [221].

OPT is capable of producing very high resolution images and reconstructions due to the optical clearing process prior to imaging [218]. The clearing process matches the index of refraction of the sample tissue with that of the surrounding medium and renders samples transparent [222]. The raw data obtained in OPT must be then be mathematically transformed to recreate the original object [220, 223]. OPT imaging is performed in both transmission and emission modes; transmission is used for studies of anatomy, whereas emission mode is used to image the distribution of fluorescently labelled samples. In transmission mode, a wide field (diffuse or non-diffuse) light source illuminates a sample which is angularly rotated stepwise; the light transmitted in this stepwise fashion is captured using a CCD microscope. Similarly, in emission mode, wide field illumination of an appropriate wavelength excites the same rotating sample and the emitted fluorescence is captured using in the same manner as in transmission mode.
Generally, transmission and emission modes are used concurrently to render fluorescent visualisations against a morphological backdrop. As is the case with many optical imaging techniques, tradeoffs exist in their operation in terms of resolution and depth of field. For instance, partially closing the iris of the system in both transmission and emission modes of OPT increases the depth of field of the system, thus degrading the image resolution [218]:

\[ DOF_{\text{max}} = \eta_{\text{bath}} \left( 1.305 \frac{\lambda}{NA^2} \right) \]  

(28)

where \( DOF_{\text{max}} \) is the maximum depth of field achievable by the system; \( NA \) is the system numerical aperture; \( \eta_{\text{bath}} \), the refractive index of the material in which samples are suspended. Organic solvents which act as index-matching agents are, however, generally toxic. Consequently, analysis of live specimens is restricted to those that are already adequately transparent, e.g. small embryonic tissue. Use of a wide-field CCD in OPT operation causes some of the pixel noise to be generated by multiply scattered photons that were emitted from regions of the tissue that do not lie on the intended projection for that pixel. Use of a laser scanning approach renders less noise at a given pixel, in a manner analogous to the use of laser scanning in confocal imaging [224].

In addition to its use in developmental biology, OPT microscopy has the potential for use in medical applications for which knowledge of the 3-D structure may be useful, but not readily accessible from histopathological sections (see §2.3.1). Given OPT’s versatility in its ability to simultaneously record both absorption and emission profiles, it is particularly useful in performing gene expression analysis [221, 224, 225].

OPT has been shown to reveal superb sectioning ability, useful for reconstructing vertebrate embryos and in the examination of the 3-D anatomy of developing organs [221, 225, 226]; modelling of early human brain development [227]; and in the 3-D imaging of isolated cell nuclei [228]. With regard to contributions this technique has made to the knowledge of flow and related dynamics, OPT has been shown to be applicable in studies of gas-solids flow to analyse particle concentration [229]; general flow rate analyses [230, 231]; cell tracing [232]; and the spatiotemporal analysis of zebrafish [233].

2.3.2.2 Stimulated emission depletion microscopy

Since the research of Abbé, it has been considered that the resolution limit of light microscopy based on focusing optics had been reached [234]. Einstein’s notion of de-exciting a quantum system by stimulated emission has not only facilitated the invention of the
laser but also of stimulated emission depletion (STED), a super-resolution microscopy technique which utilises the non-linear de-excitation of fluorescent dyes to overcome the resolution limit imposed by diffraction. This has resulted in resolution improvements by a factor of 10-12 [235-237] with a focal plane resolution greater than 6 nm achievable [238], surpassing that of current high-end x-ray microscopy [239].

The intensity distribution of the excitation light in the focal plane of the lens is determined by diffraction and described by the point-spread function (PSF) [240],

\[ h_{\text{exc}}(v) = \text{const}. \left| \frac{2J_1(v)}{v} \right|^2 \]  \hspace{1cm} (29)

where \( J_1 \) is the first-order Bessel function; \( v = \frac{2\pi r NA}{\lambda_{\text{exc}}} \); \( r \) is the distance from the focal point; \( NA \), numerical aperture; and \( \lambda_{\text{exc}} \) is the excitation wavelength. One possible way to reduce the spatial extent of \( h_{\text{exc}}(v) \) is to inhibit the fluorescence in its outer regions; this is equivalent to an increase in resolution [235]. Employment of an additional STED laser beam (see Figure 16(b, center)) inhibits fluorescence by being focused with small lateral offsets \( \pm \Delta v \) with respect to the excitation beam and overlaps with the excitation beam on either side. Thus, the STED beam depletes the excited state before fluorescence takes place via stimulated emission (see Figure 16(a)) and only the innermost region of \( h_{\text{exc}}(v) \) remains which contributes to the fluorescence signal (see Figure 16(b, right)).

![Figure 16](image)

**Figure 16:** (a) Energy diagram of spontaneous emission, non-radiative decay and stimulated emission processes for a typical four-level energy system [241]; (b) Measured focal spots for excitation (left: 470 nm; blue), STED (center: 603 nm, orange) and effective spot (right: 22 nm, green) [237].

The first experimental confirmation of the superior lateral resolution of this non-invasive STED technique was demonstrated by the separation of adjacent Pyridine nanocrystals [242] that are otherwise indiscernible.
Chromophores, such as haemoglobin, absorb but have undetectable fluorescence because the spontaneous emission is dominated by their fast non-radiative decay. STED competes effectively with this non-radioactive decay, providing a new contrast mechanism which has been investigated in the vascular network of a nude mouse ear [241]. In addition, STED has been implemented in nanofluidic studies [243, 244] (with reported spatial resolutions better than 70 nm) and in protein flow cytometry research [245].

![Figure 17: Maximum intensity projection (red channel) stimulated emission ex vivo imaging of mouse ear microvasculature based on endogenous haemoglobin contrast. In the magnified image, individual RBC rouleaux are visible within a single capillary [241].](image17)

### 2.3.2.3 Micro-computed tomography

The basis of all computed tomography techniques is the ordered collection of x-ray projected data from multiple angles in space [246]. Many comparisons may be drawn between the working principles of micro-computed tomography (micro-CT) and those of OPT (see §2.3.2.1). 3-D images of vasculature are obtainable by micro-CT imaging, with resolution on the micron scale.

![Figure 18: High-resolution micro-CT vascular tree 3-D volume rendering of ligated hindlimb of the mouse; skeletal structure is included for clarity [247].](image18)

A micro-CT system comprises three basic elements: an x-ray source; the conversion electronics; and a method for either rotating the specimen or rotating the scanner around the specimen. This experimental setup is very similar to that of Figure 15(a), substituting bright-field illumination with an x-ray source. To generate 3-D volume data, methods such as cone
beam reconstruction [248] are used to convert the projection data into a stack of slices [246]; this may be further extended into filtered back projection methods, as is the case in OPT.

As an x-ray penetrates an object in transmission mode, it is exponentially attenuated by the material along its path in the same manner as described by the Beer-Lambert law (see §1.1.1). However, the main limitation of micro-CT relates to contrast; as the absorption of x-rays by soft tissues or optical coloured stains is low, this causes difficulties in the simultaneous imaging of tissue structure and in the analysis of gene expression, respectively [249]. In addition, image quality is inherently related to x-ray dose, a concern that is particularly relevant for in vivo studies (e.g. the potential for radiation damage to the specimen, thereby altering the physiology). High spatial resolution requires high doses, and a reduction in dose causes higher image noise and reduced image contrast [250].

The modulation transfer function (MTF) of an imaging system is a common comparator for micro-CT spatial resolution [246, 251]:

\[ MTF(\xi) = \left| \int_{-\infty}^{+\infty} e^{-j2\pi \xi x} [LSF(x)] dx \right| \]  \hspace{1cm} (30)

where \( \xi \) represents spatial frequency; and LSF is the integral of the point spread function. In any real imaging system, the MTF is non-ideal and values drop progressively at higher spatial frequencies. Consequently, small vessel diameters appear to broaden and this causes inaccuracies in CT-values. However, improvements in scanner components have made bench-top nano-CT units feasible, having the ability to image at cellular and subcellular levels with voxel resolutions on the order of 500 nm [250].

Micro-CT provides high resolution 3-D volumetric data, suitable for analysis, quantification, validation and visualisation of vasculature. Reported studies for the use of micro-CT include the assessment of induced or implanted disease models [252, 253]; screening for anatomical abnormalities and/or changes in live animals [254]; assessing angiogenesis during fracture healing [255]; in morphological studies, for example, the rodent renal vasculature [256, 257]; and the assessment of cancer staging for predictions of metastatic relapse [258]. Micro-CT has also branched into the realm of fluid dynamic simulations in, for instance, the mouse aorta [259, 260] and assessment of coronary artery disease [261]. Although in vivo micro-CT imaging is still in its infancy, the possible implications and applications of its use in biomedical research is potentially far-reaching.
2.3.2.4 Confocal & two-photon imaging

The previously described methods of optical projection tomography and stimulated emission depletion microscopy (§2.3.2.1, §2.3.2.2) both utilise fluorescence in their operation. However, a severe problem with epi-fluorescence is the unwanted contribution of signals from structures above and below the focal plane; this can produce a background glow that can cause image degradation [262]. This is surmounted by employing the principles of confocal imaging.

Geometrically speaking, confocal means having the same foci. In the confocal régime, an object is illuminated with a small (diffraction limited) spot, usually derived from a focused laser beam. An objective lens replaces that of the conventional microscope condenser and the field of illumination is limited by a pinhole positioned on the microscope axis. This spatially restricts the optical system so that only signals emanating from the focal point are detected. Both the intensity of illumination and the sensitivity of the detector fall rapidly with distance away from the focal plane. Thus, light scattered from parts other than the illuminated point on the specimen is rejected from the optical system (by the exit pinhole) resulting in reduced blurring from light scattering, improved effective resolution and signal-to-noise ratio [263]. This technique may be used in both transmitting and epi-illuminating modes, and an image of the complete specimen is built up by a raster scanning. Reported resolutions have been of the order of 100 nm [19]; however, with 4-Pi methods, an axial resolution of 100 nm and a localisation accuracy of < 10 nm in fixed and immune-labelled specimens [264].

![Microscope schematic for (a) confocal and (b) two-photon methods.](image)

**Figure 19**: Microscope schematic for (a) confocal and (b) two-photon methods.

As described above, confocal microscopy dramatically reduces the contribution of fluorescence from the out-of-focus regions of a specimen. However, regions above and below the focal plane are still exposed to the full intensity of the excitation light and ‘parasitic’ fluorescence in every direction, converging and diverging from the illuminated
spot in the focal plane. Consequently, biological specimens still succumb to photon-induced damage and rapid fluorescent bleaching. These shortcomings are circumvented by two-photon microscopy [265, 266], and have been extended to the practice of multiphoton (i.e. more than 2) fluorescence microscopy [263].

The challenges posed by the confocal methods may be surmounted by outputting an intense infra-red (IR) laser pulse which induces fluorescence in the blue or UV excitable fluorophore at the focal spot of the system only. At this position, the coherent electromagnetic field strength is so high that its acts non-linearly to excite chromophores at twice the frequency of the IR pulse, inducing an electronic transition equivalent to the absorption of a single photon, possessing twice as much energy [267]. The excited fluorophore can then emit a single photon of fluorescence. As a result, fluorophores above and below the focus do not experience the fluorescence effects of the two-photon methods and are therefore neither excited nor damaged. Additionally, two-photon microscopy permits confocal imaging of planes much deeper in the tissue (new approaches have led to depths of almost 1 mm [268]) with considerably higher light coupling efficiency, with less bleaching and damage outside of the focal plane [265, 269, 270]. For very thin objects such as isolated cells, confocal microscopy produces images with higher optical resolution due to the shorter excitation wavelengths. Nonetheless, the superior optical sectioning and light detection capabilities of the two-photon modality results in better performance in highly scattering tissue.

The biggest advantage of 3-D optical sectioning approaches such as confocal microscopy and two-photon microscopy [271], is their ability to visualise biologically significant molecular distributions within a single specimen. However both techniques do suffer some shortcomings. Although the confocal principle allows clear imaging within semitransparent tissue, turbid media causes scattering and absorption of light thus diminishing the ability to image with depth. This limits the actual useful depth of confocal imaging to around 100-200 μm [263] even with the use of near IR illumination which is more penetrating than visible light [272-274]. Moreover, use of IR illumination for imaging itself reduces image resolution, due to the longer wavelength for a given numerical aperture [272]. The pulsed lasers required for two-photon excitation are much more expensive than the continuous wave versions used in confocal microscopy. In addition, despite the fact that the in vivo resolution provided by multiphoton imaging is reportedly superior to that offered by MRI and PET, the necessity of either thinning or removing a portion of the skull for imaging the brain surface impedes the potential clinical applicability of this technique [275].
Yet, despite these limiting factors, confocal and two-photon imaging have been used in a wide variety of applications: rodent cerebral microcirculation investigations [276-279]; used in conjunction with particle image velocimetry (see §2.5.1) to investigate micro-channel blood flow [280]; as a quantitative method in the study of embryonic vasculature dynamics [281, 282]; dynamic studies of Alzheimer’s disease and epilepsy [275]; and in cytometric studies of the relationships between stem cells [283], tumour cells [284] and their tissue environments [285, 286].

2.3.2.5 Photoacoustic tomography & microscopy

Purely optical imaging in biological media is hampered with the either imaging depth or spatial resolution trade-offs, owing to strong optical scattering. Purely ultrasonic (USn) imaging may improve upon these limiting factors in the optical (quasi-) diffusive regime, due to the fact that USn scattering is 2-3 orders of magnitude weaker than optical scattering [251]. Photoacoustic tomography (PAT) refers to imaging based upon the tenets of the photoacoustic effect. If an object is irradiated with a short-pulsed laser beam, some of radiation is absorbed and partially converted into heat. This heat causes a pressure rise via localised thermoelastic expansion, and this pressure rise propagates as a USn or photoacoustic (or optoacoustic) wave. Detection of these signals by an array of transducers allows reconstruction of the light absorption distribution and deduction of information on the distribution of absorbing inclusions in tissue. A typical PAT system uses an unfocused US detector to acquire the photoacoustic (PA) signals, and the image is reconstructed by inversely solving the photoacoustic equations (e.g. backprojection algorithms based on time-reversal methods [287-290]).

PAT is an optical absorption based modality, with contrast and resolution based on the PA excitation phase and PA emission phase (derived from the USn detection), respectively. In PAT, NIR light is used as it is in this wavelength range (and that of the visible wavelengths) that haemoglobin dominates soft tissue optical absorption, producing functional network maps of haemoglobin oxygen saturation in the vasculature [291]. If an isosbestic point is selected for imaging, the PA signal represents total haemoglobin concentration, irrespective of oxygenation.

The generation and propagation of a PA wave in an inviscid medium may be described by the following [251]:

\[
\left( \nabla^2 - \frac{1}{v_s^2} \frac{\partial^2}{\partial t^2} \right) p(\vec{r}, t) = -\frac{\beta}{\kappa v_s^2} \frac{\partial^2 \Pi(\vec{r}, t)}{\partial t^2} \quad \text{(31)}
\]
where $v_s$ is the speed of sound; $t$, time; $p(\vec{r}, t)$, the acoustic pressure at location $\vec{r}$ and time $t$; $\beta$, thermal coefficient of volume expansion; $\kappa$, isothermal compressibility; and $T$, temperature rise. The spatial resolution ($\Delta x$) of a PAT system is determined by the frequency bandwidth of the PA signal detectors ($\Delta f$) [287]:

$$\Delta x \sim \frac{v_s}{\Delta f} \tag{32}$$

PAT combines the advantageous aspects of optical diffusion tomography and conventional US imaging, with achievable spatial resolutions on the order of 0.1-10 mm (frequency bandwidth dependent) and imaging depths of several cm.

Intravital microscopy is the current ‘gold standard’ for imaging of dynamic microvascular regulation [292]; however, it is generally invasive and lacks crucial morphological parameters such as depth information. PA microscopy (PAM) overcomes these limitations by being reliant on endogenous optical contrast, working non-invasively in reflection mode with time-resolved depth detection.

![Figure 20](image)

**Figure 20**: (a) Annular illumination of functional PAM system [293]; (b) A representative microvascular network in a nude mouse ear imaged in vivo by OR-PAM [294].

In contrast to the PAT technique, a PAM system uses a spherically focused ultrasound detector with 2-D point-by-point scanning, requiring no reconstruction algorithm. Conventional reflection-mode confocal PAM is implemented using both dark-field pulsed-laser illumination [295, 296] and high-NA USn detection. A pulsed laser beam (e.g. Q-switched pulsed Nd:YAG\textsuperscript{1} laser) delivered by a multimode optical fiber passes through a conical lens forming annular illumination and is then weakly focused into biological tissues. Due to this illumination type, the PA signal is greatly minimised. A concave acoustic lens is attached to the transducer, which in turn is immersed in a water container for added USn coupling efficiency. Light from the optical fiber is expanded by a conical lens and focused

\textsuperscript{1} Nd:YAG: neodymium yttrium aluminium garnet
through an optical condenser. The optical dark-field illumination and USn detection are confocal, as the optical and USn transducer foci are overlapped. On each laser-pulse excitation, the emitted PA wave is recorded as a function of time (thus forming an A-scan); raster scanning of the PAM probe head yields 3-D volume data sets. The lateral resolution ($\Delta r$) depends on the USn parameters, the source center wavelength ($\lambda_0$) and the transducer numerical aperture ($NA$):

$$\Delta r = 0.71 \frac{\lambda_0}{NA}$$

(33)

In order to resolve fine structures, spatial resolutions of 10 μm or higher are desired. Applying this to the dark-field methodology, a USn transducer with center frequency of 400 MHz is required; however, this limits the penetration depth to $< 100$ μm. Spatial resolution in dark-field PAM is limited by USn parameters ($< 50$ μm), and is thus unable to resolve artifacts on the arteriole, venule and capillary scale [293, 297]. By employing techniques such as diffraction-limited optical focusing (OR-PAM) [298], resolution improvements on the order of 5 μm or greater are obtainable.

The PA Doppler (PAD) effect [299-301] is the combination of the production of an acoustic wave from a light-absorbing medium and the shifting of this wave when it has a motion relative to a detector. Consider a light absorbing-particle suspended in a liquid, moving at a velocity $\vec{v}$ and an intensity-modulated laser beam illuminates the particle at an angle $\alpha$. The particle undergoes the PA process and results in the formation of an acoustic wave which is collected by a USn transducer. Doppler shifts values depend the angle between the flow path of the particle and the transducer. The PAD shift ($f_{PAD}$) of the PA wave along a typical angle $\theta$ may be expressed as [301]:

$$f_{PAD} = f_M \frac{v \cos \theta}{v_s}$$

(34)

where $f_M$ is the modulation frequency; and $v_s$ is the velocity of the moving scattering object. In §2.2.2, the method of laser Doppler flowmetry was outlined; a principle disadvantage of this method is its limited measuring depth and loss of flow directional information due to multiple light scattering. With the PAD methods, however, this is no longer an issue. Additionally, as light-absorbing particles are used as flow tracers, lower background noise and higher detection contrast result.

PA techniques are frequently used in cerebral microvascular imaging [302] (see Figure 20(b)) and cancer detection. Enhanced angiogenesis is typical for malignant tumours from
onset and therefore PAT allows for early tumour detection and diagnosis [303, 304]. These methods have also been utilised in the assessment of absorption contrast differentiation between metastatic and normal breast tissue [305, 306]. In addition, PA techniques have been used alongside complementary multimodal modalities such as fluorescence imaging to yield in vivo functional molecular information [307, 308].

2.4 Cytometric methods

Cytometry is the quantitative term for whole single cell analyses with a relatively low numerical aperture. Cells in suspension are sucked or pressed into a cytometer apparatus by overpressure or the use of mechanical pumps. Covered with sheath fluid, the cells undergo separation (see Figure 21). Solid tissue, however, cannot be analysed by flow cytometer means without some form of initial tissue disintegration [309]. For analysis of solid tissues, slide-based cytometry is implemented by lamp illumination [310] and camera based detection, or laser excitation and fluorescence detection [311].

![Figure 21: Illustration of flow cytometric cell sorting][312]

In conventional flow cytometry (FCM), forward (cell size and refractive index dependent) or orthogonal (provides granularity information) light scatter is used for label-free analysis of rough leukocyte discrimination, for instance [313, 314]. Application of fluorescent dyes to label specific markers or cellular functions paved the way for gleaning additional cellular information and the characterisation of biomarkers [315]. The feasibility of simultaneous analysis of many parameters lies in the multicolour approach of fluorescent dyes. Lasers or modern LEDs excite cells impregnated with fluorescent dye and the emitted fluorescence is detected by photodiodes. This allows for analysis of complex cellular
structures, cell subtypes, cell-cell interactions, biological processes and identification of rare cell types. The use of fluorescence is particularly useful in cytomics, which characterises single cells to unravel their interactions within cell systems and so to get a better insight into biological processes in organisms [309]. Polychromatic cytometry produces a complicated pattern of fluorescence to examine many parameters simultaneously in order to find cell interactions and causal connections; this reduces costs as it prevents repetitive use of markers for identifying populations. For example, cytometric systems utilising 17 dyes simultaneously have been reported, for immunological analysis of disease patterns [316].

Cytometric procedures have enabled the separation and sorting of targeted cells by employing laser tweezers [317, 318] and lab-on-a-chip concepts [312, 319, 320]. Cells may be isolated from the larger cell collective by applications of single laser pulses or deflection in a continuous laser beam, and stored in a reservoir for further analysis, e.g. in the determination of relative concentration and purification.

As previously mentioned in §2.3.2.4, the use of fluorescent sources induces photobleaching with prolonged exposure therefore affecting analytical results [321]. However, this bleaching affect has itself been used as a tool for single-cell fluorescence spectroscopy [322, 323] in the discrimination of DNA information at the single-molecular level [324].

Flow cytometry may be incorporated in a wealth of different studies, notably: cell cycle analysis [325, 326]; apoptosis§ [327-329] and stem cell investigations [330, 331]; and drug effect trials [332, 333]. Furthermore, flow cytometry has been used in conjunction with other complementary modalities such as: photothermal and photoacoustic applications for the study of blood and lymph flows (see Figure 22) [334-336]; applications in tumour analysis and fluorescence based breast cancer imaging [337-340]; functional brain imaging [341]; and utilised with contrast enhancing fluorescence nanoparticles for HIV screening [342, 343]. Flow cytometry has also been used in a number of clinical studies for example sickle cell anaemia, with the potential for discriminating between sickled and non-sickled cells, as the sickled variety have reduced oxygen-carrying ability and more slowly through the microcirculation [344].

§ Apoptosis: cell death
2.5 Correlation based mechanisms

2.5.1 Particle tracking velocimetry

Capillary video-microscopy was one of the first means by which abnormal capillary vasculature and dynamics in, for example, wound sites and port wine stains, was documented [345-347]. Video cursors were placed along the length of a capillary, one at an upstream and one at a downstream position, separated by a known distance.

![Capillary Loop and Video Cursors](image)

**Figure 23:** Illustration of television microscopy methods of capillary velocity estimation [348].

The temporal variations in light intensity subjected to cross-correlation computations yield transit times for photometric events moving from one cursor to another, thereby allowing the computation of red blood cell velocity (RBC\(_V\)) values. Currently, videocapillaroscopy is the clinical ‘gold standard’ for capillary imaging and analysis and is in routine clinical use by rheumatologists. However, the depth of tissue penetration is a limiting factor and restricts capillaroscopic techniques to the nutritive portions of the skin [349].

Particle image velocimetry (PIV) is a non-intrusive measurement technique, used to measure full-field velocity data within a 2-D plane of a particle-seeded flow field [350]. The fluid velocity is inferred from the motion of tracer particles which constitute a random pattern that is ‘tied’ to the fluid, whose motion is visible through tracer pattern changes. In PIV, two
sequential images are subsampled at one particular area via an interrogation window. Within these samples, an average spatial shift of particles may be observed from one sample to its counterpart in the other image [351], provided flow is present. The displacement of a tracer particle \( d \) in a finite time interval \( \Delta t = t'' - t' \) may be given by [352]:

\[
d(x; t', t'') = \int_{t'}^{t''} v[x(t), t]dt
\]

where \( v[x(t), t] \) is the tracer particle velocity. The tracer pattern does not necessarily have to be composed of discrete tracer particles; the above interpretation is likewise applicable to continuous tracers such as dyes. PIV tracer velocity values are deduced by calculation of the expectation value of the auto-correlation function for a double-exposure continuous image [352]. The temporal auto-correlation function of the scattered light intensity yields measurements of velocity gradients in a steady flow [353], thus providing a way of determining shear gradients quantitatively.

Performance of PIV methods in measuring instantaneous velocity fields were improved upon by the introduction of direct cross-correlation of image fields in place of multiple-exposure auto-correlation methods [354]. Cross-correlation methods of interrogation of successive single-exposure frames can be used to measure the separation of pairs of particle images, resulting in improvements in spatial resolution, detection rate, accuracy and reliability. A high cross-correlation value near unity is observed when many particle images in the initial image match their corresponding spatially equivalent shifted partners in the subsequent image, i.e. the higher the correlation coefficient, the greater the accuracy of the velocity data obtained [350]. PIV has been associated with certain limitations, such as directional ambiguity [352], and its use of microbeads for contrast have resulted in severe obstructions and distortion of the flow profiles [355], thus limiting its clinical application.

The clinical applicability of the PIV technique has been reported in small animal models and noteworthy is the analysis of arterioles with both bifurcation and confluence in the rat mesentery [356]. The most successful approach for employing advanced velocimetry for measurements in microfluidics (see §2.4) is microparticle image velocimetry (μPIV) using fluorescently tagged polystyrene particles [243, 357, 358]. PIV has been used as a complementary technique to a wide variety of modalities. Recently, it was coupled with OCT (see Figure 24) for quantitatively characterising the microfluidic-scale flow generated by epithelial motile cilia of *Xenopus tropicalis* embryos [359]. PIV is not restricted to optically acquired images only; US imaging has been used to measure the velocity
distribution in the porcine left ventricle [23], with reported spatial and temporal resolutions of 0.4 mm and 0.5 ms, respectively [360]. In addition, X-ray velocimetry has been suggested as a means of in vivo wall shear stress measurement, as it is a highly influential factor for the progression of arterial disease [361].

![Figure 24: 2-D, two-component flow velocity field of OCT-based particle tracking velocimetry of non-recirculatory cilia-driven fluid flow [359].](image)

2.5.2 Correlation spectroscopy

Scanning laser image correlation (SLIC) is an optical correlation technique which traces a laser beam rapidly with respect to the motion of particles. It applies the use of pair-correlation [362] to analyse reflected fluctuations from moving particles, thus yielding temporal information between sequential images. SLIC is capable of discriminating among multiple populations of particles travelling simultaneously through the same channel at different velocities [363, 364].

In SLIC, a laser follows a trajectory across a channel, illuminating a series of (many) small regions (see Figure 25). The reflected intensity for each region is measured with an optical detector.

![Figure 25: Illustration of the SLIC method for flow measurement. A laser scans many small regions, the reflected intensity is detected and pair-correlation provides a flow measurement. Image adapted from [363].](image)
In order to perform velocity measurements, temporal comparisons need to be made of a particle initially and at a later time via cross-correlation. For SLIC computation, normalised cross-correlation is used [363] and is defined as:

\[ G_{ab}(\tau) = \frac{(\delta I_a(t) \cdot \delta I_b(t+\tau))_I}{((\delta I_a(t))_I (\delta I_b(t))_I)_I} \] (36)

where \( a \) and \( b \) refer to the columns in Figure 25; \( I \), intensity; \( \tau \), temporal correlation shift; \( \langle \, \rangle_I \), temporal average; and \( \delta I(t) = I(t) - \langle I(t) \rangle \). 2-D plots may be deduced via multidistance pair-correlation [365]. Spatio-temporal image correlation spectroscopy (STICS) is fundamentally different from other optical correlation techniques, e.g. speckle, as it does not require the use of a model and is a purely statistical method [366]. However, it is limited in the range of velocities it can measure, the maximum velocity being limited by the acquisition speed of detection [363]. SLIC has been widely implemented in assessments of vascular dynamics in, for example, zebra-fish embryos [41, 367].
3 Coherence domain optical methods – Optical Coherence Tomography

This chapter outlines the basic principles of Optical Coherence Tomography (OCT) and gives a short historical overview from its initial inception to current, modern day advancements. System characteristics are outlined, in addition to comparisons between the different variants and functional extensions of OCT in terms of SNR, sensitivity and imaging speeds.

3.1 Michelson interferometry: the basis of OCT

Optical Coherence Tomography (OCT) is based on a classic optical measurement technique known as low-coherence or white light interferometry, first described by Isaac Newton. Interferometry is a powerful technique capable of measuring the magnitude and echo time delay of remitted light with very high sensitivity. One of the earliest uses of interferometry was in photonics to measure optical echoes and backscattering in optical fibers and waveguide devices [368, 369]. Its first introduction into the biological realm was reported by Fercher et al. in 1988, where it was applied for the non-invasive measurement of axial eye length [370].

Many of the aforementioned optical techniques (Polarisation Spectroscopy, Optical Projection Tomography and Photoacoustic Imaging) are based on the diffuse scattering of light with turbid media. OCT, on the other hand, falls into the category of ballistic (or quasi-ballistic) optical imaging and is analogous to ultrasound pulse-echo imaging [371], employing coherence gating. Ballistic scattering refers to photons that have undergone no scattering; whereas a diffusive regime refers to photons that have suffered many scattering events and as such have completely lost memory of their original incidence direction.

The intensity of singly backscattered light is given by [251]:

\[ I(z) = I_0 e^{-2\mu z^2} R_B \]  

(37)

where \( R_B \) denotes the portion of backscattered light received by the detector; \( z \) is the ballistic pathlength in the scattering medium; the factor of two in the exponent results from the round-trip propagation. Ballistic imaging rejects non-ballistic photons; the retained ballistic photons
have better collimation, a more well-defined wavefront, and retain their incident polarisation state (in a non-birefringent medium). OCT detection is based on classical interferometric principles and can provide clear visual boundaries between different vessel wall layers in a non-contact manner. Contrast in OCT images originates from the varying intensity of backreflected light, and signal differences originate from subtle variations of refractive index.

In interferometry, a monochromatic light source beam is emitted towards a beamsplitter and is split into two, with one half backreflected from a reference mirror and the remaining backreflected by an object surface. These split beams are then recombined by the original beamsplitter and received by a detector. The intensity received by the detector may be written as [372]:

\[
I(\varepsilon) = E_{R0}^2 + E_{S0}^2 + 2E_{R0}E_{S0}\cos\left(\frac{2(kS_lS - kR_lR)}{\Delta\phi}\right)
\]

where \( R \) and \( S \) denote the reference and sample arms, respectively; \( E_0 \) is the incident electric field; \( k \), the propagation constant; and \( l \) is the arm length from beamsplitter to the back reflection surface(s). The time-varying phase difference, \( \Delta\phi \), results in the interference term becoming an alternating current which produces interference fringes. Thus, the recorded is referred to as a spectral interferogram, varying periodically with (round-trip) optical pathlength difference (or mismatch) \( 2\eta(l_S - l_R) \). The sample under interrogation is characterised by its depth-dependent electric field reflectivity profile along the sample beam axis; the reconstruction of this profile as a function of sample depth from non-invasive interferometric measurements is the goal of low-coherence interferometry in OCT. The method by which such axial profiles are extracted is dependent upon the mechanism with which it is imaged (time/Fourier-domain). For example, in the case of FdOCT, direct application of inverse Fourier transformation to the spectral fringe signal enables reconstruction of the axial structure of the measured object along with the coherence noise terms and zero frequency term (DC). The resulting spectral interferograms include three distinct components: (a) ‘DC’ component: a pathlength-independent offset with an amplitude proportional to the power reflectivity of the reference mirror plus the sample reflectivities; (b) ‘auto-correlation’ terms: represent interference occurring between the different sample reflectors and appear as artifacts; and (c) ‘cross-correlation’ terms: present for each sample reflector and are the desired components for OCT imaging, dependent on both light source wavenumber and pathlength differences.
If a coherent (narrow linewidth) light source is used, interference will be observed over a wide range of path length differences. OCT, however, utilises low coherence light sources (e.g. superluminescent diodes) to detect optical echoes and may be implemented in free-space or (single-mode) fiber-based systems. Confocal microscopes have the advantage of slightly improved lateral resolution over conventional bright-field microscopes and the ability to perform ‘optical sectioning’ due to their peaked axial response (unlike bright field microscopy, for which out-of-focus light is blurred, but not attenuated). The fundamental quality that differentiates OCT from other forms of optical microscopy is that the predominant axial component of image formation derives from an interferometric ranging measurement. The sample arm of an OCT system may be treated as a reflection-mode scanning confocal microscope, in which the single-mode optical fiber serves as a pinhole aperture for both illumination and collection of remitted light from the sample. The lateral point spread function of an OCT system at its focal plane as a function of lateral position may be written as [372]:

\[ I(u) = \left( \frac{2J_1(u)}{u} \right)^4 \]  

(39)

where \( J_1(u) \) is a first-order Bessel function; \( u \) is the normalised lateral range parameter, \( u = \frac{2\pi x \sin(\alpha)}{\lambda_0} \), \( x \) is lateral distance from the optic axis; and \( \alpha \) is the subtended half-angle of the objective aperture. Likewise, the axial response of an OCT system from a planar reflector as a function of position along the optic axis is given by:

\[ I(u) = \left( \frac{\sin(u/2)}{u/2} \right)^2 \]

(40)

where \( u \) is the normalised axial range parameter, \( u = \frac{8\pi x \sin^2(\alpha)}{\lambda_0} \). The axial peaked response provides the depth sectioning capability.

Low-coherence light may be characterised as having statistical phase discontinuities over a distance known as the coherence length, which is inversely proportional to the bandwidth of light. When such light is used, interference is only observed when the path lengths of the reference and sample arms are temporally/spatially matched to within the coherence time/length of the light. The coherence length \( (l_c) \) of light is defined as the spatial extent along the propagation direction over which the electric field is substantially correlated and is
equal to the width of the field auto-correlation function [373]:

$$l_C = \frac{4\ln(2) \lambda_0^2}{\pi \Delta \lambda} \tag{41}$$

where $\lambda_0$ is the source center wavelength; and $\Delta \lambda$ is the full-width at half maximum (FWHM) bandwidth. The coherence length is related to the coherence time by $l_C = c \tau_C$, where $c$ is the speed of light. Therefore, the axial resolution may be determined by the source coherence length (i.e. the arrival of time echoes), or more accurately, $\Delta z = \frac{l_C}{2}$. Although the above expression for $l_C$ is for a Gaussian lineshape, it is the most common form used in OCT literature as it approximates the spectral shape of actual light sources and has useful Fourier transform properties [370]. The inverse proportionality between axial resolution and bandwidth is illustrated in Figure 26; micrometer scale axial resolution requires extremely broad optical bandwidths.

![Figure 26: Graphical illustration of theoretically achievable values for axial resolution $\Delta z$ with light source bandwidth $\Delta \lambda$; the central wavelength values used are shown in the legend.](image)

In contrast to conventional microscopy, the axial and transverse resolutions in OCT are decoupled from each other. However, as is the case in traditional microscopic methods, the transverse resolution in the OCT imaging system is determined by the diffraction limited spot size of the focused optical beam [374]:

$$\Delta x = \frac{4\lambda_0 f}{\pi d} \tag{42}$$

where $d$ is the spot size on the objective lens and $f$ is its focal length; the transverse resolution results from the confocal mechanism. Thus, fine transverse resolution can be obtained by
using a large numerical aperture (NA) objective lens, focusing the beam to a small spot size. Similarly, transverse resolution is also related to the depth of field (DOF) or the confocal parameter $b$ or twice the Rayleigh range $z_R$:

$$b = 2z_R = \frac{\pi \Delta x^2}{\lambda}$$ 

Improving the transverse resolution decreases $b$, similar to that observed in conventional microscopy. Thus, an inherent trade-off exists between (transverse) resolution and the DOF. The relationship between focused spot size and DOF for low and high numerical aperture focusing is illustrated in Figure 27.

**Figure 27**: Illustration of the influence of transverse resolution in relation to achievable depth of field. Increasing the transverse resolution decreases $b$, similar to that observed in conventional microscopy [372].

Recent advancements have enabled transverse resolution improvement by software means, e.g. digital refocusing [375], and by hardware amendments, e.g. 4-fold resolution improvement by lateral oversampling [376]. Typically OCT imaging is performed with low NA focusing to achieve a large DOF (i.e. $b > \Delta z$) and the axial resolution is governed by the measurement resolution for echo time delays of light. However, the low NA used in this instance limits the transverse resolution. The axial-transverse resolution decoupling feature is particularly powerful for ophthalmic and/or catheter/endoscopic imaging where high NAs are not available. Conversely, if very high NA focusing is used, fine transverse resolution is achievable; however, the resulting DOF can be comparable to, or shorter than the coherence length (i.e. $b < \Delta z$). This operating regime is known as optical coherence microscopy (OCM) [377], in which en face imaging can achieve improved imaging depth and contrast compared with confocal microscopy as it rejects unwanted scattered light more effectively [378, 379].
3.2 OCT variants

Generally referred to as the first generation OCT system [380], time-domain OCT (TdOCT) systems employ mechanical reference arm scanning to achieve depth-resolved imaging from various depths in a biological tissue sample [371, 381]. The movement of the reference arm induces a Doppler frequency shift in the reflected reference beam. Detection of beams of this type may be categorised as heterodyne detection owing to the Doppler shift. Consequently, such systems are capable of only achieving an imaging speed of several thousand A-lines/s, thus preventing the realisation of 3-D scanning for in vivo applications. In a TdOCT system, increasing the light source bandwidth for high resolution imaging capabilities decreases the SNR as it requires increased electronic detection bandwidth. In order to maintain the same SNR, either the A-scan rate or depth scan range has to be decreased, or the sample illumination intensity has to be increased. However, as high scan rates and large depth scan ranges are desirable features in OCT systems, and considering the ANSI limits for incident optical power, e.g. ophthalmic imaging, such resolutions require a more sensitive technique.

![Figure 28](image-url) Schematic of free-space (a) TdOCT and (b) FdOCT systems.

The Fourier-domain equivalent of TdOCT (FdOCT), which encompasses both spectral and swept source variants [102], avoids the varying of the reference optical path by considering all multiple partial waves for all sample beam backscatterers along a single A-scan simultaneously. It accomplishes this by dispersing the recombined beam into its spectral components by a spectrometer and performs an inverse Fourier transformation of the detected spectrum, yielding an A-scan image in its entirety (see Figure 29). Although FdOCT methods require more complex post-processing, they have great potential in terms of speed of acquisition and sensitivity. This sensitivity enhancement is approximately given by the ratio of the axial imaging depth to the axial resolution. In general this corresponds to a 50- to 100-fold sensitivity increase in imaging speeds.
Figure 29: (a) 2-D (and 3-D) cross-sectional images are constructed by imaging at different transverse positions; (b) 1-D representation of the backscattering in a cross-sectional plane of the tissue as indicated by the line through tissue in (a) [374].

For a given \( N \) pixel-based Spectral-domain OCT (SdOCT) system with a depth scan range of \( x_{depth} = \frac{N}{2} l_c \) (given that the highest detectable spectral periodicity before aliasing is \( 0.5N \)), a TdOCT system acquiring signal over the same total time duration as an SdOCT system will have a signal-to-noise (SNR) ratio of [382]:

\[
SNR_{TdOCT} = 10\log \left( 4P_S T \left[ \frac{2\epsilon}{Nh\nu} \right] \right) = SNR_{SdOCT} - 10\log(\frac{N}{2}) \quad (44)
\]

where \( P_S \), total detected sample power; \( T \), total signal collection time; \( \epsilon \), the quantum detector efficiency; and \( h\nu \), the photon quantum energy. Therefore a SdOCT system is intrinsically more sensitive than a TdOCT by a factor of \( 0.5N \) [383]. For a Gaussian source this SNR advantage is expected to be \( 0.25N \); thus, for a given source power and A-scan rate, a SdOCT system with \( N = 2048 \) can possess a ~20 dB to 30 dB sensitivity advantage over its time-domain counterpart [384].

In addition to sensitivity and resolution considerations, the nominal axial imaging depth of an OCT system is derived by the following expression [385]:

\[
z_{max} = \frac{1}{4\eta_{av} \Delta\lambda} \frac{\lambda^2}{N} \quad (45)
\]

where \( \eta_{av} \) is the average refractive index of the sample under study; \( N \) is the number of detector array elements; all other terms are as described previously. As the depth-to-resolution ratio of OCT is > 100, it qualifies as a high resolution imaging modality [251], and is capable of doing so down to millimetre scale depths.
3.2.1 Fourier-domain: sensitivity & imaging speed advances – a comparison

As will be discussed in full in §3.2.2.1, rapid scanning optical delay (RSOD) lines are used to balance second order dispersion between sample and reference arms [386]. Phase wrapping, the characteristic ring appearance on vessels with high velocities, occurs due to complex numbers having unique phase only modulo \(2\pi\) and may require correction if phase differences have absolute values greater than \(\pi\). Utilising a stationary reference arm mirror with a RSOD line has been reported to yield a system dynamic range (i.e. ratio of minimum and maximum detectable Doppler shifts, before wrapping) of over a factor of 600 [387]. Scanning galvanometers used with time-domain RSOD mirrors cannot provide perfectly uniform movement and the associated deviations in angular velocity are a source of phase instability not found in Fourier domain systems.

Second generation Fourier domain systems (FdOCT), achieve depth resolved imaging through the application of fast Fourier transform computation to the detected spectral interferogram without the necessity of mechanical scanning. FdOCT methods have been reported to exhibit a SNR gain of several hundredfold [388], a typical sensitivity advantage of 20-30 dB [389] and therefore deliver imaging speeds which far surpass their time-domain counterparts [390], achieving great success in the research, clinical and commercial arenas. FdOCT scanning enables the capture of volumetric datasets of \(512^3\) sample points, equating to \(~130\) million voxels, within a time frame of 10-30 s [372]. Currently, increased efforts are being paid to third generation OCT systems, i.e. ultrahigh speed systems, \(> 100\) kHz A-line rate.

Two FdOCT strategies exist: spectral-domain (SdOCT, employing a broadband light source and high speed spectrometer) and swept source (SsOCT, employing a high speed tunable laser and photo detector; also known as optical frequency domain reflectometry, wavelength tuning interferometry or optical frequency domain imaging). However, FdOCT methods are not one and the same; although both measure all optical echoes simultaneously rather than sequentially as in TdOCT, their operation differs and there are certain advantageous/disadvantageous aspects associated with each. For example, in SdOCT, the interference of the two beams will have a spectral modulation as a function of frequency, which is measured using a spectrometer. The periodicity of this modulation is inversely related to the echo time delay – therefore different time delays will produce different frequency modulations. The echo delays can be measured by rescaling the spectrometer
output from wavelength to frequency and then Fourier transforming the interference signal, resulting in an axial scan measurement of the magnitude and echo delay of the light signal from the tissue. On the other hand, in SsOCT the output from a narrow bandwidth, frequency swept light source is divided into a sample and reference beam. The sample beam is backscattered from the specimen at different depths, whereas the reference arm is at a fixed delay. The sample and reference arms have a time offset determined by the path length difference, related to the depth of the structure encountered in the specimen. When the sample and reference arm signals interfere, an intensity beat is produced at a frequency which is given by this frequency offset; different echo delays will produce different frequency modulations. The resulting photodetector signal is digitised and the beat frequency signal is Fourier transformed resulting in an axial scan measurement of the magnitude and echo delay of the light signal as in SdOCT.

FdOCT images are subject to two primary sources of artifacts. First, a complex conjugate ambiguity arises because the Fourier transform of the real-valued spectral interferometric signal is Hermitian symmetric. This ambiguity leads to artifactual superposition of reflectors at positive and negative pathlength differences between the sample and reference reflectors. Second, non-interferometric and sample auto-correlation terms appear at DC, obscuring reflectors at zero pathlength difference.

In TdOCT, probe or sample-related motion affects only the image pixel for which the signal is being acquired. If the pixel acquisition time is very small, the degradation in SNR and spatial resolution due to motion may be considered negligible. However, with SdOCT systems in which the signal is acquired over time from various depths within a sample, motion-induced signal degradation may be significant. SdOCT detection must be operated at high speeds because specimen motion produces averaging of the interference fringes if the acquisition speed is too slow. High-speed acquisition has other outward advantages; increasing the number of axial scans delivers superior resolution in the transverse direction and thus yields higher-definition images.

The imaging depth achieved using FdOCT systems is limited by two mechanisms: the spectral sampling interval (which limits the maximum depth observable) and the system spectral resolution, which leads to a falloff of SNR with depth (see Figure 30; the SNR decay, as measured with the db-SdOCT system, was -4.62 dB over a depth of 60 μm (extrapolated this is 115.39 dB/1.5 mm)). Regarding the spectral sampling interval, in SdOCT systems this is limited by the pixel spacing of the CCD, whereas for SsOCT it is limited by the sampling rate of the temporally sweeping source frequency. In SsOCT, the sampling interval should be
smaller than the instantaneous linewidth of the source; otherwise the amplitude of the coherence function will decay in depth, thus limiting the usable ranging depth [391].

Figure 30: SNR falloff measurements for the db-SdOCT system described in this work. The degradation of sensitivity with depth is clearly seen as a mirror is translated from the coherence length zero position in steps of 10 μm by a precise translation stage. (Top) Corresponding fringe patterns.

SNR falloff, on the other hand, describes how the exponential fall-off of sensitivity of a FdOCT system degrades as a function of the imaging depth due to fringe washout (decreasing visibility of higher fringe frequencies corresponding to large sample depths). For SsOCT systems, the spectral resolution is defined as the instantaneous linewidth of the swept laser source. As such, although SsOCT systems exhibit comparable performances to SdOCT, SsOCT systems also feature reduced excess noise. The issue of SNR falloff in SdOCT systems can be ameliorated by either decreasing the pixel size of the CCD camera or moving the interested region of the sample near the equal-pathlength point. However, since spectral sampling and spectral resolution are coupled in spectrometer-based SdOCT systems, they are more limited by falloff compared with SsOCT methods. In addition, heterodyne detection in SsOCT enables the resolution of complex conjugate ambiguity, the removal of non-interferometric and auto-correlation artifacts, and falloff induced by finite source linewidth.
By introducing a carrier frequency to the fringe signal, complex conjugate artifacts may be removed by quadrature detection, in addition to doubling the achievable imaging depth when samples are shifted to large pathlength differences [392, 393].

Some other factors comparing SdOCT and SsOCT systems are outlined in Table 3. Despite the obvious disadvantageous factors associated with the use of Fourier-domain based OCT systems, the research is ongoing. Recently, efforts have been put forth into the expansion of the imaging depth of SdOCT systems. Through the use of a 1050 nm light source and an InGaAs line scan camera (120 kHz, 2048 photodiode array), this resulted in a two-fold increase in spectral resolution and extending the imaging depth to ~6.1 mm [380]. With the greatly increased data input rate which FdOCT modalities provide and the computationally intensive FTs they require, high speed signal processing modules have been implemented to acquire and process the OCT signal in real time for true video-rate imaging [394, 395].

Table 3: Comparison between swept source and spectral-domain FdOCT systems in terms of detection, acquisition and clinical translation [380].

<table>
<thead>
<tr>
<th>Issue</th>
<th>SsOCT</th>
<th>SdOCT</th>
</tr>
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| Detection mechanism          | • ~5mm imaging depth, and ~6dB/3 mm sensitivity fall-off  
○ May use a balanced detection scheme for enhancing dynamic range | • Highly non-linear interferogram capture as a function of frequency  
○ Auxiliary MZI** required                                             |
| Data acquisition             | -                                                                      | • Requires a high speed A/D converter with stable performance  
- very expensive  
- Poor phase stability due to imperfect synchronisation between wavelength sweep and data acquisition  
○ Additional hardware/numerical approaches required | -                                                                      |
| Clinical translation         | -                                                                      | • Employ inexpensive Camera Link® interfaces                             |
|                              | • Existing technical and regulatory issues                             | • More cost effective for clinical use, e.g. ophthalmic analysis        |
The Wiener-Khinchine theorem links time domain with frequency domain interferometry. It is possibly the single most important Fourier relation related to OCT theoretical formulation. The depth (Δz-) domain and wavenumber (k-) domain representations of the OCT signal are equivalent because they are Fourier related. The superposition of individually backscattered fields together with the reference waves will form interference fringes only if the temporal delays between the different light fields are smaller than the range of temporal coherence.

The coherence time τc is defined as the full-width at half maximum (FWHM) of the auto-correlation function G₁(τ) of the electric field E(t) [251]:

\[ G₁(τ) = \int_{-∞}^{+∞} E(t)E(t + τ) dt \]  \hspace{1cm} (46)

According to the Wiener-Khinchin theorem, both the coherence length and time are inversely proportional to the frequency bandwidth for a given spectral shape. If S(ω) is the power spectral density distribution of the light source considered (and is Gaussian-shaped):

\[ \int_{-∞}^{+∞} G₁(τ)e^{-iωτ} dτ = |E(ω)|² = S(ω) \]  \hspace{1cm} (47)

where E(ω) is the Fourier transform of E(t). Therefore, the Wiener-Khinchin theorem (a special case of the cross-correlation theorem) states that the auto-correlation function of the electric field and the power spectrum are a Fourier transform pair.

**Figure 31**: Illustration of the Fourier transform relationship between a Gaussian-shaped coherence function γ(z) (characterised by the coherence length l_c) and the light source spectrum S(k) (characterised by the central wavenumber k₀ and wavenumber bandwidth Δk) [372].

### 3.2.2 Ultra-high resolution OCT

The original intention of OCT was to enable non-invasive optical biopsy, i.e. the *in situ* imaging of tissue microstructure with a resolution approaching that of histology, but without the need for tissue excision and post-processing. Improving axial resolution values by one order of magnitude to the submicron region (and two orders of magnitude compared to conventional ultrasound), ultrahigh resolution OCT (UHR OCT) has enabled the superior...
visualisation of tissue microstructure, e.g. cellular resolution imaging in opaque media. As seen in §3.1, to improve axial resolution, the spectral bandwidth must be either increased or the central wavelength decreased; therefore novel light sources are necessary [396-398]. As previously discussed, the light source not only determines the axial resolution via its bandwidth and central emission wavelength, it also influences both the penetration depth achievable in the sample and the transverse resolution. Hence, the light source is the key technology and the appropriate choice of such is imperative. As the wavelength in a material of higher refractive index becomes shorter, the actual axial resolution may be determined by $\frac{\Delta z}{\eta_G}$, where the group refractive index $\eta_G$ is typically 1.35 – 1.4 for most biological tissue. Nevertheless, the axial resolution is not only limited by the sample dispersion, but also by absorption and scattering within the sample.

In order to be deemed successful as an in vivo optical biopsy tool in opaque media, micron-scale resolution and millimetre range penetration depth is required. In the wavelength range 800 nm to 1.8 μm, scattering is the predominant mechanism limiting image penetration depth. As scattering depends strongly on wavelength and decreases for longer wavelength, a much higher image penetration depth in opaque tissue can be achieved with light at 1300 nm than at e.g. 800 nm; optimum wavelengths for imaging in non-transparent biological tissue have been shown to be in the 1.3 – 1.5 μm range [399]. Imaging at the 1000 nm range represents an attractive compromise in terms of resolution (compared with 1300 nm range) and image penetration (compared with 800 nm range). At 1000 nm, water dispersion is near zero and it is therefore possible to eliminate the influence of depth-dependent tissue dispersion.

3.2.2.1 Dispersion compensation of extended bandwidth sources

Utilising ultra-broadband light sources in an OCT setup can provide much improved values of axial resolution; however, they are also prone to chromatic dispersion in optically-dense materials such as glass tissue and water. As the speed of light is dependent on the refractive index ($\eta(k)$) of the material, certain spectral components are slowed down to a greater extent than others, hence causing light dispersion. A dispersion mismatch can occur if different lengths of optical fiber or other dispersive media are present in the sample and reference arms. Considerable amounts of dispersion can be tolerated if the dispersion present in the interferometer arms is equal, thus creating a coherence function (see Figure 31) free from dispersion artifacts. If dispersion is present, the coherence function will not only broaden due
to the dispersive imbalance but its peak intensity will decrease also, and therefore degrade the axial resolution and reduce sensitivity (dynamic range). A dispersion imbalance introduces a frequency-dependent phase shift $e^{i\theta(k)}$ in the complex spectral density as a function of the wave vector $k$, resulting in a FWHM increase in the corresponding interferogram. Material dispersion, $k(\omega) = \frac{n(\omega)\omega}{c}$, can be expanded into many different orders and is best described by a Taylor series expansion [400]:

$$k = k_0^* + \frac{\partial k}{\partial \omega} (\omega - \omega_0) + \frac{1}{2} \frac{\partial^2 k}{\partial \omega^2} (\omega - \omega_0)^2 + \cdots + \frac{1}{n!} \frac{\partial^n k}{\partial \omega^n} (\omega - \omega_0)^n \quad (48)$$

where $\omega = 2\pi v$. The first two terms are not related to dispersive broadening; $\ast$ denotes zero-order dispersion and adds a constant offset; $\circ$ relates to first order dispersion or group velocity, which changes the coherence length ($l'_c = \frac{l_c}{\eta_g}$) where $\eta_g$ is the group index and can improve depth resolution as $\eta_g > 1$; and $\#$ indicates second order dispersion (group velocity dispersion) and degrades depth resolution by a factor of $(1 - l\beta\Delta\lambda)^{0.5}$, where $l$ is the length of the dispersive path and $\beta = \frac{d\eta_g}{d\lambda}$ is the group dispersion [401]. The impact of dispersion on the A-scan signal can be derived from the effect on the coherence function.

There are several methods by which dispersive effects can be mitigated, e.g. inserting variable-thickness BK7 and fused silica prisms in the reference arm [402]. Second order or group velocity dispersion can be compensated for by implementing rapid scanning optical delay lines [403, 404]; however, higher orders of dispersion are not compensated for. An alternative is dispersion compensation in software by various numerical methods [405, 406]; e.g. dispersion can be removed by multiplying the dispersed cross-spectral density function with a phase term $e^{-i\theta(k)}$, which can be derived experimentally [407]. Compensation of higher order terms is important only when an ultra-broadband light source is used, which can significantly degrade resolution with increasing bandwidth. In addition to dispersion caused by a disparity due to optical material differences in the reference and sample arms, a second source of dispersion results from the non-linear function of the phase dependency of wavelength as the signal may be equidistant sampled in $\lambda$-space but not in $k$-space, causing additional broadening of the coherence envelope after DFT. This can be resolved by resampling and spectral calibration (see §4.2.2). Further to the reasons outlined above, additional dispersive non-linearities can be apparent due to the diffraction grating, surface imperfections of the optics and misalignment.
3.3 Functional extensions of OCT

OCT provides images similar in scale and geometry to histology and just as various histological stains can be used to enhance contrast, various extensions of OCT allow for visualisation of features which may not be readily apparent.

3.3.1 Polarisation sensitive OCT

The specificity of conventional OCT can be improved by providing measurements of the polarisation properties of the probing radiation as it propagates through tissue. The criterion of pathological changes in tissue is a measured decrease in tissue macroscopic birefringence. Biochemical composition has a tendency to change polarisation state due to the high degree of molecular organisation. Pathological processes are characterised by the changes in the amount of collagen fibers and their spatial organisation. Therefore, a comparative analysis of polarization backscattering properties of normal and pathological tissues may be used for early diagnosis of neoplastic processes and biochemical tissue composition [408]; this is performed by polarisation-sensitive OCT (PsOCT).

There are three separate mechanisms for the alteration of polarisation state in biological tissue: birefringence, dichroism and optical rotation. Dichroism can generally be neglected for biological tissue and the round-trip nature of OCT cancels the effect of any circular birefringence. A material is described as birefringent if the real part of its refractive index is polarisation dependent resulting in the decomposition of it into separate beams which travel at different speeds and experience phase retardation. Organised linear structures exhibit form birefringence, e.g. tendon, muscle, bone, cartilage, collagen and teeth.

Typically in a PsOCT system, collimated short coherence light passes through a polariser and is then split into two as it passes through a regular beamsplitter as per the usual interferometric process. Reference arm and sample arm light then passes through a quarter wave plate each and upon recombination after reflection from the mirror/specimen, the detector arm light is split into its orthogonal components via a polarising beamsplitter. The transmitted polarisation state ($\vec{E}$) as a result of the optical system represented by a Jones matrix ($\vec{J}$) acting on an incident polarisation state ($\vec{E}$) can be determined by [372]:

$$\vec{E'} = \begin{bmatrix} E'_{\parallel} \\ E'_{\perp} \end{bmatrix} = \begin{bmatrix} J_{11} & J_{12} \\ J_{21} & J_{22} \end{bmatrix} \begin{bmatrix} E_{\parallel} \\ E_{\perp} \end{bmatrix} = \vec{J}\vec{E} \quad (49)$$
The detected intensity in each polarisation channel can be described by the 2-D intensity vector \( \vec{I} \) [409]:

\[
\langle \vec{I}(\Delta z) \rangle = \left( \begin{pmatrix} E_{r\|} & E_{r\perp}^* \\ E_{s\|} & E_{s\perp}^* \end{pmatrix} \right) + \left( \begin{pmatrix} E_{r\|}^* & E_{r\perp} \\ E_{s\|}^* & E_{s\perp} \end{pmatrix} \right) + \left( \begin{pmatrix} E_{r\|} & E_{r\perp}^* \\ E_{s\|} & E_{s\perp}^* \end{pmatrix} \right) + \left( \begin{pmatrix} E_{r\|}^* & E_{r\perp} \\ E_{s\|} & E_{s\perp} \end{pmatrix} \right)
\]

(50)

where \( \vec{E}_r \) and \( \vec{E}_s \) are the Jones vector representations of light returning from the reference and sample arms, respectively; and \( * \) denotes the complex conjugate. The Jones formalism is unable to describe partially polarised light and the processes which lead to depolarisation. These shortcomings are addressed by Stokes parameters [410] and Mueller matrices [411].

\[\text{Figure 32: OCT and PsOCT images of thermally damaged ex vivo porcine skin; a clear change has occurred in the birefringence of the tissue collagen [412].}\]

PsOCT has been used in a wide variety of applications including correlating burn depth and burn severity (i.e. degree) with a decrease in collagen birefringence [413, 414], monitoring the onset and progression of caries lesions [415], analysis of the retinal fiber layer for potential early detection of glaucoma [416], examination of articular cartilage for detection of osteoarthritis [417], identification and delineation of basal cell carcinoma, and in the assessment of dermal photo-ageing.

### 3.3.2 Spectroscopic OCT

Spectroscopic OCT (SOCT) analyses depth localised absorption spectra or spectral backscattering of native or foreign chromophores in order to enhance OCT image contrast or to extract functional information. The relevant optical properties are the refractive index, absorption coefficient, scattering coefficient, and scattering anisotropy (see §1.3). Due to scattering and absorption in a sample, the spectral content of the OCT signal changes with depth. Therefore, to obtain localised spectroscopic information, confined spectral analysis
methods [418] must be used otherwise depth varying information will be lost. The absorption coefficient ($\mu_a$) of the medium may be deduced by the following [372]:

$$\kappa(\omega) = \frac{c\mu_a(\omega)}{2\omega}$$  \hspace{1cm} (51)

where $\omega$ is frequency; $\kappa$ is the imaginary part of the complex refractive index, $m(\omega) = \eta(\omega) + i\kappa(\omega)$; and $c$ is the speed of light in vacuo.

One of the primary applications of using SOCT is to exploit its capability of providing enhanced OCT image contrast through the use of various agents [419-421]. SOCT has also been particularly successful in the assessment of developmental biology specimens in vivo [422].

![Figure 33: SOCT images of a developing Xenopus Laevis tadpole in vivo. (top) Standard OCT intensity image, (bottom) auto-correlation SOCT imaging [422]. SOCT exhibits increased contrast between tissue types.](image)

Molecular imaging holds a pivotal role in medicine due to its ability to provide invaluable insight into disease mechanisms at molecular and cellular levels. Molecular contrast OCT is capable of providing contrast agent distribution profiles in a sample by measuring the agent’s spectral differential absorption [423]. Many areas of medicine would benefit from a technique that allows determination of spatially resolved tissue oxygenation, e.g. intensive care management – SOCT has been shown to be successful in this respect [424].
3.3.3 Methods of dynamic assessment

Owing to its reliance on interferometry, OCT is more limited than confocal imaging in terms of signal detection. As light reflected from the specimen must be coherent with a reference beam, it cannot detect fluorescence (as this requires the illumination and detection beams to be of different wavelengths). As such, OCT appears unsuitable for analysis of immunohistochemistry or other standard gene expression assays [224]. Although OCT may be likened to US in its theoretical formulation, it also is victim to some of the associated consequences, for instance, conspicuous speckle artifacts [251]. The use of all photons as in photoacoustic imaging (see §2.3.2.5) is of considerable advantage over OCT techniques which are reliant on (quasi-) ballistic interactions, due to the fact that a useful percentage of (diffuse) photons penetrates several cm into tissue. Additionally, diffuse, random, light scattering can perform uniform illumination of microvessels which may otherwise be obscured.

Some very active research areas at present include: diagnosing dermatological diseases e.g. psoriasis [425]; assessment of arteriosclerotic plaques [426, 427]; and determining burn extent and tissue viability by defining tissue barrier layers characteristic of scarring (wound healing and graft flow) [428, 429]. However, in truth, OCT has ubiquitous applications, having emerged in a variety of clinical fields: ophthalmology [430-432], intravascular imaging in cardiology [433, 434], oncology [435, 436], gastroenterology [437-439], general dermatology [440-442], dentistry [443, 444], and gynaecology [445, 446], to name a few. Functional extensions of OCT have emerged: polarisation-sensitive systems used, for example, in the diagnosis of neoplastic†† processes; differential phase-sensitive methods for use in photorefractive surgery; full-field advancement of the conventional single-point OCT detection technique; and endoscopic OCT. Combining OCT with complementary techniques such as OCT with confocal microscopy (optical coherence microscopy), amalgamates the spatial sectioning and pin-hole rejection capabilities of confocal microscopy with the additional longitudinal sectioning provided by OCT coherence gating. Another asset of OCT is that it can be used to separate structure and function in 3-D and to measure flow in individual microvessels. Methods which embody this ability are outlined in the following subsections.

†† Neoplasm: a new or uncontrolled growth of abnormal tissue, e.g. a tumour
3.3.3.1 Doppler

Scanning of the reference mirror of an OCT system at a velocity $v$, produces a Doppler signal at a frequency $f_D = \frac{2v}{\lambda_0}$; the dynamic constituents of the tissue (with velocity $v_s$) will also produce a Doppler signal ($f_{Ds}$). Hence, the signal of the Doppler OCT (DOCT) will be proportional to [4]:

$$A(t) \cos[2\pi(f_D - f_{Ds})t + \phi(t)]$$

where $A(t)$ is the reflectivity and $\phi(t)$ is the phase shift defined by the scatterer position. By simply measuring the frequency shift of moving particles in a defined vessel, it is possible to characterise blood flow and surpasses that of Laser Doppler Flowmetry (see §2.2.2) due to OCT’s ability to resolve structures in depth (see §6.2 for a detailed outline).

Doppler ultrasound (US), the fundamental principle upon which DOCT is based, was developed as a tool for vascular disease assessment and monitoring. However, despite the advancements of DOCT techniques, a principal limitation still exists: the Doppler method is inherently 1-D. The ‘true’ velocity is extracted from this 1-D information by assuming the direction of blood motion, resulting in an inaccurate determination of the flow velocity [448]. Application of Doppler techniques have been shown to be more appropriate for larger blood vessels with faster flow rates, in which Doppler shifts are easier to detect [366]. Furthermore, as a Fourier transformation is usually performed in sliding short-time windows, tradeoffs between axial and velocity resolutions exist in the flow estimation [251]. DOCT is also inherently sensitive to the phase stability caused by both the system and the measurement environments [449]. The preliminary experiments regarding DOCT involved investigating...
picoliter blood volumes [96]. Since then, DOCT has had great success in the visualisation of ocular media and related flow dynamics [450-453], in addition to cerebral [454], cardiovascular and urological applications [455].

3.3.3.2 Speckle variance

Speckle variance (SV) techniques based on structural image intensity have been used in tumour microvascular imaging with high-frequency ultrasound [456]. The interframe speckle variance ($SV_{ijk}$) images of structural image of the OCT intensity ($I_{ijk}$) may be calculated as follows:

$$SV_{ijk} = \frac{1}{N} \sum_{i=1}^{N} (I_{ijk} - \bar{I})^2$$  \hspace{1cm} (53)

where $N$, the number of B-mode frames SV is calculated across; $i$, slice index; $j$ and $k$, lateral and depth indices of the images; and $\bar{I}$, the average over the same set of pixels. SV-OCT images accentuate blood vessels as flowing portions induce localised speckle patterns and thus are uncorrelated between frames [457].

![Figure 35](image)

**Figure 35:** (a) SV-OCT microvascular projection image of an implanted gliosarcoma tumour in the dorsal window chamber of a mouse (scale bar = 250 μm). Colour represents relative vessel depth; (b) Magnified region of interest from (a) (750×750 μm²) [458].

One of the most outward advantages of SV is its independence of the Doppler angle for flow velocity computations and its simplicity, with little additional computational complexity. In addition, as it is intrinsically based on contrast, SV techniques have a potential benefit over fluorescence microscopy, especially when neovasculature exhibits increased permeability. However, there are issues relating to the interpretation of the variance results obtained. The calculated variance is on the range of $\pm \infty$ and is dependent on the chosen window size. Thus, variance itself does not directly indicate flow and *a priori* structural knowledge is required to separate static and dynamic regions. In addition, SV-OCT suffers from multiple scattering induced artifacts and interframe bulk tissue motion.
[459]; this can lead to spurious values of artificial contrast wrongly indicating the presence of vessels beneath real blood vessels.

SV-OCT imaging has been reported in the dynamic monitoring of antivascular treatment effects (e.g. photodynamic therapy) [460]; analysis of the developing embryonic mammalian environment [461]; and with spectroscopic OCT for blood oxygen saturation analysis of the chorioallantoic membrane [457].

3.3.3.3 Optical micro-angiography

Since the spectrum acquired in SdOCT is a real function, the Hermitian symmetric complex conjugate ambiguity which arises from Fourier transformation causes a mirror image that limits the overall scan range. Extracting the full range complex signal would effectively double the imaging range. Optical micro-angiography (OMAG) is a recently developed technique reliant on endogenous contrast, which effectively separates the moving and static scattering elements of tissue to achieve 3-D high-resolution blood flow images approaching that of conventional histology, allowing for \textit{in vivo} perfusion assessment.

![Figure 36](image)

\textbf{Figure 36:} In vivo cerebral blood flow in cortical mouse brain using OMAG. \textit{(a)} 3-D microstructural image (8 mm$^3$) where the skull bone, meninges, and cortex are delineated; \textit{(b)} x–y projection image of directional blood flow network within the scanned volume in \textit{(a)} [462]. (Scale bar = 500 μm.)

In OMAG, spectral interferograms are modulated by a constant Doppler frequency (e.g. by a piezo-translation stage), thereby making separation of the moving and static scattering components within the sample feasible. The mathematical analysis involved essentially maps velocities moving into the tissue away from the surface into one image and velocities moving out of the tissue toward the surface into a second image by means of the Hilbert transform and subsequent fast Fourier transform [449]. This effectively separates the signals from moving constituents and bulk static tissue in frequency space. A further advancement of the OMAG modality came in the form of Doppler OMAG (DOMAG), a method which encompasses the advantages of both OMAG and the phase-resolved OCT methods [463]. In DOMAG, a digital frequency modulation approach is implemented to provide high-resolution 3-D bidirectional flow mapping within highly scattering media. It effectively eliminates the requirement of capturing two 3-D interferogram data sets, thereby reducing the computational
load and thus increasing the potential temporal imaging resolution for *in vivo* imaging studies [462].

As OMAG does not make use of OCT signal phases to assess the blood flow, it tolerates inevitable sample movement and tissue optical heterogeneity, thus limiting noise production [462]. OMAG and DOMAG have been demonstrated in the non-invasive assessment of cerebrovascular circulation in mice [449, 462, 464]; the retina [465, 466]; cutaneous skin assessments [467]; and in the diagnosis of renal pathologies using ultrahigh-sensitive optical microangiography (UHS-OMAG), with detectable velocity values ranging from ~4 μm/s to ~30 mm/s [468]. However, due to the inherent oversampling of data required for OMAG operation, recent computational advances using a modified Hilbert transform without the use of spatial frequency modulation have permitted volumetric bidirectional flow mapping without manual segmentation [469].

### 3.4 Advancements in OCT flow assessment

Recently, techniques have been proposed for flow velocity investigations that are distinct from those which utilise phase information, frequency shifts or Doppler broadening of bandwidth. Time-varying speckle is manifested as a change in OCT image spatial speckle frequencies; methods have utilised this fact to provide quantitative flow information *in vitro* [470].

Auto-correlation methods for quantitative mapping of transverse particle-flow velocity have been proposed [471], which employ the statistical nature of the intensity fluctuations of backscattered light modulated by (stochastically) flowing particles [472]. These methods are derived from the theoretical analysis of fluorescence correlation spectroscopy [473]. Such methods are clearly advantageous, e.g. phase-resolved Doppler OCT which has a limited velocity dynamic range caused by phase wrapping [474]. Such methods have been further applied as a means of non-invasive measurement of concentration in non-flowing particle suspensions governed by Poissonian distributions [475].

Means of calculating absolute flow velocities without explicit knowledge of vessel angles have recently been reported by employing the use of surface integrals along the x-y plane [476, 477] (see Figure 37) and by maximum intensity projection correlation of coincident pixels in multiple OCT B-scans [478].
OCT techniques which encompass cross-correlation have recently been introduced [479], in which a 2-D cross-correlation between two spatially distinct images was performed. However, in practical application, the measured velocity range was smaller than predicted; this error was attributed to the quasi-synchronous manner of image acquisition. This may be improved upon by continuously analysing the specimen using multiple, spatially disparate imaging points [480]. By performing cross-correlation analysis of flow induced fluctuations of the OCT signal, transit times may be obtained and ergo velocity values for particle flow. Methods which utilise the time scale of random fluctuations in the dynamic scattering component related to RBC\textsubscript{v} have recently been reported in quantitative studies of capillary haemodynamics [481].

Figure 37: (a) OCT maximum intensity projection angiogram of the rat somatosensory cortex vasculature at 12 μm transverse resolution; (b) Magnified area as shown in (a) [476].
4 Instrumentation & theoretical development

The present chapter will outline the development of the db-SdOCT system in addition to an in-depth overview of the theoretical development of the associated cross-correlation algorithm for the quantification of blood flow velocity. The optical design considerations are discussed and compared with pre-existing dual-beam optical modalities. A detailed description of the working principle of temporal correlation mapping is provided in addition to the general OCT processing method and system software automation. The associated protocol for system calibration (i.e. spectral and dual-beam) is also presented.

4.1 Introduction: Primary aim of research

Non-invasive in vivo imaging modalities have obvious advantages for the clinical realm; noteworthy are their ability to provide clinically relevant information without disturbing the normal biological environment. Great attention has been paid in the past two decades to the measurement of flow velocity on the micrometer scale and many techniques for assessing the blood supply have been investigated. This is borne out of recognition of the vitally important role that blood flow plays in the health of the individual [482].

The delay and magnitude of backscattered or remitted light detected by an Optical Coherence Tomography (OCT) system carries information pertaining to the axial structure of semi-transparent objects. OCT has a ubiquitous presence, having emerged in a variety of clinical fields: ophthalmology [430, 431], intravascular imaging in cardiology [433, 434], oncology [435, 436], gastroenterology [437-439], general dermatology [440, 441], dentistry [443, 444], and gynaecology [445, 446] to name but a few. Functional extensions of OCT have emerged: polarisation-sensitive systems are used, for example, in the diagnosis of neoplastic processes; differential phase-sensitive methods for use in photorefractive surgery; full-field advancement of the conventional single-point OCT detection technique; and endoscopic OCT. Fundamentally, OCT is an important clinical asset as it can be used to separate three-dimensional (3-D) structural and functional information in individual microvessels.
### 4.1.1 Doppler angle-dependent dynamic assessment

The use of the Doppler functionality has been the predominant force for the quantification of moving particles within media. Doppler ultrasound, the underlying principle upon which Doppler OCT (DOCT) is based, was developed as a tool for vascular disease assessment and monitoring. However, despite the advancements of DOCT techniques, a principal limitation still exists: the Doppler method is inherently 1-D. The “true” velocity is extracted from this 1-D information by assuming the direction of blood motion, resulting in an inaccurate determination of the flow velocity [448]. In many applications, a precise estimation of the Doppler angle is difficult particularly when the flow is embedded in a highly scattering medium, e.g., *in vivo* blood flow monitoring [483]. However, Doppler phase shift assessment of velocity values requires that the angle between the incident light source and the vessel in question be known *a priori* (see § 6.2). In conventional DOCT systems, accurate determination of flow velocity is limited; the Doppler shift simply vanishes for flows in the transverse direction. Proskurin *et al.* described a method which utilised the temporal fluctuation of the remitted interference signal (transversal component) and the Doppler shift (longitudinal component) to determine the fluid flow velocity vector. The method was capable of quantifying the velocity perpendicular to the flow direction but was error prone at low velocities. In addition, Doppler bandwidth and power Doppler techniques do not discriminate flow direction [35].

Due to the extensive tortuosity of the microvasculature (especially if ailments which alter the physiology are present), the Doppler angular dependency may lead to incomplete vascular maps *in vivo*. For some angular regions close to the perpendicular, a minute change in vessel angle with respect to the incident beam can impact greatly on the value of the measured velocity [484]; however, some progress has been made in this area. For example, circumpapillary scanning of the retina has enabled direct extraction of angles from the measurement volume [485]; nonetheless, this increases the computational requirements of the processing. It has been shown that Doppler techniques are only appropriate for larger blood vessels with faster flow rates, in which Doppler shifts are easier to detect [366]. Furthermore, as a Fourier transformation is usually performed by sliding short-time windows (see §6.2.1), tradeoffs between axial and velocity resolutions exist in the flow estimation [251]. DOCT is also inherently sensitive to the phase stability caused by both the system and the measurement environments [449].
In an effort to surmount the restrictions imposed by angular uncertainties, the subject of these studies pivots upon the design and development of an in-house cross-correlation dual-beam SdOCT (db-SdOCT) system. The proposed method operates by quasi-simultaneous illumination and measurement of two distinct planes; this forms a miniature time-gate. By analysis of light intensity fluctuations at two points a known distance apart, transit times may be deduced via temporal cross-correlation, thereby yielding velocity values irrespective of vessel tortuosity. As DOCT methods are sensitive to motion normal to the incident beam, this technique eliminates the need for phase sensitive detection and instead utilises the temporally evolving phase itself as a metric for quantifying velocity by statistical means. A comparable may be drawn between this method and that of single cell cytometric analyses [309].

4.1.2 Dual-beam methods

Several investigations have reported the use of a dual-beam optical imaging modality [486, 487]. The classic interferometric setup of partial coherence interferometry (PCI), and hence OCT, has the drawback of being sensitive to longitudinal object positions, which can result in signal degradation and the insufficient suppression of mirror terms [488]. Efforts to overcome such limitations in ocular media have been reported, in which the cornea was used as a reference surface in a common path dual-beam interferometric arrangement [489]. A dual-beam Doppler spectral domain OCT (SdOCT) system which eliminated the ambiguity associated with unknown orientation of local velocity vectors in the blood vessels of zebrafish has been reported [490].

Recent work has been proposed [491, 492], which outlines a dual-beam scan Doppler optical coherence angiography method (see Figure 38).

![Figure 38: Mosaic of en-face images of the retinal vasculature captured using dual-velocity optical coherence angiography [491]; two different scanning beams with spatial and temporal separation provide two measurable velocity ranges enabling higher sensitivity to very low velocity flows.](image-url)
A similar technique has also been reported [493], in which two laterally displaced sample beams make it possible to apply phase-resolved Doppler analysis. However, these methods are sensitive to involuntary sample motion. Regardless, dual-beam methods have been reported to yield an increased dynamic range for detectable velocities [102] and are capable of providing useful microstructural information, in addition to providing high spatial resolution velocity measurements [494].

4.2 Optical design considerations of dual-beam OCT (db-SdOCT) system

The SdOCT system used in these studies was an in-house built, dual-beam design as depicted in Figure 39. The light source primarily used (see §5.4) was a super luminescent diode broadband source (*Denselight, Singapore*), with a center wavelength of 1310 nm and a spectral width (FWHM) of 130 nm (axial resolution, Δz = 5.83 μm). The system was split into two separate sub-systems via fiber beamsplitters (50/50 and 99/1 varieties; 99% of this was fed to the sample arms). Each sample arm had its own independent reference arm. The two sample arms were then coupled onto a bulk optic beamsplitter (50/50) and then to a 2-D galvo arms system (sGM) for raster scanning through the system objective lens (LSM03; *Thorlabs GmbH*). Polarisation controllers were used to match the polarisation of the reference and sample arms by stress birefringence and to optimise fringe contrast [495]. Conventional lenses have a focal length that varies with wavelength and thus focuses ultra-broadband light to different planes. This variation in focal position for different wavelengths alters the local effective bandwidth and therefore degrades resolution [372]. For specially corrected achromatic optics, different imaging distances introduce a wavelength-independent attenuation of the whole spectrum, thereby maintaining the spectral shape, bandwidth and therefore axial resolution.

A single galvo arm (aGM) was utilised in one of the arms preceding the bulk beamsplitter for precise positioning for relative beam separation calibration. An optical switch (see §4.2.1) was employed as a means of discriminating the interferometric data of each channel for detection by a digital line scan camera (InGaAs, f_{max} ≈ 47 kHz; *Goodrich, CA, USA*), providing full depth ranging for each channel. The camera was preceded by a spectrograph centred at 1310 nm (*BaySpec, NJ, USA*). Automation of the system was implemented in LabVIEW (*National Instruments, UK*), which controlled sequential system triggering, data acquisition and collection.
Sensitivity to flow is inversely proportional to the acquisition speed. Hence the flow sensitivity of Doppler FdOCT becomes low owing to high acquisition rates [492]. To improve sensitivity, acquisition speeds have to be decreased or multiple scans taken of the sample position; neither is an attractive prospect as both reduce the scanning speed of the system. However, by utilising a dual-beam configuration, the same area of illumination as in conventional OCT may be approximately scanned in the same time (as the achievable switching time approaches zero). Yet, applying this in hardware requires a number of considerations.
In order to implement the previously discussed optical system, the alignment, data acquisition and processing for two separate interferometric set-ups needs to be considered. This outwardly increases the complexity of the system and as such a means of discriminating the data obtained by both planes of illumination is required. In the previous discussion regarding dual-beam methods (see §4.1.2), the modalities considered both common-path arrangements and separate hardware two-beam systems. However, a common trend present in all of the optical implementations was the use of either: (a) multiple light sources [494], spectrometers [493] and cameras [491, 492] for separate analysis of both sample signals; or (b) modulation such that separate polarisation states may be analysed from Wollaston prisms [496]. Although this enables the simultaneous acquisition of data from both sides of the dual-beam configuration, it greatly increases the costs involved in addition to the bulk and size of the system which, if implemented in a clinical setting, could cause issues. In this work, a single light source, spectrometer and camera are utilised and a solid state (2 × 1) MEMS optical switch (model: OSW12-1310E-APC-SP; Thorlabs, GmbH) is used to discriminate the optical data from each plane of illumination, which exhibited exceptionally low insertion loss (0.7 dB) and channel cross-talk (75 dB). In order to successfully separate data using such a set-up requires appropriate triggering, supplied by LabVIEW automation of the system; see §4.4.

4.2.2 Dispersion correction and system calibration – remapping to k-space

As outlined in §3.2.2.1, in general, the SdOCT spectra obtained are not necessarily evenly spaced in k-space [372]. This implies that any depth profile computed without calibration would yield inaccuracies, due to the unevenly sampling of data. Spectral calibration means undertaking an accurate assessment of the wavelength corresponding to each spectral element. Determination of this wavelength mapping is of vital importance; in addition to the implications of the depth profile, incorrect wavelength mapping generates a depth-dependent broadening in the structural OCT image.

Recently there have been trends in calibration published procedures which describe the use of extraneous equipment (optical spectrum analysers, light sources, etc.) in determining the mapping relationship of the obtained spectrographs. A sophisticated calibration method using specific spectral lines and a third-order polynomial fitting to determine the wavelength-pixel number relationship has been reported; the method is simple and accurate, but requires an additional light source [497]. Although the use of calibration light sources is a reasonably
straightforward method, the introduction of such equipment may ultimately lead to flaws in
the obtained data, as incorporation of instrumentation with its own calibration procedure,
limitations and elements of noise may affect the results. A parametric iteration method alters
the wavelength assignments until the intensity modulation is a perfect sinusoid as a function
of $k$; however, this process is of low efficiency [498]. Employing calibration methods which
make use of only the equipment present can eliminate the prospect of errors arising from
extraneous sources. In addition, the calibration procedure used may be necessary for each
measurement session because of thermal and mechanical instabilities of the spectrometer.
This is not the most practical means of assessment, especially in a clinical setting where
processing times are at a premium and bulky calibration equipment would be a hindrance.

When represented as a function $k$, interferogram fringes should be perfectly periodic. In
order to achieve this, the phase (or argument of the sinusoidal oscillation) is linearly related
to $k$. A conversion of raw spectrographs from $\lambda$-space to $k$-space is essential to avoid
deterioration of axial resolution and signal-to-noise ratio (SNR) [390, 499]. The detected
mutual interference signal between the sample and reference arms at the spectrometer of a
fiber-based Michelson interferometer may be expressed as:

$$I_m(\lambda) = 2a_Ra_SS(\lambda)\cos(2kz + g(\lambda))$$  \hspace{1cm} (54)

where $k$ is the wavenumber; $S(\lambda)$ is the spectral density of the light source; $a_R$ and $a_S$ are the
amplitude reflecting coefficients of the reference and sample mirrors respectively; $z$ is the
relative optical path difference between the two arms; and $g(\lambda)$ represents dispersion
mismatch. The Hilbert transform extracts the phase of the spectral signal; however, if a
dispersion mismatch between the sample and reference arms exists, the phase retrieved via
Hilbert transform would be incorrect if the dispersion was wavelength dependent. After
Hilbert transformation, the complex interference signal may be represented as:

$$\hat{I}_m(\lambda) = 2a_Ra_SS(\lambda)e^{j\phi(\lambda)}$$  \hspace{1cm} (55)

where $\phi(\lambda) = 2k\Delta z + g(\lambda)$. If two interferograms are obtained with a known optical path
difference between them, subtraction of the phases from the two interferometric signals can
remove the influence of dispersion mismatch, provided $g(\lambda)$ is not changed with respect to
the optical path difference subtraction. There are $2\pi$ ambiguities in the Hilbert transform
because solutions of the arc-tangent function are limited to a value ranging from ($-\pi$ to $\pi$).
A simple phase unwrapping method can be implemented, which adds multiples of $\pm 2\pi$ when
absolute jumps between consecutive elements of wrapped phase obtained by the Hilbert
transform are greater than the default jump tolerance of $\pi$. If the phase ($\phi(\lambda)$) of every pixel is known and the data are separated by a known distance $z$, the wavelength distribution may be determined by the following equation:

$$
\phi(\lambda) = \frac{4\pi}{\lambda} \Delta z \tag{56}
$$

where $g(\lambda)$ has been compensated for. It can be seen from Figure 42 that upon calibration, in addition to being evenly sampled in $k$-space, the data results in narrower width and high amplitude point spread functions (PSF) than the uncalibrated data. The calibration coefficients which result from this linearisation process become a standard portion of the OCT processing algorithm; this is discussed in full in §4.4.

**Figure 40:** (Top) Wrapped phases of data corresponding to two different optical pathlengths and (bottom) unwrapped phases of experimental calibration data.
Figure 41: Values for the real phase as determined by the dispersion subtraction method (top), from which the corresponding wavelengths are determined. (Bottom) Calculation of the wavelength-pixel number relationship, computed using the calibration coefficients.

Figure 42: Sample A-scans resulting from the iFFT of calibrated and uncalibrated data for the different mirror positions. The calibration procedure resulted in axial points spread functions (PSFs) with higher amplitudes and narrower widths (red, blue), compared to uncalibrated data (green, teal).
4.3 Correlation mapping OCT

As outlined in §2.5, optical correlation techniques quantitatively measure flow, are insensitive to detector noise and are non-invasive [500-502]. For example, fluorescence correlation spectroscopy (FCS) was originally developed as a microscopy method suited for measuring macromolecular transport properties and concentrations [503]. The time variation in collected photon counts directly reflects fluctuations in the concentration of fluorophore molecules within the defined beam observation volume. Image correlation methods are imaging analogues of FCS.

Our group recently proposed a non-invasive high sensitivity imaging technique capable of visualising capillaries without the use of exogenous contrast agents; this was termed correlation mapping OCT (cm-OCT) [478]. The technique demarcates static from transient and altering aspects of the microvasculature by calculating correlation values between adjacent A-scans (and in fact maps the decorrelated regions) of structural images. In addition to successfully revealing flow regions from structural data, this method was shown to be capable of detecting Brownian motion and extracting parameters such as capillary density and vessel diameter. Although the method was successful in a qualitative sense, it was not, however, capable of providing a quantitative view of the flow involved and was therefore limited. Thus, the construction of a new system model and the advancement of correlation based assessment of OCT data for the analysis of subject dynamics and velocity quantification was required.

4.4 Cross-correlation algorithm development for dynamic analysis

A univariate signal is a single observed variable that varies as a function of time or position. Stochastic (or random) implies that the measured signal changes with every repetition of an experiment; however, the fundamental process which generates the signal in question does not change. As such, OCT data may be considered as a stochastic univariate time series with chronologically ordered observations at regular intervals [504]. The covariance between two random variables $X$ and $Y$ may be defined as:

$$
\text{cov}(X, Y) = E[(X - \mu_X)(Y - \mu_Y)]
$$

(57)

where $E$ is the expectation operator; and $\mu$ is the respective mean.
The correlation coefficient of the normalised covariance may be expressed as:

\[
\rho_{X,Y} = \frac{\text{cov}(X,Y)}{\sigma_X \sigma_Y}
\]  

(58)

where \(\sigma\) is the respective standard deviation; and \(|\rho_{X,Y}| \leq 1\). A negative correlation coefficient has a tendency for the signs of \(X\) and \(Y\) to be opposite, whereas a positive correlation is obtained by a pair with the same sign. Independence implies that the correlation coefficient equals zero. Flowing particles induce modulations in the magnitude of remitted light detectable by an OCT system. The durations of these modulations vary inversely with transverse velocity \([471]\). In addition, for the case of slow particle flow, random Brownian motion and the stability aspects of the experimental set-up may influence the backscattered light received. Consequently, given the statistical nature of these fluctuations, this information may be harnessed and utilised to provide functional information pertaining to a dynamic system.

An image time series recorded from detection channel \(A\) and \(B\) may be considered to be a matrix of raw OCT intensity values stored as a function of pixel location \((j)\) and of image number \((n)\):

\[
I_{A|B}(n) = OCT_{A|B}(j,n)
\]  

(59)

where \(OCT_{A|B}\) refers to the raw OCT data obtained for channel \(A\) and \(B\) of the db-SdOCT system; \(j\) ranges from \((1:1024)\) pixels of the linear CCD; \(n\) ranges from \((1:N)\), \(N\) being the total number of A-scans acquired. An equivalent representation of this discrete intensity matrix is as a function of image space and capture time for each channel of the dual-beam system:

\[
I_{A[i+(i-1)]} = OCT_A(j,T/2) \quad I_{B[2i]} = OCT_B(j,T/2)
\]  

(60)

where \(i\) ranges from \((1:N/2)\); and the total time for acquisition of both channels is \(T = N \cdot \tau\). As can be seen from Figure 39, only one camera and spectrograph is utilised in the system. In order to alternately present each channel’s data for detection, it was necessary to apply appropriate triggering to the system (LabVIEW; National Instruments, UK) to ensure no channel overlap, which would otherwise render unusable data. To do this it was necessary to take into consideration the switching time between measurements. This switching time \((\tau)\) was deduced by:

\[
\tau = T_{OSWF} + t_A
\]  

(61)

where \(T_{OSWF}\) is the reciprocal of the optical switching frequency and \(t_A\) is the acquisition time. The transit time, \(t_t\), is the theoretical time taken for a particle to pass through both
planes of illumination of the db-SdOCT system a fixed distance apart (\(\delta\)), at a particular velocity; this is \(t_t = \delta/v\). As a general rule, the total number of A-scans acquired was taken to be \(10(t_{t,min}/\tau)\) to ensure sufficient data was obtained for correlation measurements, where \(t_{t,min}\) is the transit time for the smallest experimental velocity value considered.

All data was acquired via a TDMS\(^\ddagger\ddagger\) file in LabVIEW, a file type which is portable to other applications such as Excel. Due to the optical switching, the data was collected in alternating columns and as such was easily separated for correlation computation. All odd and even columns were grouped together as two individual datasets representing the OCT data acquired by Channel A and B (ChA, ChB), containing the information about either channel’s structural (and therefore functional) information. Upon acquiring each image of \((y \times 1024)\) pixels (where \(y\) is the number of vertical pixels per image), this was averaged along \(y\) to mitigate the effects of noise. The dynamic aspects of the intraluminal data were considered, as outside of the lumen of the vessel, e.g. at the vessel walls, there (ideally) occurs no change in state with time thereby rendering high correlation values irrespective of the flow within (see §5.1.3, Figure 52). The haemodynamic relevance of intraluminal measurements particularly in cardiovascular high-risk patients with type 2 diabetes has recently been reported [505]. The intraluminal column data of each channel was cross-correlated in time by means of the following expression:

\[
\frac{\sum_{i=1}^{p}(l_{Ai} - \bar{l_A})(l_{Bi} - \bar{l_B})}{\sqrt{\sum_{i=1}^{p}(l_{Ai} - \bar{l_A})^2 \sum_{i=1}^{p}(l_{Bi} - \bar{l_B})^2}}
\]

where \(c_{AB}\) refers to the relative correlation values obtained with between ChA and ChB; \(k\) is the range from \((1:N/2)\); \(p\) is the total number of intraluminal pixels chosen for correlation testing; \(\bar{l}_{A/B}\) implies the average of the intraluminal pixels considered. The resulting correlation values are of the range of \((0 \pm 1)\) indicating weak and strong correlation, respectively. As the flow rates applied generated laminar flow within the capillary (calculated as having \(Re < 2000\), where \(Re\) is the Reynolds number) it is anticipated that for a specific flow rate, values of high correlation to occur at a certain time difference between channels. As each correlation calculation represents a different point in time, instances of maximal correlation between channels would occur at a later time for slower values of flow.

\(^\ddagger\ddagger\) TDMS: a binary file format which is easily exchangeable, inherently structured, and capable of high-speed streaming
and vice versa. Consequently, temporal correlation maps (see Figure 43) were computed which illustrate relative correlation values with time providing a visual means of correlation assessment with time for different flow rates.

After computation of a correlation map, the temporal locations of correlation maxima for each column of the resulting map were averaged for the intraluminal data considered, yielding the transit time, $t_t$, for flow passing between both illumination planes in succession. As for Doppler related processing procedures in which the centroid of the power spectrum was taken as the Doppler frequency [96], analogously the mean of the transit time obtained for all A-scans using the cross-correlation method was used to compute the velocity value. Lastly, division of the relative separation distance $\delta$ by this value of $t_t$ yields a value for the transverse velocity.

### 4.4.1 Processing outline of OCT data prior to correlation analysis

Prior to the correlation procedure outlined above, a preceding computational OCT analysis is performed. To eliminate the reference power term, the spectrum from only the reference arm is detected and subtracted from the interference spectrum. This spectrum is acquired at the beginning of every image acquisition to account for fluctuations in the source between measurements. The interference of sample with reference arm light generates a modulation of the spectrum that is associated with structural properties of the sample. In addition, pixel response in the CCD camera and spurious etalons in the interferometer can cause modulations of the spectrum, leading to structural artifacts which may be constant or fixed over many spectra. This fixed pattern noise is removed by generating a reference spectrum by averaging (or obtaining the median, as suggested by [506]) over spectra (two reference spectra are computed, specific to either ChA or ChB) and each individual spectrum is divided out by the relative averaged background spectrum.

A limitation of SdOCT is the broadening of the point-spread function with increasing imaging depth. As such, a spectral calibration method as mentioned in §4.2.2 is implemented to optimise system sensitivity and signal quality; if not performed, a negative effect on system resolution would be evident, compromising the effective field of view in the axial direction and may induce artifacts known to hinder e.g. diagnostic sensitivity and specificity. After spectral calibration of each side of the db-SdOCT system is performed and coefficients computed, a $k$-space interpolation and sequent FFT was applied. Light passing through a vessel containing moving particles may induce forward scattering of light and thus
shadowing artifacts in the resulting velocity image and may make Doppler images noisy at deeper depths due to high scattering [507] – see Figure 44.

Figure 43: Illustration of computational analysis flowchart of intraluminal capillary data. Cross-correlation analysis of each channel yields a temporal correlation map, from which regions of maximal correlation may be seen. Computation of the maximal correlation values between channels renders a time difference if multiplied by \( \tau \). Division of this into the predetermined value for the beam separation \( \delta \) yields values for transverse velocity.
For these studies, both amplitude and phase information were saved for further comparative analysis. OCT signal phase information denotes the argument of the sample structure terms and contains the optical path length difference between the reference and sample interface, as well as any phase shift due to scattering or reflection. By using a defined sample interface as a phase reference, one can exploit the potential of having the exact, quantitative phase information at hand. It has been shown that by comparing the phases of successive depth profiles at the same sample location, the conventional SdOCT setup has sufficient sensitivity to measure depth-resolved vibrations with nanometer and even picometer precision [508-512]. The phase ($\theta$) was deduced by the following:

$$\theta(j,n) = \tan^{-1}\left(\frac{\text{Im}[\text{OCT}_{\text{Air}}(j,n)]}{\text{Re}[\text{OCT}_{\text{Air}}(j,n)]}\right)$$  \hspace{1cm} (63)

where $\text{Im}$ and $\text{Re}$ indicate the imaginary and real components respectively. All custom computational processing was performed using Matlab (R2007b, The Mathworks Inc.).

### 4.4.2 Effects of dual-beam sample arm spatial separation

Microcirculatory blood flow measurable using laser Doppler flowmetry has been shown to be influenced by tissue optical properties and probe geometry. In such investigations, it has been found using both theoretical Monte Carlo simulation models [511, 513] and sophisticated tissue phantoms [514, 515] that the sampling depth increases with increasing separation between the emitting and the receiving fiber. Consequently, such systems could be used to discriminate between superficial and deeper blood vessels. In addition, it has been found that fluid velocity, turbulence, Brownian motion, and probe beam geometry contribute to broadening of the Doppler spectrum in coherence based methods. Broadening, due to
particle Brownian motion, dominates at low fluid velocities. At high velocities, Doppler spectrum broadening is dominated by the temporal fluctuation of the interference signal caused by the scattering particles passing through the probe beam [516].

In the vasculature, it is commonly accepted that RBCs are not evenly distributed, but are rather aggregated into rouleaux, i.e. resembling stacks of coins [483, 517, 518]. Stemming from Taylor’s frozen turbulence approximation (see §5.5) [23, 519], it may be implicitly assumed that such structures (e.g. particles) move en masse along vessels. In the case that a particle turns in an arbitrary direction, the probing points must be arranged closely such that a particle passes through the two scattering regions in succession without decorrelation. Additionally, if the beam separation was large and a vessel was particularly tortuous, the separation between the two beams could not be stated with certainty, as the distance would be a projection with respect to the angular position of the vessel (see §6.2.2). By keeping the beam separation small, adjacent sections may be considered parallel and velocity values may be estimated. Therefore, an informed δ value is required. Deducing this would involve: (a) maximising the value of δ such that stray reflectance from either sample arm did not induce false intensity fluctuations in the opposing beam; and (b) minimising the value of δ such that within that distance flowing particles underwent ‘flow without flip’.

As such, a calibration is performed using a beam slit profiler to track beam separation values such that the impact of this varying separation on velocity values obtained by correlation may be characterised (see §5.1); details regarding this calibration procedure are outlined in the next section.

4.4.2.1 Calibration of sample arm separation by beam profiler

In order to quantify distances of separation (δ) between the two beams of the db-SdOCT system, a calibration of movement was performed (see Table 4, Figure 45 and Figure 46) using a slit scanning beam profiler (model: BP109-IR; Thorlabs GmbH) placed at the focal plane of the objective lens and a single galvo mirror scanning arm (aGM) used for angular control. The movement of aGM (see Figure 39) was controlled via a USB connector block (National Instruments, UK) and an applied voltage. The values for the centroid position of the intensity of each beam with increased voltage yields the relative separation values (δ). As the input voltage values supplied were small, the voltage across the terminals of aGM was also monitored for consistency, as a small change in voltage could render an incorrect value of separation (in this case this was found to be 10 μm/10 mV).
Figure 45: Screenshot of BP109-IR beam profiler used for the calibration of dual-beam separation distances.

Table 4: Sample experimental values for beam separation calibration. Voltage applied to ChA (highlighted) via aGM ($V_{\text{input}}$). The voltage across aGM is monitored independently ($V_{\text{meas}}$) for any differences. The resulting movement is tracked vs. voltage and a relationship between $\delta$ and voltage is established (see Figure 46).

Theoretical values are then computed (shown in bold) and used in subsequent experimentation.

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<th>$y$ (μm)</th>
<th>$x$ (μm)</th>
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Figure 46: (a) Graph of voltage vs. beam separation $\delta$; voltage values were thus calibrated for specific sample beam separation values. Inset: Graph of input voltage to aGM vs. the measured voltage across its inputs. (b) Illustration of the 2-D reconstruction of each sample arm channel on the beam profiler for analysis.

4.5 Summary

Presented in this chapter is a detailed outline of the construction, application and processing procedures associated with the described db-SdOCT system. An optical switch is employed as a means of discriminating the interferometric data of each channel for detection by a digital line scan camera and in this way did not necessitate the requirement of having separate equipment for each side of the dual-beam system, thus substantially reducing costs and bulk system size.

Statistical information discernible from OCT data has been used for other purposes. For example, in FCS, the mean-squared intensity fluctuation divided by the squared mean image intensity is equal to the reciprocal of the mean number of independent fluorescent particles per laser beam volume [520]. Qualitatively and quantitatively, transient intensity changes have been used previously to discern various metrics concerning in vitro and in vivo environments [521].

Doppler optical coherence tomography (DOCT) methods have an inherent trade-off in flow sensitivity with acquisition time, with Doppler frequency resolution increasing and higher spatial frequency content (i.e. slower flow values) for a longer time between successive pixels. Statistical analysis of OCT image dynamics offers an advantage over
DOCT methods because subwavelength motion of a scatterer along the axial dimension is necessary to influence the phase of a signal. However, with regards statistical methods, it is necessary for a scatterer to enter and leave a focal volume in the lateral dimension to induce fluctuations in intensity and/or phase. This is advantageous as statistical based analysis is also capable of yielding transverse flow data, normal to the incident beams’ optical axis. It has been shown that this is ca. one order of magnitude more sensitive to motion parallel to the optical axis [522]. Temporally variant speckle analyses have been shown to be essentially equivalent to Doppler related techniques. However, speckle methods are unable to discern directionality, whereas Doppler measurements can. In an effort to surmount these obstacles and to make use of the outward advantages offered by statistical based OCT methods, the db-SdOCT method has been further extended in functionality to also discern directional data (see §5.3) in addition to providing values for velocity by utilising the OCT signal intensity and phase information in a different manner.

It is true to say that the smaller the separation value $\delta$, the smaller the possibility of erroneous velocity values caused by improperly quoted separation values and by unknown angles within the vasculature. Optimisation of this separation value takes into consideration: (a) Taylor’s frozen turbulence approximation ($x_t$), (b) (i) possibility of decorrelation of fluctuations if over too long a distance, (ii) possibility of inducing erroneous signal fluctuations in either beam ($x_d$). This thus forms a range to optimise the beam separation value to account for both laminar and turbulent flow rate assessment patterns, $x_d \leq \delta < x_t$. Taylor’s frozen turbulence approximation implies that an advected quantity remembers its initial conditions as it is being transported by the Eulerian mean velocity of a larger scale flow [470]. As such, if this distance were to be optimised for the db-SdOCT system in turbulent and laminar conditions, then flow rate assessment would be possible in either scenario (see §5.5). However, to completely disregard the possibility of angularly induced errors occurring is unrealistic, especially given that biological orientation is random, unpredictable and unknown a priori. Nevertheless, by careful optimisation of system parameters, such effects may be mitigated as much as possible and incorporated into computations as standard.

In highly scattering turbid media, spectral broadening [523] may cause the calibrated values for the beam size and ergo relative sample beam separation values to induce errors, especially if the specimen is not placed approximately within the focal plane. In addition, the initial choice of focal volume size (i.e. $l_c \times \Delta x$) may also play a part in the introduction of
unnecessary errors to the assessment of flow dynamics by statistical means. Thus, the use of extended broadband sources which produce superior values of axial resolution may prove advantageous; however, these also induce higher order dispersion effects, although which can be readily corrected [524, 525], may prove to be more computationally expensive (see §5.4).

Spectrometer-based FdOCT systems usually show very good phase stability because no moving parts are used in the system. Although it is generally true that SsOCT systems do not exhibit comparable phase stability as SdOCT versions, improvements have been made in the last several years to the stability of such systems. Reduction of the time-induced phase error due to trigger jitter and improved phase stability [526, 527] has been performed and a modified Doppler algorithm to calculate the Doppler variance for phase unstable situations has been demonstrated [528].

The complete characterisation and application of the db-SdOCT system and algorithm for optimisation of factors such as beam separation, speed and focal volume is the focus of the next chapters, in addition to investigations regarding the application and advancement of the db-SdOCT system for the analysis of pulsatile flow patterns, in vivo research and its potential implementation for endoscopic analyses of deeply situated organs.
5 System characterisation & *in vitro* studies

*Presented in this chapter are the preliminary results utilising the method of temporal correlation mapping applied to in vitro capillary models with 2% Intralipid solution and blood used as the flowing media for the quantification of velocity values and computation of axial flow profiles. The application of auto-correlation techniques to the analytic data to generate directionality data is also presented. In addition, functional aspects associated with system construction and applications are examined: light source focal volume assessment serves to optimise the correlation mechanism and in vitro turbulent models studies the systems’ capability of discerning non-laminar flow behaviours.*

5.1 Preliminary flow velocity experiments

As a prelude to system and cross-correlation algorithm optimisation for *in vivo* purposes, various *in vitro* studies were conducted which evaluated the feasibility of application to such. In an effort to bridge the gap between theoretical formulation and experimental validation, capillaries of various cross-sectional size, materials of varying refractive index, and a broad velocity flow rate range were utilised and examined in the following investigations.

5.1.1 10% Intralipid

Phantoms that model the transport of visible and IR light are needed to evaluate techniques, to calibrate equipment, to optimise procedures and to test theoretical predictions experimentally. Tissue-like phantoms have also been adopted in areas of research connected with therapeutic implementation of optical radiation, e.g. light dosimetry, laser ablation and photodynamic therapy [529, 530]. Phantoms consist of a scattering medium, an absorbing medium and in some cases fluorophores. Some examples of common scattering media are Intralipid, Nutralipid and Liposyn. Absorbing media include biological stains e.g. Indocyanine green, and ink [531, 532]. An optical phantom is constructed by mixing the correct proportions of the scattering and absorbing media in a diluent (usually deionised water), so that the resulting suspension has the desired intrinsic optical properties of simulated tissue.
Intralipid is an intravenous nutrient consisting of an emulsion of phospholipid micelles and water. As Intralipid is turbid, has no strong absorption bands in the visible spectral regions, and is readily available and inexpensive, it is often used as a tissue simulating phantom in light dosimetry experiments [533]. Additionally, Intralipid is versatile and can be used in both solid and liquid forms for dynamic and bulk optical property measurements. As such, solutions of Intralipid are used in flow studies as their motion induces fluctuations in the resulting backscattered light. For example, Brownian motion of Intralipid particles in aqueous suspension have been used for simulated dynamic light scattering for the modelling of skin burns [534]. The scattering media used was 20% Intralipid solution (*Fresenius Kabi Ltd., UK*) diluted to 2% as a means of imitating blood flow within silica glass capillaries of various internal diameter size (see Figure 47).

**Figure 47:** Illustration of experimental configuration of capillaries aligned in a Petri dish.

The capillaries were analysed in a parallel phantom holder; this was a Petri dish, which had minute holes drilled in both sides. Each capillary was fed through these holes and held in place with epoxy resin. Fluid was fed to each capillary by an independent network of tubing. Initial experimental investigations utilised these bare capillaries containing flowing Intralipid as phantoms. To test the behaviour and feasibility of application of the db-SdOCT system, various capillary sizes were used (50:50:200 μm, 200:100:500 μm) in addition to a range of test velocities. Each capillary was positioned in the focal plane of the objective lens (for completeness the angles between the impinging light and the capillary samples were estimated with a goniometer stage; measured as varying between 83.18°-86.03°), illuminated by both sample arm beams.
To provide flow to the capillaries, a syringe pump was used (model: PHD2000, *Harvard Apparatus Ltd.*, USA; accuracy: ±1%, reproducibility: ±0.1%, min flow rate 0.0001 μL/hr). The Intralipid solution was pumped through each capillary with average velocities ranging from 0.5 to 10 mm/s; these values are half the anticipated peak velocity assuming a parabolic flow rate and were converted to the appropriate analogue syringe pump rates (mL/min) (see Table 5). Varying distances of dual-beam displacement were also investigated for such velocity values (see Figure 49 and §4.4.2.1). Applied flow rates within the capillaries were allowed 5 minutes settling time before measurement acquisition via the db-SdOCT system in order to avoid any unnecessary turbulent effects which may arise and may ultimately affect the resulting correlation computation.

Table 5: Example experimental values for a 50 μm capillary; calibrated beam separation (δ) and the respective input voltage (V_input); and desired velocity values (v̂) converted to syringe flow rates (Q) for pump infusion.

<table>
<thead>
<tr>
<th>δ (μm)</th>
<th>V_input (V)</th>
<th>v̂ (m/s)</th>
<th>Q (m²/s)</th>
<th>Syringe pump flow rate (mL/min)</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td>0.0334</td>
<td>0.0005</td>
<td>9.817E-13</td>
<td>0.00006</td>
</tr>
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<td>10</td>
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<td>0.0010</td>
<td>1.963E-12</td>
<td>0.00012</td>
</tr>
<tr>
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<td>0.0015</td>
<td>2.945E-12</td>
<td>0.00018</td>
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<tr>
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<td>0.0020</td>
<td>3.927E-12</td>
<td>0.00024</td>
</tr>
<tr>
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<td>0.0025</td>
<td>4.909E-12</td>
<td>0.00029</td>
</tr>
<tr>
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<tr>
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<td>0.0035</td>
<td>6.872E-12</td>
<td>0.00041</td>
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<tr>
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<tr>
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<tr>
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<td>1.178E-11</td>
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<tr>
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<td>0.0065</td>
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<td>0.00077</td>
</tr>
<tr>
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<td>0.0070</td>
<td>1.374E-11</td>
<td>0.00082</td>
</tr>
<tr>
<td>140</td>
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<td>0.0075</td>
<td>1.473E-11</td>
<td>0.00088</td>
</tr>
<tr>
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<tr>
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<td></td>
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<tr>
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<td>0.00112</td>
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<tr>
<td></td>
<td>0.0100</td>
<td></td>
<td>1.963E-11</td>
<td>0.00118</td>
</tr>
</tbody>
</table>

5.1.1.1 Results

As discussed previously, to test the feasibility of the application of the db-SdOCT system and cross-correlation algorithm, various capillary sizes, test velocities and dual-beam separation values were investigated. Both amplitude intensity and phase information was saved and
computed for further comparative analysis. For clarity, all capillary sizes tested and their respective velocity values yielded by cross-correlation computation are truncated into one graph (see Figure 48). As can be seen from the resulting velocity values, utilising the phase as the metric of interest in the cross-correlation based assessment consistently yielded more accurate representations of the theoretical velocities (flow rates) applied; i.e. the average percentage error for intensity-based and for phase-based correlation analysis was 5.13% and 4.65%, respectively.

The effect of dual-beam separation is examined and highlighted in Figure 49. In these experiments, a consistent flow velocity value of 10 mm/s was chosen (flow rates were adapted for the varying capillary cross-sections considered) and the separation was varied in accordance with the voltage calibration values, e.g. see Figure 46. It is evident that for the intensity and phase-based values of Figure 49(a), that smaller values of dual-beam separation value $\delta$ deviate greatly from the imposed flow rates, irrespective of capillary diameter size (a fourth order polynomial fit to the data is used for trend clarity). This graph shows that for a tolerance of $\pm 10\%$ of 10 mm/s (indicted by the dotted lines), the resulting deviations decrease for larger values of $\delta$; in fact, velocity data from all capillary sizes resides within this tolerance for $\delta$ values greater than $\sim 60 \, \mu m$. In an effort to further emphasise this trend, the resulting relative differences obtained for the velocity values were subtracted from unity and analysed with respect to their $\delta$ values; see Figure 49(b) (a fifth order polynomial fit to the data is used for trend clarity). It is clearly evident that for larger $\delta$ values, the data converges towards unity; this coincides with the behaviour seen in Figure 49(a), in that for $\delta$ values $\geq 60 \, \mu m$ the relative difference in error between the theoretically imposed flow rates and those gleaned from correlation assessment are $\leq 10\%$. It is apparent that although most capillary sizes follow a common trend, the data obtained from the 50 $\mu m$ capillary was somewhat removed.
Figure 48: Graphical results of flow experiments using 2% Intralipid solution in in vitro flow phantoms. These phantoms were comprised of silica capillaries of varying internal diameter and thus flow rate. Each capillary was subjected to the same range of velocities provided by the syringe pump to test the feasibility of application of the cross-correlation method in different environments; (a) intensity and (b) phase-based correlation analysis. The velocity test range was 0.5:0.5:10 mm/s and was the same for each capillary; the values are shifted for clarity. Error bars: ±10% of values provided by syringe pump. $\tau = 1.48$ ms.
Figure 49: Graphical representation of the investigation of distance $\delta$ and velocity values obtained by (top) intensity and (bottom) phase-based correlation analysis of 2\% Intralipid flow. (a) A velocity value of 10 mm/s was chosen and correlation was performed for a range of separation distances. It can be seen that lower values of $\delta$ yield greater deviations from the theoretically anticipated flow rate value. (Fourth order polynomial fit used). (b) The relative difference between the data from (a) was obtained; this difference was subtracted from unity. As for (a), increasing $\delta$ values yield values more representative of the flow rate value. (Fifth order polynomial fit used.)
5.1.2  Agar-blood flow phantom

In an effort to create a more realistic version of a tissue phantom than was previously described, the same experimental measurement procedure was repeated for an in vitro phantom of whole blood flowing in capillaries surrounded by a solid tissue mimicking phantom. Blood plasma is the yellow liquid component of blood in which blood cells are suspended and constitutes 55% of the total blood volume. It is composed of mostly water (93%) and contains dissolved proteins, glucose, clotting factors, minerals, hormones and carbon dioxide [535] – see §1.1.2. Whole blood components for this research were supplied by the Irish Blood Transfusion Service (see Appendix C relating to the ethical use of blood products).

The use of solidifying agents to construct phantoms has been documented in various studies, for example, concerning optical mammography [536]. The use of agar as a solidifying agent is desirable as it allows higher hardening of the sample; agar powders are highly pure and are available with almost no absorption and very low turbidity; and as they are used as standard in biological routines, they are well characterised and controlled. A 1% sample of highly purified agar powder (type: A-7049; Sigma, USA) was dissolved in distilled water and heated to a melting temperature of 95°C. As agar has negligible absorption and low turbidity, appropriate amounts of Intralipid as a scattering medium were added to mimic skin tissue. Previous work has shown that tissue phantoms composed of a 2% Intralipid solution aptly imitate skin optically over the wavelength range of 1000-2200 nm [507, 537]. The optimal temperature for adding Intralipid to the warm agar solution is non-critical in the range from 40°C-80°C, so as to avoid any change in optical properties that can occur due to extremes of hot and cold [538]. As such, the addition of Intralipid was done when temperatures were approximately at the 60°C, monitored by a glass thermometer in the solution. The solution was stirred continuously while cooling to 40°C to ensure good uniformity. At this temperature, the solution of agar and Intralipid was poured into the Petri dish containing the array of capillaries (see Figure 47) and carefully added until all capillaries were submerged an arbitrary distance. The solution was solid and usable within an hour.

5.1.2.1  Results

As for the 2% Intralipid experiments, both amplitude intensity and phase information was saved and computed for further comparative analysis of their correlation efficacy. Likewise, all capillary sizes tested encased in the skin mimicking phantom and their respective velocity
values yielded by cross-correlation computation are truncated into one graph (see Figure 50). As for the Intralipid flow experiments, utilising the phase as the metric of interest in the cross-correlation based assessment consistently yielded more accurate representations of the theoretical velocities (flow rates) applied; i.e. the average percentage error for intensity-based and for phase-based correlation analysis for the blood flow phantom was 5.55% and 4.85% respectively.

The effects of dual-beam separation were also examined for these blood flow studies; see Figure 51. As for the Intralipid experiments, a consistent flow velocity value of 10 mm/s was chosen and the dual-beam separation value \( \delta \) was varied. It is evident that for the intensity and phase-based values of Figure 51(a), that smaller values of dual-beam separation value \( \delta \) deviate greatly from the imposed flow rates, irrespective of capillary diameter size (a fourth order polynomial fit to the data is used for trend clarity). This graph shows that for a tolerance of ±10% of 10 mm/s (indicted by the dotted lines), the resulting deviations decrease for larger values of \( \delta \); in fact, data from all capillary sizes resides within the tolerance for \( \delta \) values greater than ~60 \( \mu \)m, as it did for the Intralipid studies. In an effort to further emphasise this trend, the resulting relative differences obtained for the velocity values were subtracted from unity and analysed with respect to their \( \delta \) values; see Figure 51(b) (a fifth order polynomial fit to the data is used for trend clarity). It is clearly evident that for larger \( \delta \) values, the data converges towards unity; this coincides with the behaviour seen in Figure 51(a), in that for \( \delta \) values \( \geq 60 \mu \)m the relative difference between the theoretically imposed flow rates and those gleaned from correlation assessment are \( \leq 10\% \). It is apparent in these studies however, that the behaviour of the 50 \( \mu \)m capillary was consistent with the other capillary sizes. It is also clear that for the phase-based relative difference trend that the phase analysis for all sizes followed a more definite common trend than that displayed by the intensity correlation analysis. The reasoning behind the behaviour is discussed in §5.1.3.
Figure 50: Graphical results of flow experiments using in vitro flow phantoms. These phantoms were comprised of silica capillaries of varying internal diameter, through which whole blood was pumped at known rates. The capillaries were submerged in an agar mixture containing 2% Intralipid and thus forming a skin mimicking model. Each capillary was subjected to the same range of velocities provided by the syringe pump as a preliminary means of testing with a view to in vivo applications. Data was obtained and analysed using (a) intensity and (b) phase-based correlation analysis. The velocity test range was 0.5:0.5:10 mm/s and was the same for each capillary; the values are shifted vertically for clarity. Error bars: ±10% of values provided by syringe pump. τ = 1.48 ms.
Figure 51: Graphical representation of the investigation of distance $\delta$ and velocity values obtained (top) intensity and (bottom) phase-based correlation for various in vitro blood phantoms. (a) A velocity value of 10 mm/s was chosen and correlation was performed for a range of separation distances. It can be seen that lower values of $\delta$ yield greater deviations from the theoretically anticipated flow rate value. (Fourth order polynomial fit used.) (b) The relative difference between the data from (a) was obtained; this difference was subtracted from unity. As for (a), increasing $\delta$ values yield values more representative of the flow rate value, and seem to converge about 100-120 um separation. (Fifth order polynomial fit used.)
5.1.3 Discussion

The present studies demonstrate the potential of using an in-house spectral-domain OCT system with dual-beam configuration (db-SdOCT) for velocity estimation by quasi-simultaneously measuring two planes of illumination. By intensity fluctuation assessment it is possible to derive information pertaining to the motion of transient scattering particles. This method utilises the statistical aspects of OCT data to yield velocity values. The use of the phase of the OCT signal as the input to the correlation computations outperformed that of intensity in terms of robustness, sensitivity and accuracy. However, despite this, the intensity information is also of interest as it can be applied, for example, in cellular systems which can take advantage of the fact that it is sensitive to the aggregation of biomolecules [539].

According to Taylor’s frozen turbulence hypothesis, particles move *en masse* along vessels and if probing points are arranged closely, a particle may pass through these two illumination planes in succession without decorrelation. Characterisation of such beam separations has been shown previously in Figure 49 and Figure 51. As can be seen in Figure 52(a), different flow behaviours within capillaries reveal different patterns in the resulting temporal correlation maps. Slower velocity values find higher instances of correlation at a later time in the opposing channel due to the longer time taken to traverse the dual-beam separation distance $\delta$. At the faster flow rates, a temporal aliasing effect evident in the correlation maps may be attributed to the quasi-simultaneous method of acquisition, dictated by the camera speed and optical switching time. Faster acquisition speeds would allow a larger volume of correlation data to be captured and thus better distinguish statistical similarities in the phase values – this is investigated in §5.4. In addition, by utilising faster acquisition speeds, these high correlation regions may be even more visually apparent. Ideally when performing correlation based analysis, the larger the dataset set used gives a more statistically sound value, as a single spurious data point may influence the resulting computation considerably if the dataset used was very small.

As the experimentation performed in these studies primarily concentrated on the averaged intraluminal data within the capillary to yield velocity values, demarcation of the capillary from its surrounding turbid medium was required. In an effort to test how rigorous this processing procedure was for discerning dynamic from static regions, a region outside of the capillary was segmented (see Figure 52(b)), which was volumetrically the same size as for the intraluminal data investigations; this yielded correlation values of 0.97, indicating the
absence of dynamic constituents. The demarcation of flow from static regions is also highlighted by the techniques’ ability to yield axial flow profiles, §5.2.

![Figure 52](image.png)

**Figure 52:** (a) Examples of temporal correlation maps obtained by the cross-correlation technique for velocity quantification in a 300 μm capillary with flowing 2% Intralipid; both x and y axes indicate time (i.e. $T/2$ for both channels) and the coloured scale indicates normalised correlation values. For slower flow rates, it is evident that correlation between channels occurs at a later time and as flow rates increase, localised regions of high correlation emerge. (b) Application of the temporal correlation method to a region outside of the capillary as in (a). The region size chosen for correlation was the same as the intraluminal data; the mean correlation value for this map was found to be 0.97, i.e. static medium.

A clear emergent trend from these in vitro experiments is the increasing accuracy of the cross-correlation mechanism (irrespective if the correlation metric used is the intensity or phase) at yielding velocity values with increasing capillary size. In terms of justifying this trend, sufficient transient speckle OCT data must be obtained in order to succinctly identify like instances of correlation between the two channels of the db-SdOCT system. As such, increasing the volume of data available for correlation analysis is therefore scaled with the capillary size, as a larger volume of intraluminal data is considered for larger diameters. However, as the segmentation of the intraluminal data is somewhat subjective, the influence of the focal volume of the light source may also play a part in the associated correlation analysis; this is investigated in §5.4.

Media containing solutions of different optical properties influence light differently in terms of absorption and scattering. As such it was instructive to consider the effect such optically variant solutions have on the cross-correlation based method of yielding velocity data. The large scattering coefficient and anisotropy of blood ($\mu_s = 650$ cm$^{-1}$ and $g = 0.995$ [507], respectively) can cause multiple forward scattering thus causing a distortion of the deep speckle pattern [540]; Intralipid ($\mu_s = 4.4$ cm$^{-1}$ and $g = 0.350$ [470]) does not show this effect. The average difference between intensity and phase related velocity error values
obtained via 2% Intralipid flow experiments and blood skin-phantom flow experiments is 0.41% and 0.2%, respectively. Thus, multiple scattering effects which may be caused by solutions with such highly anisotropic capabilities have neither shown to be influential nor impeding in the assessment of velocity values by the techniques outlined here. These effects are more likely to be a problem wherein the probability of a multiplied scattered photon masquerading as a local ballistic photon in deep tissue is significant due to the overwhelming numbers of scattered versus ballistic photons. Multiply scattered photons will show a low correlation as their interaction with the tissue is random.

In general, the results obtained indicate a tentative first step in developing a robust tool for flow velocity quantification by means of cross-correlation. In order to investigate the applicability of the method for in vivo studies, a full characterisation of the system is required in terms of beam separation, functional extensions, and optimisation of the optics involved; this is explored in the following sections.

5.2 Application of cross-correlation method to yield depth resolved velocity profiles

5.2.1 Background

The computation of cross-sectional axial profiles of velocity enables the analysis of dynamic and temporal changes which can occur due to e.g. an obstruction. For example, cross-section blood flow velocity profiles of cerebral haemodynamics of rat pial microvessels have been examined in response to neural activation [541]. In addition, axial profile computation has also played an instrumental role in gleaning knowledge and understanding of biological developmental models, e.g. the chicken embryo [542].

For Newtonian fluid within a vessel, the velocity profile is the well-known parabolic variety (Hagan-Poiseuille flow), and consequently volume flow rate may be inferred from measurement of the centreline velocity. As such, amendments were made to the existing cross-correlation algorithm outlined in §4.4, enabling the windowing of the spectral data to yield depth-dependent velocity distributions. Throughout the course of this work, the feasibility of applying this velocity profiling algorithm was investigated and applied. In §5.2.2, a detailed outline of the computational considerations is provided and in §5.2.3 the results of some of the resulting profiles is provided, stemming from the in vitro Intralipid and blood phantom studies of §5.1.
5.2.2 Computational considerations

As previously documented, velocity values are computed by obtaining the mean of the intraluminal correlation-derived OCT data. However, as this provides a single value indicative of the dynamics present, a means of discerning axial values in order to visually represent the spatial dynamics taking place is desired. In an effort to achieve this, a computational extension was considered based on the original cross-correlation code described (see Figure 53). In place of an intraluminal average value, axial values were computed by windowing segments of the intraluminal data and these segments were cross-correlation processed individually. A range of window sizes are considered for each computation and the values are plotted corresponding to their respective axial positions in depth for the capillary under test. Data was obtained in the usual manner adopted in these studies by acquiring $N$ A-scans at the same transverse position.

As illustrated in Figure 43, velocity values are obtained via intraluminal cross-correlation analysis by computing the average of the A-scan number difference which yields the corresponding maximal correlation values between channels, obtaining the product of this and the time between A-scans, $\tau$, and finally the quotient of this temporal value and the beam separation value, $\delta$:

$$
\dot{v} = \left( \frac{\delta}{\sum_{i=1}^{N} \frac{A(i)-B_{\text{maxcorr}}(i)}{N/2}} \right) \left( \frac{1}{\tau L} \right)
$$

where $N$ is the total number of A-scans acquired; $L$ is the total capillary lumen length along which the intraluminal averaging occurs; $A$ and $B$ refer to the A-scans in question of each of the dual-beam system channels and indicate the number of A-scans between each channel until a maximal relative correlation value was achieved. For the axial assessment however, only a minor amendment of this expression is required:

$$
\dot{v}_{\text{axial}}(M) = \left( \frac{\delta}{\sum_{i=1}^{N} \frac{A(i)-B_{\text{maxcorr}}(i)}{N/2}} \right) \left( \frac{1}{\tau L} \right)
$$

where $M$ denotes the number of segmented regions of $L$ considered to yield an axial distribution of velocity values. The number of segments scales linearly with the capillary lumen size as dictated by the associated axial and transverse resolutions of the system.
Figure 53: Illustration of the axial velocity processing algorithm, an extension of the original cross-correlation velocity algorithm. The OCT data is segmented axially in windows scaled with respect to the capillary lumen size. Velocity values from these co-similar segmented regions are obtained via cross-correlation in the usual manner and plotted versus their respective axial positions.
5.2.3 Results

It has been shown previously (§5.1.3) that when solutions of different optical properties are considered, multiple scattering effects caused by solutions of varying anisotropy values were not influential regarding the assessment of velocity via cross-correlation. However, the flow phenomena in this scenario considers that which is flowing transversely to the incident light, and as such it is practical to consider if any differences may be obtained for studies of their respective axial velocity profiles.

The method outlined of yielding axial velocity profiles via cross-correlation was applied to flowing solutions of Intralipid and blood in capillaries of varying diameter. In an effort to test the feasibility of discerning profiles for differing situations, data was examined as a function of capillary size and flow rate for larger flow rates in smaller capillaries and vice versa (i.e. Intralipid: 150 μm, 300 μm, 500 μm at 5 mm/s, 2.5 mm/s and 1 mm/s, respectively; Blood: 50 μm, 100 μm, 200 μm at 0.5 mm/s, 3 mm/s and 6 mm/s, respectively). The flow rates and capillary sizes examined had Reynolds numbers far below the laminar-turbulent cut-off of $Re \equiv 1800$. As can be seen from Figure 54 and Figure 55, the resulting profiles exhibit laminar flow profiles, with second order polynomial fits to the data for clarity.

Blood displays a variable viscosity attributable to cell agglomeration or deformation [503]. Although it has been shown that for vessels of inner diameter (155-2000) μm this effect can be ignored [519], this cannot be disregarded for smaller vessel sizes. However, axial velocity flow profiles were computed for blood flow in 50 μm capillaries at 0.5 mm/s (see Figure 55(a)) and the resulting in a parabolic profile did not highlight an inability of registering such flow profiles or exhibit any behaviour which departs from the laminar regime due to cell agglomeration. However, as the phantom created in this situation is an idealised case without bifurcation and consists of a single capillary size, investigations into the effects of turbulence is discussed in greater detail in §5.5.
Figure 54: Axial plot profiles obtained for flowing 2% Intralipid solution using (left) intensity and (right) phase-based correlation analysis for (a) 150 μm, (b) 300 μm and (c) 500 μm, with flow rates 5 mm/s, 2.5 mm/s and 1 mm/s respectively. (Fitted curve: 2nd order polynomial.)
Figure 55: Axial plot profiles obtained for flowing blood encased in an opaque agar skin mimicking phantom using (left) intensity and (right) phase-based correlation analysis for (a) 50 μm, (b) 100 μm and (c) 200 μm, with flow rates 0.5 mm/s, 3 mm/s and 6 mm/s respectively. (Fitted curve: 2nd order polynomial.)
5.3 Feasibility study on the application of db-SdOCT in bidirectional media

5.3.1 Background

Doppler flow monitoring is based on the principle that Doppler shifts in light backscattered from moving objects in a biological sample either add to or subtract from the fixed Doppler frequency, depending on the direction of flow. This advancement in flow characterisation has been implemented in both the TdOCT and FdOCT regimes [543].

The application of FdOCT techniques in vivo does not permit the measurement of absolute flow velocities because the angle between the incident laser beam and moving matter is unknown. Yet, this issue may be overcome by illumination/detection using different angles. For instance, the bidirectional measurement of velocities has been demonstrated using a calcite beam displacer which splits the linearly polarised light into two orthogonal components. It has been shown that this method is successful at computing velocity values independent of the Doppler angle provided the angle of incidence is close to \( \pi/2 \); however, the birefringence of the cornea and retinal fiber layers of ocular media have resulted in errors in the measured velocity caused by channel cross talk [544-546]. In addition to alterations of the optics set-up, computational advancements have been introduced, permitting volumetric bidirectional flow mapping without manual segmentation [547].

The ability to discern flow direction in addition to providing values of velocity has potential applications in non-invasive analysis of microscopic blood flow in several situations of clinical and research interest. These situations include the evaluation of dermal vessel structure in the presence of bifurcation and sudden alteration in vascular size; blood vessel proliferation in laser treatment planning for dermatological conditions; and endoscopic identification of small blood vessels underlying gastrointestinal ulcers [469].

5.3.2 Experimental design & implementation

In an effort to provide a fully characterised assessment of flow using db-SdOCT methods, inspiration for this was taken from recent work by Wang et al. [96]. As mentioned in §3.4, auto-correlation methods for quantitative mapping of transverse particle-flow velocity employ the statistical nature of the intensity fluctuation of backscattered light modulated by (stochastically) flowing particles. When a particle traverses a probe beam, relatively strong backscattered light with a pulse width identical to the traverse time, \( t_0 = \frac{w}{\hat{v}} \), results
(distinct from the transit time between beams used in cross-correlation computation, \( t_\tau \) – this difference is illustrated in Figure 56), where \( w \) is the transverse size of the probe beam. This results in modulations of the remitted light which may be approximated as a sequence of rectangle functions [471]. This concept has been used to deduce particle-flow velocity [473]; however, it is unable to discern directional data. As the system described in this work may be likened to a miniature time gate, by utilising the concepts of auto-correlation and knowing the relative temporal separation (\( \tau \)) of these beams, it is possible to distinguish which beam the particulate matter under investigation encounters first and thus direction may be discerned.

The autocovariance \( (r(k)) \) is a measure of the covariance between pairs of observations of a stationary stochastic process at a lag \( k \). A long autocovariance function indicates slowly varying data, whereas short autocovariance functions denote that at small distances the data is not related or uncorrelated. Likewise for the covariance of two variables, \( r(k) \) can also be normalised to yield the auto-correlation function \( \rho(k) \) [548]:

\[
\rho(k) = \frac{r(k)}{\sigma_x^2} = \frac{r(k)}{\sigma_y^2}
\]

The value for the auto-correlation at lag 0 is \( \rho(0) = 1 \). Most physical processes have an auto-correlation function that damps out for greater lags.

Prior to commencing any experimentation, the initiating channel (i.e. either \( A \) or \( B \)) is selected (see Figure 56; in this case, \( A \) was chosen for illustration as the starting channel for acquisition and switching although in general this is arbitrarily chosen).

**Figure 56:** Illustration of the parameters involved when utilising auto-correlation data for bidirectional studies. \( A \) and \( B \) refer to each channel of the db-SdOCT system; \( w \) denotes the respective beam width; \( t_\tau \) is the traverse time for each probe beam and \( t_\tau \) is the transit time used in cross-correlation computation (included here for differentiation only, not used in auto-correlation computation); \( \delta \) is beam separation distance; and \( \delta \) indicates velocity direction.

For these studies, the transit time may be calculated from the reciprocal of the slope (calculated before the first auto-correlation zero) of the normalised auto-correlation function
of the raw OCT data. From the calibration outlined in §4.4.2, the beam width size is readily obtained from the beam slit profiler (this is the mean of size in the x- and y-direction). If one channel was larger than the other in terms of beam width, then this would outwardly affect the value of transit time obtained. As such, the multiplication of the transit time via auto-correlation by a factor of $\beta = \frac{w_{A/B}}{\delta}$ (where $w_{A/B}$ is the size of the width of sample arm beam $A$ or $B$) eliminates the possibility of any temporal delay associated with beam size (the division by $\delta$ is done as it is common to each sample arm and introduces a dimensionless quantity). Both auto-correlation functions of channel $A$ and $B$ are performed on the same time scale. The addition of $\tau$ (see Eq. 61) to the latter channel only serves to account for the time difference due to the camera integration and optical switching; as this value is small, it has no impact on the resulting auto-correlation computation. Taking these elements into consideration, the channel which takes longer to reach the first auto-correlation zero ($ACF(0)$) indicates which channel received data at a later time, thereby discerning direction. The results for directionality testing in various experimental environments are described in the following section.

5.3.3 Results

As auto-correlation computation utilises the same raw OCT data as for cross-correlation analysis, both algorithms can be implemented simultaneously, resulting in only marginal increases in processing time. As with the cross-correlation data, both intensity and phase were tested for their ability to yield directional data. In all auto-correlation experiments, a temporal compensation delay factor ($\beta$) was incorporated, computed from beam calibration. For instance, in the case of a 500 μm in vitro sample with flowing blood in a solid agar based skin mimicking phantom, ChB initiated data capture; however, the actual simulated flow direction was from ChA to ChB. If the method is capable of discerning direction, Channel B would take longer to register the flowing matter, despite the fact that this channel initiated acquisition; this was found to be the case (see Table 6). Although the intensity-based method was successful with some of the datasets for yielding directional data, the phase proved capable of discerning the correct flow direction (this was known a priori) in every instance. It was evident from the experiments performed that modulation duration varied inversely with transverse velocity. This is demonstrated in Figure 57, where the time to reach the first auto-correlation zero is longer for flow at 1 mm/s as opposed to 8.5 mm/s.
Table 6: Sample experimental values for a bidirectionality investigation of a 500 μm in vitro sample with flowing blood in a solid agar based skin mimicking phantom. In this case, Channel B initiated data capture; however, the simulated flow direction was from ChA to ChB.

<table>
<thead>
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<th>Acctcorr_intensity</th>
<th>Acctcorr_phase</th>
<th>Intensity</th>
<th>Phase</th>
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<td>ChA</td>
<td>ChB</td>
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</tr>
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<td>0.01005</td>
<td>0.00943</td>
<td>0.01113</td>
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</tbody>
</table>

Figure 57: Experimental data indicating that the duration of modulations varies inversely with transverse velocity. 2% Intralipid flowing in a 400 μm capillary at (top) 1 mm/s and (bottom) 8.5 mm/s.
A variety of different experimental configurations were investigated – velocity ranges, capillary size and medium (flowing blood within opaque agar, Intralipid etc.). A selection of this data is shown in Appendix B for clarity and completeness. It was previously assumed that the larger the capillary diameter, the larger the amount of statistical information that would be present for auto-correlation analysis, and thus a more clear indicator for velocity direction detection. This is evident in Appendix B, which reveals that for decreasing capillary diameter size, the (intensity-based) auto-correlation analysis becomes steadily unreliable. In the case of the data of Table 6, an unusually high prevalence of intensity-based inaccuracy is present at a large diameter value of 500 μm, which can only be attributed to the fact that the initiating channel was not the first to experience flow. However, despite the shortcomings of the intensity-based analysis, phase-based auto-correlation analysis proved very effective in predicting the flow direction. This may be attributed to the inherent stability associated with the system in relation to the phase, thus providing a more consistent and reliable variable for correlation based analysis.

Bidirectionality studies were conducted in all *in vitro* and *in vivo* studies in this work. Where more relevant to the subject at hand, this information is included (i.e. see § 6.1.1). In conclusion, utilisation of the auto-correlation function of each sample beam provided a simple but effective means of discerning directional data without the need for excessive computation and may be performed simply on the raw OCT data, concurrent to the cross-correlation means of discerning velocity metrics.

5.4 Impact of coherence focal volume on velocity value

5.4.1 Background

The means by which velocity data is obtained using the db-SdOCT algorithm and the associated optics are quite distinct from the computational aspects of Doppler related processing. As such, investigating the effects of a variety of light sources (with various spectral characteristics) on the effectiveness of the cross-correlation algorithm is an instructive exercise, especially with a view to full system characterisation and optimisation.

As seen in §3.1, to improve axial resolution, the spectral bandwidth must be either increased or the central wavelength decreased. As previously discussed, the light source not only determines the axial resolution, it also influences both the sample penetration and the transverse resolution. In this investigation, three different light sources were tested with varying values of probing coherent volume. A relatively inexpensive super luminescent
diode (SLED) broadband source (model DL-BX10; *Denselight, Singapore*) and two sources of an extended broadband SLD source (model LS2000B; *Thorlabs GmbH*) were investigated. All relevant specifications for these light sources are shown in Table 7. In the LS2000B, a multiplexed dual SLD arrangement of two fiber-pigtailed SLDs provides a single extended bandwidth (200 nm, typical) light source. Considering the higher order dispersive effects evident in ultra-broad bandwidth sources (see §3.2.2.1), the variation in longitudinal and transverse resolution values, and penetrative depth of field differences, the effects of these various characteristics are assessed in the following subsection.

**Table 7:** Outline of the spectral characteristics of the various light sources used to investigate their effects in db-SdOCT cross-correlation assessment; \( \Delta x \) is the transverse resolution; \( V_{\text{coherence}} \) is the probing coherent volume; \( b \) is the confocal parameter.

<table>
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<th>LS2000B</th>
<th>SLD B</th>
<th>SLD A+B</th>
</tr>
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<td>1.34E-06</td>
</tr>
<tr>
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<td>1.35</td>
<td>1.35</td>
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<td>( \Delta x )</td>
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<td>( l_c )</td>
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<td>( V_{\text{coherence}} )</td>
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<td>( mm )</td>
<td>( b ) or DOF</td>
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<td>2.84</td>
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### 5.4.2 Results

Given the aforementioned higher order dispersive effects evident in ultra-broad bandwidth sources, the variation in longitudinal and transverse resolution values, and penetrative depth of field differences, the effects of these various characteristics were assessed individually using the db-SdOCT system and subsequent cross-correlation processing. Three different capillary sizes were investigated (500 \( \mu m \), 300 \( \mu m \) and 50 \( \mu m \)), at various flow rates of 2% Intralipid solution (0.5:1:10 mm/s) and different acquisition rates of the line scan camera (OPR33 = 16846 Hz; OPR32 = 31680 Hz). All light sources underwent a separate spectral calibration (see §4.2.2) to yield the appropriate coefficients for equidistant sampling from \( \lambda \)-space to \( k \)-space for subsequent FFT processing. All data was cross-correlation processed in the same manner to yield velocity values and directionality data.

In terms of the analysis of acquisition rates, OPR32 outperformed OPR33 with almost twice the accuracy (approximately 1.94 times increased accuracy at higher rates) at emulating the desired velocity values obtained by both intensity and phase-based analysis. As such, decreasing the db-SdOCT system acquisition rate negatively impacts on the results of
computed values of velocity in cross-correlation analysis. Longer exposure times (i.e. OPR33), although they permit an increased SNR, can result in motion induced blurring and consequently the information captured within that instant is not as distinct for correlation purposes at it would be if a higher acquisition rate (OPR32) was used. However, precisely how low an exposure time can be implemented and successfully supply the user with adequate velocity data remains to be seen.

As per Figure 58, the deviation of values from their theoretically predicted values increases with decreasing capillary diameter; errors over all velocities and acquisition rates for capillary sizes 500 μm, 300 μm and 50 μm were, respectively, 7.88%, 7.92% and 8.41%. The primary purpose of this experiment was to investigate whether correlation based results performed better with increased or decreased focal volume metrics. It was found that the deviation from theoretically anticipated velocity values varied as follows for averaged values of all phase and intensity-based velocity values, and all capillary sizes (errors in parentheses): 1310 nm SLED (7.77 %), 1340 nm SLD B (8.14 %) and 1300 nm SLD A+B (8.30 %). The reasoning behind this trend is outlined in §5.4.3.

Directionality studies were also performed on all velocity data, acquisition rates and capillary sizes. It was found that although all light sources could predict the correct (known a priori) directional behaviour (for phase analysis), SLD B performed the best in terms of intensity-based directional predictions. A sample of bidirectionality focal volume experimental values for a 500 μm capillary using all three light sources at OPR33 can be seen in Table 8.
Figure 58: Graphical data showing velocity values obtained by (a) intensity and (b) phase correlation analysis. Three different light sources were considered, tested on three different capillary sizes, 500 μm (top), 300 μm (center) and 50 μm (bottom), and at two different acquisition speeds (OPR33 = 16846 Hz; OPR 32 = 31680 Hz). Error bars: ±10% of values provides by syringe pump.
Table 8: Experimental values for a bidirectionality investigation of a 500 μm in vitro sample with 2% Intralipid solution for a (top) SLED 1310 nm, (middle) 1300 nm SLD A+B and (bottom) 1340 nm SLD B light sources at OPR 33.

<table>
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<th>Velocity (m/s)</th>
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<th>Autocorr\textsubscript{phase}</th>
<th>Intensity</th>
<th>Phase</th>
<th>ChB to ChA flow direction?</th>
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5.4.3 Discussion

Speckle is the result of the superposition of many random wavelets, whose amplitudes and phases are statistically independent, phases are uniformly distributed over \((-\pi, \pi)\) (only fulfilled if there are sufficient scatterers in the coherence volume) and are perfectly polarised [549]. Speckle properties are affected by sample properties like structure and motion, and by properties of the light source and the sample beam optics. In OCT, the noise aspect of speckle dominates and considerable attention has been paid to methods devoted to speckle reduction. In clinical analyses, OCT speckle can mask diagnostically relevant image features and reduce the accuracy of segmentation algorithms [372]. There have been many attempts to decipher sample attributes from speckle properties. In industrial metrology, speckle has played an important role in the measurement of surface roughness, strain, and deformation [550]. Generally, however, speckle is treated as an insidious form of noise in OCT and is thus suppressed in most instances. The most straightforward technique to remove speckle would require differentiation between statistical speckle and sample-specific speckle; this is typically not possible. Hence, techniques of suppression/removal of speckle have been reported, manifesting as hardware and software implementations [551, 552]. Methods of speckle averaging have been implemented which significantly improve the quality of the images and appearance of the subsurface structure [553-555]. However, second-order temporal speckle statistics have also been shown to carry information about scatterer motion whose fluctuations have a dependence on the mean velocity [556]; coupled with OCT, this offers the capability of depth resolved flow profiles with the added benefit of being able to detect motion normal to the OCT axis. However, although Doppler methods can discern directional information, speckle flow measurements cannot.

Different velocity phenomena are present in the previously described experimental investigations: an increase in cross-correlation velocity value accuracy for increased capillary size, acquisition rate and certain focal volume considerations. In terms of justifying the trend of increasing accuracy of velocity values gleaned with increasing capillary diameter, this may be understood using the above rationale of speckle OCT. Regions of movement within a real-time OCT image capture are apparent by the intermittent flickering of the speckle present. As such, in reference to cross-correlation analysis, a sufficient amount of transient speckle OCT data must be present and detectable within the coherent probe volume (see Figure 59) of the impinging light beams such that clear computation of like events of high correlation may be realised.
It is evident from Figure 58, an increased deviation from theoretically applied flow rates occurs for decreasing capillary size using cross-correlation analysis. Insufficient OCT speckle data within the probing coherent volume at faster velocities makes discernment of instances of high statistical correlation more difficult. However, for the lower values of velocity applied in e.g. 50 μm sizes, such errors are not as prominent and the reduction in sample data from this reduced coherent volume is compensated for by the slower flow rates. Although this may indicate a theoretical upper limit in terms of the assessable velocity range for smaller capillary values, capillaries of this size occurring naturally have much smaller flow rates than those represented by the upper velocity range assessed here. Reported values for red blood corpuscle velocity (RBCv) within capillaries indicate a mean value of 1.85 mm/s [434]; the experimental flow range was less than and much greater than this value as a test for the capability of the system for biomedical applications in, for example, turbulent conditions for which larger velocity values may be present. Thus, although this is a possible limiting aspect to the db-SdOCT method and algorithm, the naturally occurring in vivo biological would permit its use as in vitro analogues are non-ideal.

As discussed above, it is evident from the experimental data that there is a fall off in accuracy for faster flow rates in both the intensity and phase regimes, dependent upon capillary size. Recent imaging applications in wide-field fluorescence and confocal microscopy have increasingly centered on the demanding requirements of recording rapid transient dynamic processes that may be associated with a very small photon signal and which often can only be studied in living cells or tissues. Improvements in camera, laser, and computer hardware have contributed to many breakthrough research accomplishments in a number of fields. As high-performance camera systems typically employing low-noise cooled CCDs have become more capable of capturing even relatively weak signals at video rates and higher, certain performance factors necessarily take on greater importance. The influence of the acquisition time as investigated in §5.4.2, concluded that increasing the line
scan rate positively influenced the ability of the cross-correlation algorithm to yield increasingly accurate velocity values. In clinical terms, reducing acquisition times may improve patient throughput, increase camera efficiency, and reduce costs; however, reducing acquisition time also increases image noise [557], and therefore the ability of the intensity-based methods; thus, the utility of the db-SdOCT method in such instances may prove advantageous.

The detectable velocity dynamic range ($VDR$) of a phase-resolved Doppler OCT (PrDOCT) system is governed by a detectable Doppler phase shift, a flow angle and the acquisition interval:

$$VDR = \frac{\lambda_0}{4T\eta} \left( \frac{\Delta\phi_{err}}{\min} \cdot \frac{1}{\max} \right) \quad (67)$$

where $\Delta\phi_{err}$ is the phase error which can be statistically quantified as the deviation from the mean of the phase difference measured from a stationary sample. Shortening the acquisition duration will increase both the maximum and minimum detectable velocity values; however, this may lead to the invisibility of slow flow. Both the upper and lower limits of the VDR depend on the flow angle when the absolute flow is concerned. In practice, in in vivo flow monitoring, the flow orientation may vary from $0^\circ$ to $90^\circ$ degrees relative to the incident beam, leading to a wide dynamic range of axial flow velocity. Invisibility of slow flow will be more severe when the flow angle approaches $90^\circ$, which produces extremely slow axial flow. In an effort to surmount the obstacles of VDR using PrDOCT, multi-scale measurement protocols have been reported [558], in which the Doppler phase shifts have been computed along both the fast and slow scanning axes thus yielding different ranges of detectable flow velocity simultaneously. Methods such as moving-scatterer-sensitive SdOCT have reported improved sensitivity over conventional PrDOCT techniques by disregarding the influence of stationary scatterers by subtracting adjacent complex axial scans before calculation of the Doppler frequency shift [559]. However, in terms of db-SdOCT analysis, cross-correlation between two channels (whose data length are both $N$) can provide a maximum time delay of $\pm (N - 1)\tau$ and a minimum time delay of $\pm \tau$ ($\pm$ denotes possible opposing directions). As such, the velocity measurement range is $\pm \left( \frac{\delta}{(N-1)\tau} \sim \frac{\delta}{\tau} \right)$, whose parameters could be flexibly set to meet the requirements of a variable velocity measurement range.
The errors associated with SLD A+B and SLD B for cross-correlation velocity assessment may be, respectively, attributable to (i) higher order dispersion effects and (ii) the comparable degradation of the transverse resolution, resulting in an insufficient amount of data gleaned for correlation purposes. Conventional lenses have a focal length that varies with wavelength and thus focus ultra-broadband light to different planes. This variation in focal position for different wavelengths alters the local effective bandwidth and therefore degrades resolution [557]. For specially corrected achromatic optics, different imaging distances introduce a wavelength-independent attenuation of the whole spectrum, maintaining the spectral shape, bandwidth and therefore axial resolution. In an effort to compensate for higher orders of dispersion prominent in the extended SLD A+B source, achromatic lenses were employed in addition to dispersion compensating blocks in the sample and reference arms. However, multiplexed SLD light sources have the disadvantage of having spectrally modulated emission spectra that can produce side lobes in the coherence function, resulting in image artifacts. Therefore, without numerical compensation of such artifacts, ultra-broad bandwidth sources are naturally degraded in terms of resolution. Nevertheless, if such compensation was implemented, the computational requirements for all sources would not be equal and thus could not be compared appropriately for focal volume influence in correlation assessment.

In terms of directionality, it is evident from Table 7 that the resulting transverse resolution of 1340 nm SLD B (theoretically calculated, 34.80 μm) is 2.29% and 3.08% larger than those of the 1310 nm SLED and extended bandwidth SLD A+B, respectively. The increase in the probing coherence volume may be attributed to the improvement seen in the clear distinguishing of direction, considering either intensity or phase-based correlation analysis. As flowing media passes transversely through the probing beams, the time taken to traverse these probing lengths is justifiably longer both transversely and axially, enabling an increased accuracy regarding the discernment of direction via auto-correlation analysis.

To conclude, implementing cross-correlation analysis to yield velocity data was best achieved by using an inexpensive light source, and doesn’t require additional hardware amendments or computational additions to compensate for dispersion effects, etc. As such, adaptation of this method into existing OCT set-ups is relatively straightforward and a cost-effective means of dynamic assessment, free from angularly induced artifacts. Although it has been shown that an increase in coherent focal volume yields more easily discernible directional data, this is true in either intensity or phase-based analysis cases. However, as a clear increase in accuracy is obtained implementing phase-based correlation velocity analysis
compared with intensity-based analysis, adequate directional data may be discerned without intentional degradation of transverse resolution values.

5.5 Assessment of turbulent flow models

The study of the flow dynamics in blood vessels with complex geometry is important in rheology and cardiology to better understand the effect of blood flow on the possible rupture of atherosclerotic plaque [372] and aneurism [560, 561]. Atherosclerosis is the formation of plaques which bulge into a vessel, thus forming a constriction. Coronary artery disease is caused by a build-up of atheromatous plaques within the walls of the coronary arteries (see Figure 60), which may rupture initiating a clotting cascade causing ischemia and/or infarction [562-564]. These anatomical changes induce deviations of the normal blood flow patterns and consequentially a modification on the shear stress acting on vessel walls, and have been recognised as one of the possible factors of plaque localisation and growth [484]. As laminar flow is characterised by velocities that vary spatially in magnitude but not direction, this is juxtaposed to turbulent flow which is both temporally and spatially variant [565]. Turbulent flow occurs at vessel branch points (bifurcations) in diseased and stenotic arteries and across stenotic heart valves. The existence of turbulent flow behaviour increases the energy required to drive blood flow (i.e. perfusion pressure) due to frictional losses and occurs when a critical Reynolds number is exceeded (see §1.1.2). Identification of turbulent flow and understanding of its underlying cause(s) is important from the perspective of prompt and effective patient treatment.

![Figure 60](image-url): (Top) IVUS longitudinal reconstruction of an atherosclerotic plaque lesion at vessel bifurcation [566]. (Bottom) OCT (A) and histological (B) images obtained of human coronary artery plaques obtained at autopsy. 'c' indicate locations of subintimal calcifications. (Scale bar = 500 μm.) [567]
Physical characterisation of atherosclerotic plaques is crucial for the treatment of coronary artery disease and various means of physical analysis have been reported based on texture and intensity features [568]. The possibility of the assessment of blood flow in vessels with constrictions causing turbulent behaviour presents a useful tool for clinicians and can be applied to evaluate the velocity distribution on both the proximal and distal sides of the blockage. This may also allow analysis of shear stresses on vessel walls [569-571]. Such information is highly sought after as it provides estimations of the risk of plaque rupture with potential consequent acute coronary syndrome [572] and myocardial infarction (heart attack) [573] – for example, 86% of all heart attacks are due to the rupture of a vulnerable plaque in a coronary artery [574]. In addition, assessment of turbulent behaviour may also be indicative of certain conditions, for example, anaemia which is characterised by high flow velocities and low blood viscosity [575].

In terms of assessment of dynamic disturbances by optical means, the presence of spectral broadening can serve as an indicator. Laminar flow reveals a narrow spectrum spread yielding a singular Doppler-frequency shift. The extent of turbulence in blood flow may be inferred from the variance of the spectrum [576]. In addition to the assessment of dynamic turbulence, phase-resolved Doppler variance techniques have the potential to be used to investigate the properties of particles in highly scattering media [577] and quantifying Brownian motion [578]. Traditional dynamic light scattering, a predominant method used to characterise particle size, is unable to provide depth resolved information or imaging functions. Doppler variance OCT measures the spectral bandwidth of the Doppler frequency shift due to the Brownian motion of the particles and can provide quantitative information about particle size. Spectral bandwidths of Doppler frequency shifts for various sized particles were demonstrated to be inversely proportional to the diameter of the particles [528].

Although the importance of turbulence assessment is of grave clinical importance, accurate representation of the velocity values involved is also significant. Quantification of velocity values may also proffer a means of assessment of the probability of plaque rupture, due to increased pressure and stresses imposed on the vessel walls. The presence of vascular blockages is characterised by turbulent and transient haemodynamical behaviours. Taylor’s frozen turbulence approximation is the central assumption invoked in most experiments designed to investigate turbulent media [578].
“If the velocity of the air stream which carries the eddies is very much greater than the turbulence velocity, one may assume that the sequence of changes $u$ at a fixed point are simply due to the passage of an unchanging pattern of turbulent motion over the point.”

It has been shown that the scale of turbulent motions has a strong influence on the applicability of Taylor’s approximation [579]; yet, if localised dynamics can be analysed over a range of characteristic lengths, then velocity values can be determined irrespective of the turbulences involved. This implies that although flow disturbances may be detected, an appropriate velocity value may also be computed for such situations as random fluctuating velocities with no discernible pattern yield defunct data. An experiment to test this hypothesis is proffered in the following subsection by using an *in vitro* flow model with a simulated localised plaque model and the resulting dynamic assessment is compared with a control model.

### 5.5.1 Experimental considerations and results

In order to assess turbulent behaviour, a simple *in vitro* experimental flow model utilising 200 $\mu$m and 500 $\mu$m silica glass capillaries was constructed. Two models were manufactured; one with a tightly compacted sample of sponge in the 500 $\mu$m side (an atherosclerotic plaque (AP) model) and another which served as a control and appropriate comparison for any deviations in flow behaviour between it and the turbulent model. Two different capillaries were chosen for several reasons; (a) the two different sizes were used to mimic vessel bifurcation and thus encourage the formation of turbulent behaviour, (b) as controlled input flow rates were infused to the 200 $\mu$m side, this created intentionally small resulting flow rates in the 500 $\mu$m side and thus created a challenge to discern theoretical velocity values coupled with turbulent behaviour, (c) the use of different size capillaries gives a more impartial comparison between the control and AP models.

![Figure 61](6.png) **Figure 61**: Illustration of the artificial atherosclerotic model used to mimic a vascular plaque. Turbulent flow analysis was carried out on both this model and that of a control to investigate the feasibility of utilising the cross-correlation method in turbulent environments.
A solution of 2% Intralipid solution was used as the flow medium and the input flow rates (to the 200 μm side) used in these studies were 0.001 mL/min, 0.002 mL/min and 0.004 mL/min (i.e. 0.5 mm/s, 1 mm/s and 2 mm/s). Due to the cross-sectional size difference between the inflow and outflow sides of the models, these input flow rates correspond respectively to 0.08 mm/s, 0.16 mm/s and 0.32 mm/s, to be sensed in the outflow 500 μm side. To test the influence of scale on turbulent motions and therefore the applicability of Taylor’s approximation, a range of calibrated beam separation distances were implemented using the db-SdOCT system, (15:15:150) μm. The measurement protocol for these experiments involved repetition of velocity measurements for each different beam separation value \( \delta \), in each capillary size and model. In the AP model, measurements were taken after the obstruction in terms of flow direction. The subsequent cross-correlation velocity analysis was the same as previously outlined.

The resulting data for both the control and plaque model is outlined in Figure 63; both control and plaque model data are plotted and a polynomial fit to the data was performed for clarity (dashed fit line: control; solid fit line: AP model). It is evident that velocity values computed for the 500 μm side of the in vitro models deviated quite substantially more between the ±10% tolerance than the 200 μm side for most data points, in both AP model and control cases. A stark comparison may be drawn between the behaviours of the same capillary side for the AP and control models, if their resulting cross-correlation plots are considered (see Figure 62). In this figure, the resulting temporal cross-correlation maps for the 500 μm side of both the AP and control model at the same separation value \( \delta \) are compared. Although the plots vary greatly visually due to differing levels of turbulence present, this fact did not impede the ability of yielding velocity values well within a tolerance of 10% of the theoretically anticipated velocity value in the resulting cross-correlation velocity assessment of the AP model example (Figure 62 (right)).

Figure 62: Cross-correlation map differences (intensity data) between flowing 2% Intralipid solution at 2 mm/s (0.32 mm/s) in the 500 μm capillary sides of (left) control and (right) plaque model at \( \delta = 60 \) μm.
Figure 63: Turbulence experiment using 200-500 μm control and atherosclerotic plaque models. Different velocities were tested (top: 0.5 mm/s, 1 mm/s, 2 mm/s) for differing values of beam separation, δ. The resulting flow velocities sensed in the 500 μm side are lower due to the increase in capillary diameter (bottom: 0.08 mm/s, 0.16 mm/s, 0.32 mm/s); (a) intensity and (b) phase-based correlation analysis. (Dashed fit line: control; solid fit line: AP model; dashed limit lines indicate ±10% of theoretical values.)
Although there seems to be a generally increased erratic behaviour present in the 500 μm sides due to the turbulence and/or capillary size change, it is clear that the control model deviated less from the ±10% tolerance imposed and mimicked more consistently the volume flow rates provided by the syringe pump. Furthermore, comparing the intensity and phase-based correlation analysis in terms of the 500 μm capillary side only, phase-based correlation assessment deviated less also, especially considering the lowest velocity values examined. For the 500 μm side of both the control and AP models, the intensity-based and phase-based analysis yielded theoretical deviations of 11.28% and 7.10%, respectively; for the 200 μm side of both control and AP models, the intensity-based and phase-based analysis yielded theoretical deviations of 5.38% and 4.22%, respectively.

Turbulent behaviour became increasingly more apparent with increasing dual-beam separation value; this is counter to the results obtained in §5.1.3, in which for δ values ≥ 60 μm, the relative difference between the theoretically imposed flow rates and those gleaned from correlation assessment are ≤ 10%. This reveals an opposing behaviour in response to the turbulent conditions; for δ values for which the theoretical certainty should be ≤ 10% the largest deviations from the theoretical velocity values are obtained and are therefore the best indicators of erratic dynamic behaviour. However, from the perspective of Taylor’s approximation for characteristic lengths, it is clear that the smaller δ values relayed a more accurate metric of the underlying velocities present, irrespective of the created turbulence. For the 200 μm graphs (intensity/phase) this holds true even up to a δ distance of 135 μm; this is justifiably less for the turbulent flow portions of the models, where tolerable velocity values may be quoted up to separation distances as far as 75 μm for phase-based turbulence analysis.

As previously documented, the phase-based correlation analysis yielded values of greater accuracy than that performed with intensity data; 5.66% and 8.33%, respectively, for both experimental flow models. However, it is clear that for the 500 μm side of the AP model, the values obtained by both intensity and phase correlation analysis yielded more erratic data, consequential of the turbulence created. Considering both intensity and phase-based correlation results, the deviation from the theoretical values for the 500 μm side of the control and AP model were correspondingly, 6.79% and 7.20%.
5.5.2 Discussion

The initiation, localisation, growth, composition and rupture of intracoronary atheromatous plaque (factors which define the natural history of coronary artery disease) are all dependent on inhomogeneities and irregularities of intracoronary local blood flow and endothelial shear stress [366]. As such, the reported experimental work serves to provide a means of characterisation of turbulent flow by comparison of in vitro atherosclerotic plaque and control flow models. Stemming from Taylor’s approximation, it was hypothesised that the scale of turbulent motions has a strong influence on the capability of discerning relevant velocity metrics in a turbulent and ever-changing environment. The analysis has shown that turbulence may be discerned at larger values of beam separation, as the disturbances created cause the computed velocity values to depart greatly from the theoretical velocity values. In addition, using smaller values of beam separation results in the ability to quantify the underlying velocities present, irrespective of the turbulences created. Thus it may be possible to implement both scenarios in vivo: confirming localised turbulent phenomena in addition to providing a representative value of the underlying flow velocity by using different length scales.

It has been shown that as the length scale of the turbulent structures grows, the time scale over which the Taylor’s approximation is applicable also grows [580]; in smaller, more tortuous blood vessels, turbulence is naturally more prevalent. Thus, very small values of beam separation values may be utilised so as to provide accurate velocity metrics, thereby eliminating the uncertainty and limitation involved with not having straight sections of vessels to image.

The low value of the Intralipid concentration (2%) enabled the use of the kinematic viscosity of water in the Reynolds (Re) calculations \(1.004 \times 10^{-6} \text{ m}^2/\text{s}\). The range of Re values obtained were \((0.1 \text{ -- } 1)\) and therefore very much less that the laminar turbulent transition value of \(Re \approx 1800\), indicating that flow should be nominally laminar in control regions. This suggests that the turbulence created was solely due to model construction characteristics and the presence of the simulated plaque. As such, axial velocity profiles were computed to investigate the effect of turbulence with depth; all profiles used beam separation values of \(\delta = 105 \mu m\). Approximate parabolic profiles resulted from analysis of the 200 \(\mu m\) side of both models (Figure 64). The sudden change in capillary size (which encouraged the formation of turbulent behaviour, as previously described) resulted in a deviation from a parabolic profile fit of the 500 \(\mu m\) side of the control model (Figure 65 (top)). However, at
the site of the simulated atherosclerotic plaque, an increased complexity in the in vitro vessel geometry caused irregularities to arise and the flow to turn from laminar to turbulent. A departure from a parabolic data fit (Figure 65 (bottom)) indicates that the flow, although steady and representative of the input flow velocities (theoretical deviation of 3.21% and 2.94% for intensity and phase-based analysis, respectively, at 2 mm/s on the 500 μm side of the AP model), is no longer laminar. Both second and third order fits are included for clarity in relation to his point. An increased amount of axial segments were implemented in the 500 μm side in order to completely assess the flow behaviour.

The study of the mutual effect and interactions of the blood flow with the surrounding vessel geometry is of great interest in biorheology and cardiology. The db-SdOCT technique could become a valid support for cardiologists as a means of simultaneous imaging of the vessel morphology, plaque formation, and related blood flow velocity information, in addition to providing an insight into the extent of the turbulence involved. Cardiovascular studies rely on computational fluid dynamics to determine the role and importance of factors which could be involved in the genesis of cardiovascular disease. Outlined here is a tool which is capable of imaging a real physical situation and could significantly improve the quality of new therapeutic strategies and interventional methods, such as stent design and placement [565].
Figure 64: Axial profile plots of intensity and phase-based analysis of cross-correlation related intraluminal velocity values for (top) control and (bottom) AP models (200 μm side). Although both exhibit laminar flow profiles, the AP model fit is slightly skewed representing a departure from the regular Poiseuille flow regime. (Solid fit: 3rd order polynomial.)
Figure 65: Axial profile plots of intensity and phase-based analysis of cross-correlation related intraluminal velocity values for (top) control and (bottom) AP models (500 μm side). The control model demonstrates an approximate laminar relationship – however turbulence is evident arising from the sudden change in capillary sizing. (Solid fit: 3rd order polynomial; dashed fit: 2nd order polynomial.)
6 Comparative studies and *in vivo* applications

Presented in this chapter is the application of the db-SdOCT system for *in vivo* dynamic assessment. Expanding on the system optimisation and characterisation as outlined in Chapter 5, investigations into the feasibility of application of the cross-correlation algorithm to an *in vivo* biological environment is outlined. In addition, as the functional basis of the db-SdOCT method pivots upon the unnecessary requirement of angular knowledge in its operation, a comparative study is performed on cross-correlation gleaned velocity data and that processed by the Kasai auto-correlation model for Doppler processing, in an effort to critique both methods in an appropriate manner.

6.1 Case study: Application of db-SdOCT for dynamic assessment of the nailfold plexus

Microcirculatory changes play a significant role in various vital functions. Due to their size, capillaries present subtle circulatory variations more acutely than larger blood vessels. Consequently, information about static and dynamic features of capillaries is a key indication of human physiological health.

The nailfold plexus is one of only a few locations on the body wherein capillaries advance close enough to the skin surface to be naturally visible i.e., without the use of specialised optical equipment or optical clearing agents. This makes the methods of examining them simple, non-invasive and expedient. Moreover, the fingernail is easily fixed in position, free from any movement which may be due to arterial pulsations or respirations, and the capillaries run in hairpin-like loops (6-15 μm in diameter) parallel to the skin surface. Initial microscopy techniques for capillaroscopic purposes were reliant on epi-illumination, with a magnification range of between 15 and 100 [572].

The manifestation of anomalies in vascular physiology can be used as an early indicator of the presence of a plethora of systemic ailments, enabling timely modifications to therapeutic strategies. The expression of vascular aberrations in the acral regions of the body, e.g. the nailfold plexus, represents a non-intrusive means of assessment of the presence and/or progression of a disease. This is particularly pertinent from the perspective of patient care management, as the appearance of morphological irregularities can occur prior to
clinical suspicion. Therefore, as 2-D structural computation of data and dynamic analysis is desirable, ‘on the fly’ cross-correlation processing is implemented and investigated in studies of the nailfold plexus.

Figure 66: At the nailfold, capillaries run parallel to the epidermis appearing as regular ‘hair-pin’ like loops. The above image was captured by wide-field microscopy techniques (magnification × 300) [2].

6.1.1 Results

As the data analysed in the previous studies was acquired by positioning the dual-beam system sample arms on an in vitro sample and a requisite amount data was captured, attention is now turned to the feasibility of applying correlation methodology to acquire velocity values of transient systems whilst raster scanning is performed, yielding 2-D/3-D datasets.

As cross-correlation methodology is distinct from regular OCT acquisition in terms of data capture and subsequent processing, a tailored scanning protocol is required to enable to capture of requisite data (arbitrary) for correlation analysis. Experiments of this fashion may be implemented by two different means: (a) a 2-D scan of the sample may be taken, a structural image is rendered, the dual-beam sources are positioned at a point of interest (i.e. on a vessel) and correlation analysis is performed as previously described; (b) a 3-D scan is acquired, composed of $N \times M$ A-scans ($N$: A-lines; $M$: B-mode positions) after which both structural and dynamic processing is performed. In terms of (a), this protocol may be prohibitively long, and doesn’t account for the fact that the sample may invariably move, especially if it is of biological origin. Regarding (b), although a 3-D representation of the sample may be computed quickly and over a larger area (especially considering the fact that the quasi-simultaneous dual-beam acquisition scheme enables the potential capability of scanning twice the area in the same time as conventional OCT), the large amount of A-scans required would considerably increase the computation time. However, as there is no a priori information about sample dynamics, a sufficient amount of data is required if like instances of correlation between the planes of the dual-beam system are to be detectable and therefore
yield useful velocity values. In these experiments, protocol (b) was selected. Using this regime, the time between corresponding B-scan positions must be less than the transit time of the flow involved if cross-correlation analysis is to be successful. As this is impossible to determine prior to acquisition, B-mode oversampling was performed to mitigate noise and error ($\Delta B_{\text{scan}} = 2.43 \, \mu\text{m}$).

The experiments were performed and repeated on both middle and ring fingers of a healthy female volunteer. In an effort to avoid impeding flow via occlusion [581], the finger was placed on top of a piece of double-sided tape which allowed fixation in a natural position. A few drops of oil were applied to the nailfold plexus to increase light coupling efficiency. For these studies, 15 kA-scans were acquired (i.e. 125 A-scans, 120 B-scans) with a total acquisition time of 23.9 s ($\tau = 1.59 \, \text{ms}$) using in-house custom triggering software (LabVIEW; see §4.4). After acquisition of the data, usual OCT processing was performed and to enable the discernment of capillary flow, en face images were rendered (see Figure 67). The intrinsic optical signals backscattered by the moving blood cells inside blood vessels are used as the contrast for which regions of localised flow are identified.

![Figure 67: En face OCT images of nailfold plexus measurements performed on the middle finger of left hand. Regular capillary formations are evident. Each image contains 7.5 kA-scans and B-scan oversampling was performed to assist in cross-correlation velocity quantification; total area scanned $= 0.72 \, \text{mm}^2$. The average angular difference between channels was computed to be $12.94^\circ$. (Scale bar $= 250 \, \mu\text{m}$.)](image)

In order to perform velocity processing, an arbitrary capillary location was selected and the associated axial data was truncated into a smaller dataset for correlation processing; this was performed on data acquired by both channels. Due to resolution limitations, discerning
arterial from venous side of the capillary loop was not possible, so a central region was considered (for simplicity, this fact is not evident in Figure 68). Noteworthy is the care that must be taken to ensure that the capillary location selected by either channel corresponds to the same capillary – otherwise this would render highly error prone and/or otherwise unusable data. This may be mitigated by implementing a small dual-beam separation value; however, this comes at the expense of being unable to scan as much surface area in the same time frame. Due to the known linear arrangement of the nailfold plexus capillaries and to ensure there are enough A-scans captured to enable correlation analysis of velocity, a $\delta$ value of 150 $\mu$m was chosen for these studies.

![Diagram](image)

**Figure 68:** Illustration of the processing steps taken in nailfold plexus assessment; $\delta = 150 \, \mu$m.

Cross-correlation analysis was performed on these condensed datasets as described previously. Values for velocity were analysed using both intensity and phase-based correlation methods on a number of scans taken of the nailfold plexus of both the middle and ring fingers. The range yielded by intensity and phase correlation analysis was, respectively,
(387.85 – 487.01) μm/s and (405.16 – 455.91) μm/s; the average velocity value obtained for all results was 424.73 μm/s, which is in line with previously published results [58]. Axial velocity profiles of the chosen A-scan position were computed and plotted (see Figure 69); however, due to the capillary size, only a small number of window segments were possible.

![Figure 69: Axial plot profiles for in vivo nailfold plexus assessment; (left) intensity and (right) phase-based windowed correlation analysis.](image)

As previously mentioned, the resolution of the resulting capillaries was not enough to distinguish arterial from venous side. However, for completeness, the flow was fully characterised by computing the directionality of the related capillary velocity. This indirectly enabled the discernment of which side of the capillary the measurement was taken. The 3-D raster scanning of the nailfold plexus commenced dual-beam switching with ChA, travelling downwards along the nailfold inwards towards the finger. Directional data was gleaned by inputting the phase and intensity data as per the cross-correlation analysis into in-house software dedicated to retrieving the auto-correlation of each channel, thereby deducing direction (see §5.3). The resulting data revealed that as the time taken (via auto-correlation) to traverse ChB (inclusive of the switching time, τ) was less than ChA, this indicates that the flow direction was from ChB to ChA and in the same direction as that of the raster scanning (see Figure 70). This denotes that the flow originated from within the venular side of the capillary in question, as it has been shown [582] that blood flows into the arteriolar limb, passes through the curved looped segment and flows out of the venular limb.
Bidirectionality study performed at the same locations as cross-correlation analysis; (b) enlarged portions of (a). As auto-correlation analysis revealed the direction of the in vivo flow to be the same as the scanning direction, it is deduced that the flow in question was obtained for the venular side of the capillary limb.

### 6.1.2 Discussion

Application of the cross-correlation methods to a 3-D imaging arrangement was challenging, particularly if conventional OCT scanning methods are considered. The preferential B-mode oversampling scanning enabled implementation of the db-SdOCT algorithm by acquiring sufficient data for correlation analysis. However, this had outward implications on the resolution, resulting in an inability to discern arterial from venous sides of the capillaries scanned.

Other methods have been reported which have successfully produced high resolution images of the nailfold bed structure by intensity [583, 584] and polarisation sensitive ultra-high-speed analysis (100 kA-scans/s) [585], and flow by phase variance assessment [586]. In addition, OMAG processing (see §3.3.3.3) has successfully imaged the nailfold plexus with such high resolution that the arterial and venous sides of capillaries are easily distinguishable in addition to capillary tip loops [587].

As it was known a priori that the capillaries in the nailfold align in parallel, this made it possible to allow B-mode oversampling preferentially in the y-direction. However, in cases where phenomena such as this are unknown, this introduces a possible source of conflict in the resulting processing. Timing is crucial not only to allow for software and hardware triggering of the dual-beam system but to also allow sufficient A-scan acquisition from both
channels in advance such that a correlation between dynamic aspects of the vasculature between the two illumination planes is possible. An optimised scanning protocol with a high speed camera is therefore desired, wherein sufficient data for morphological and transient analysis is available irrespective of the intravital flow configuration. In addition, as all processing was performed offline, this greatly increased the overall computational processing time. The processing consisted of en face OCT analysis, selection of an appropriate position in which to perform cross-correlation analysis and then functional extensions such as directionality and axial profiling. As the primary goal of any imaging system is possible future implementation in a clinical setting, the real-time application of such is required; a dedicated online analysis of all processing facets is desired.

The tortuosity of blood vessels is a vital medical sign. Capillaries with a high degree of tortuosity have been found in patients with psoriatic arthritis [582]. Therefore, arguments can be made that the described technique is unsuitable for regions in which straight segments do not occur. As such, vessel curvature tracking may be employed; however, this could perhaps limit the applicability of such methods for real-time applications of nailfold assessment. However, there are instances in which the above technique may be chosen preferentially over inherently 2-D methods, e.g. light microscopy. The basin area of a capillary in the nailfold is defined as the (width \times height). The size of the basin area of a capillary tends to dictate in which plane relative to the surface that flow occurs. Flow in larger basin areas occurs in the parallel plane, while smaller basin areas are suspected to flow in an oblique plane with respect to the nailfold surface. This leads to underestimations in blood flow values if flow occurs in these oblique regions. The db-SdOCT method may be applied irrespective of basin area as the angular limitations imposed by the plane inclination do not apply. As such, subjects with normal and irregular vascular beds may be compared under the same regimes.

Figure 71: (a) Irregularly sized and shaped capillaries, representing one of the most striking early indications of secondary Raynaud’s phenomenon; (b) Typical capillary haemorrhaging prevalent in connective tissue disease [582, 588].
The ease with which capillaries may be imaged does not imply that the range of ailments associated with their morphological/haemodynamical characterisation is by any means limited. Chronic illnesses such as systemic lupus erythematosus, scleroderma [53], systemic sclerosis [54, 589, 590], antiphospholipid syndrome [591], connective tissue disease [55], diabetes mellitus [592] and Raynaud’s Phenomenon [56] have all been readily identified via specific physiological markers and pathological indicators at the capillary level such as increased vessel permeability (haemorrhaging) [591], the presence of avascular areas, enlarged loops and poor circulation [27, 28]. In addition, the identification of such ailments has not been limited to solely systemic diseases. Physiological irregularities have manifested in the nailfold plexus, stemming from localised areas of discomfort and have revealed the presence of conditions such as rheumatoid arthritis [2], vasculitis, psoriasis and psoriatic arthritis [57], and migraine, in addition to psychiatric disorders such as schizophrenia [593, 594].

6.2 Use of Doppler methodology in conjunction with db-OCT – a comparative study

Initially investigated by the Austrian physicist Christian Johann Doppler, the Doppler effect is a phenomenon whereby a moving sound source seems more highly pitched when approaching an observer, and of lower pitch when receding from one. This apparent shift in frequency also occurs when the source is stationary and the observer is moving, emphasising the importance of the relative velocity between the two. The effect may be generalised beyond sound to all types of wave phenomena, having been used extensively in all branches of science, particularly astronomy and engineering [595].

Photons scattered by moving constituents result in frequency shifts due to the Doppler effect and by analysing the spectral content change of the scattered light, flow metrics may be obtained. The Doppler frequency shift of waves scattered from a moving target is proportional to the frequency of the impinging wave:

\[ f_D \approx \frac{2v_x}{c} f_0 = \frac{2v_x}{\lambda_0} = \frac{2 \eta v \cos \theta}{\lambda_0} \]  \hspace{1cm} (68)

where \( f_D \) is the Doppler shift; \( \eta \) is the target tissue refractive index; the impinging light wavelength is \( \lambda_0 \); and \( \theta \) is the angle between the probing light and the vessel investigated, which manifests as the axial component of velocity, \( v \cos \theta \), in the above expression.
Figure 72: Illustration of arbitrary interrogation geometry of a typical Doppler measurement set-up, where flow is quantified by considering the axial flow component, $v_z$.

Utilisation of the Doppler phenomenon in blood flow visualisation requires detection of the resulting frequency changes or phase shifts, in addition to the amplitude of remitted optical echoes from corpuscles. The requisite predefined Doppler angle ($\theta$) is an obstacle for velocity determination, which restricts the practical application of Doppler OCT in the assessment of microcirculation in cases where the angle could not be predetermined. Furthermore, for flow quantification, not only velocity but also flow direction is important.

The Doppler effect has been extensively used in the biomedical arena for the non-invasive assessment of tissue blood flow, the most familiar examples of which are laser Doppler flowmetry and Doppler ultrasound imaging (see §2.2.2, §2.2.6).

Figure 73: Example of dynamic data in a 200 µm capillary ($\theta = 83.8^\circ$) with flowing 2% Intralipid solution at 0.5 mm/s. (a) Doppler data was obtained by in-house code adopted from the Kasai auto-correlation method [25], utilising a 10 x 10 kernel. Symmetric velocity profiles are created about the capillary central axis. (b) 2-D axial average of velocity values from (a). Maximum value of 4th order polynomial fit to data was 0.53 mm/s (6.23% deviation from theoretical value).
Although the crux of the novelty of the db-SdOCT modality is the quantification of flow within vessels without requiring knowledge of or consideration of any angular implications on the resulting velocity values, numerous efforts have been made in advancing the Doppler methodology to attain indirect values of the Doppler angle by various means. When flow is embedded in a highly scattering medium (e.g. in vivo blood flow monitoring), accurate estimation of real flow velocity is often difficult which necessitates the precise determination of the Doppler angle. Doppler bandwidth broadening is known to be in effect in ultrasound and laser Doppler velocimetry in which the output signal bandwidth is altered by moving scatterers that traverse the probing beam. Combined use of the interference signal Doppler shift and Doppler spectrum broadening caused by transverse movement of particulate matter across the probe beam enabling computation of the longitudinal and transverse velocity components has been implemented [577]. By statistical analysis of the temporal fluctuation of a remitted interference signal, the resulting spectrum takes a Gaussian form whose width is proportional to the flow velocity [483, 596-598]. However, cessation in the Doppler spectrum broadening at slower flow values influences calculations as it results in underestimations of the Doppler angle (and therefore overestimation of computed velocity values due to the inverse proportionality). Alternative angle insensitive methods have been investigated, for example, by measuring flow signal intensity instantaneously by rejecting stationary targets by the use of self-referenced coherent demodulation followed by bandpass filtering [517]; however, this is affected by issues such as polarisation-state mismatch and dispersion disparities between the sample arm and the self-referenced arm.

In addition to angular-based discrepancies, other factors exist which are not readily incorporated into the widely-used classical Doppler analysis. For instance, in the presence of a transverse velocity component, the relation between the axial velocity component and the phase difference between consecutive A-scans is non-linear if the sample is moving obliquely [35]. Although this non-proportional relationship may be considered by taking into consideration a correlation quotient, this adds to the processing complexity and ultimately the computational time of the resulting analysis.

6.2.1 Overview of Doppler processing algorithms – Kasai autocorrelation

Time domain DOCT based spectrogram methods – StFT

The most direct means of detecting true Doppler shift is by analysing the time-domain OCT data in Fourier space, distinguishing between the constant reference arm velocity
interferometric signal and the superposed flow-induced modulation components. The mean velocity of scatterers as a function of depth is estimated from the centroid frequency of the local interferometric reflectance using overlapped short time Fourier transforms (StFT). StFTs are used to determine the sinusoidal frequency and phase content of local sections of a signal as it evolves temporally. However, this method is computationally intensive and difficult to apply to in vivo dynamics as it requires stationarity of the signal during a finite time interval, and thus imposes some constraints on the representation estimate. In addition, the StFT has a fixed time-frequency window, making it inaccurate to analyse signals having relatively wide bandwidths that change rapidly with time [599].

Spectrogram methods allow simultaneous imaging of in vivo tissue structure and flow velocity; however velocity sensitivity is limited for high speed imaging. When StFT or wavelet transformation is used to determine flow velocity metrics, the resolution is determined by the window size of the Fourier transformation of each pixel. The mean velocity of scatterers located within each axial StFT window length \(\Delta t_p\) may be estimated from the centroid of the localised Doppler spectrum magnitude. The spatial resolution of the Doppler velocity estimate is determined by the axial StFT window length (limited by the coherence length) and by the diameter of the sample beam focus in the lateral dimension, i.e. the coherence volume. It has been shown that the minimum detectable Doppler frequency shift [600] and therefore the velocity estimation precision is inversely proportional to the StFT window-size [601]. Thus, the higher the value of \(\Delta t_p\), the higher the velocity sensitivity. However, the spatial resolution, \(\Delta x_p\), is also proportional to the StFT window size:

\[
\Delta x_p = v \Delta t_p \tag{69}
\]

where \(v\) is the 1-D scanning speed of the system. Consequently, velocity sensitivity and spatial resolution are coupled; large StFT window sizes increase velocity sensitivity but decrease lateral spatial resolution metrics [96]. Conversely, increasing the image frame rate decreases velocity resolution. The inherent coupling prevents the spectrogram method from achieving simultaneously both high imaging speed and high velocity sensitivity, essential features for measuring flow in small blood vessels wherein velocity values are low. As such, sequential phase-change scanning was implemented for image reconstruction in an effort to surmount the aforementioned limitations. The phase change (obtainable via Hilbert transform [601]) in each pixel between sequential A-line scans is then used to calculate the
Doppler frequency shift. As $T$ is much longer than the pixel time window, very small Doppler shifts are detectable, and spatial resolution and velocity sensitivity are decoupled.

**Phase resolved DOCT – Fourier domain PR-DOCT**

All velocity information resides in the phase term of the demodulated signal; coherent demodulation is essential to recover the Doppler-shift frequency [25]. Local (depth-resolved) phase change analysis by comparison of sequential depth scans prevents spatial resolution and velocity sensitivity from being in direct opposition in flow assessment and without compromise increases imaging speed by more than two orders of magnitude [96]. This may be performed by, for example, obtaining a Hilbert transform of the OCT signal and by dividing the phase difference between successive A-scans with the time between successive acquisitions, thus yielding the Doppler shift. Phase-based assessment of OCT images are advantageous as they yield increased axial scanning speeds, high frame rates and reduced speckle noise [602]. Aside from Hilbert based phase assessment (recently popularised by OMAG; see §3.3.3.3), the Kasai flow estimator method has been the computational ‘gold standard’ used for Doppler shift assessment.

Denoting the power spectrum, $P(\omega)$, of a complex envelope echo signal, the mean angular frequency, $\bar{\omega}$, may be expressed as [495, 601, 603, 604]:

$$\bar{\omega} = \frac{\int_{-\infty}^{\infty} \omega P(\omega) d\omega}{\int_{-\infty}^{\infty} P(\omega) d\omega}$$  \hspace{1cm} (70)

Consequently, estimations of the mean blood flow velocity $\bar{v}$ may be deduced from the frequency spectra of echoes by the following expression [577]:

$$\bar{v} = \frac{\bar{\omega}}{\bar{\omega}_0} \cdot \frac{c}{2 \cos \theta}$$  \hspace{1cm} (71)

The Wiener-Khinchine theorem (see §3.2.1) establishes a direct relationship between the auto-correlation of the complex remitted signal and $P(\omega)$. Kasai et al. utilised this relation, yielding metrics for the mean angular frequency and its variance from the auto-correlation of complex ultrasonic pulse emissions. The total mean velocity at any pixel can be evaluated by the Kasai auto-correlation equation [605]:

$$f_D = \frac{f_a}{2\pi} \tan^{-1} \left\{ \frac{1}{p(q-1)} \sum_{z=1}^{p} \sum_{x=1}^{q} (f_{z,x+1} Q_{z,x} - f_{z,x} - f_{z,x+1} Q_{z,x}) \right\}$$  \hspace{1cm} (72)

$$\langle v \rangle = \frac{\lambda_0 \Delta \Phi}{4\pi \eta T \cos \theta} = \frac{\lambda_0 f_D}{2 \eta \cos \theta}$$  \hspace{1cm} (73)
where $f_D$ is the Doppler frequency shift; $f_a$ is the frequency of acquisition of data; $p$, $q$ denote the window size; $I$ and $Q$ denote the in-phase (real) and quadrature (imaginary) components of the complex OCT signal, respectively. The Doppler frequency shift is denoted by $\omega = \frac{\Delta \phi}{T}$, where $T$ is the time interval and $\Delta \phi$ is the phase difference between sequential A-scans at a given location. Thus, the values obtained for $f_D$ may be utilised in Eq. 73, to yield values of total mean velocity at any pixel.

Kasai-based implementation may also result in aliasing, thus placing a physical upper limit for phase unwrapping techniques. A normalised power Doppler display (i.e. the area under the Doppler spectrum, excluding DC components) removes this aliasing artifact, although at the expense of loss of directional information. The power Doppler mode does not measure flow velocity, but indicates the presence or absence of flow and as such regions which exhibit flow are uniformly bright. Images of velocity variance in contrast reveal velocity gradients and regions of higher or lower flow, albeit at the expense of direction information also. Research has shown that by performing Kasai auto-correlation in both axial and transverse directions, an extended axial velocity estimation range may be obtained [25, 606]. However, it has been shown that Kasai-estimator performance deteriorates with increasing sampling rate [607], placing fundamental limitations on its performance. This lends credence to the fact that there is no unique or optimal way of representing Doppler flow information, which necessitates choosing the method which is most suitable for the intended application.

Numerous studies exist which endeavour to optimise Doppler computation in terms of velocity estimation accuracy and robustness to noise. Fourier-transform techniques (adaptive and weighted centroid methods), sliding-windowing filtering and correlation based processing have been investigated [608] – the sliding-window filtering technique gave a demonstrably favourable performance in terms of accuracy and robustness. Consequently, in the following comparative experiment, a 2-D Kasai-estimator was utilised for Doppler shift assessment.

6.2.2 Experimental considerations and results

In order to compare and contrast the db-SdOCT method described here with previously developed Doppler methods, it is necessary to consider all of the mitigating factors associated with each method’s operation. In this way, a fair assessment of each technique may be obtained, in which neither technique is preferentially treated. However, as there is an
inherent dissimilarity with regards each technique’s operation, i.e. inducing angles, computational windowing etc., such factors were considered *a priori*.

Fundamentally, the basic Doppler methodology dictates the establishment of an angular divergence from orthogonality concerning the impinging light and the vessel being imaged. However, the primary underlying feature of the db-SdOCT methodology is that this is not a requirement, but may function irrespectively. Although both methods could be compared separately by optimising each experiment and tailoring its own individual requirements, this negates the possibility of an appropriate comparison. Regarding the data capture, a requisite amount of data was required as oversampling in DOCT methodology is considered as standard. In order to achieve this oversampling condition, a larger amount of data for the db-SdOCT method than ordinarily necessary was required. For db-SdOCT analysis, the beam separation was maintained at $\delta = 150 \, \mu m$. In order to perform both studies in the same experimental conditions, the intentional application of an angle to the experimental set-up was incorporated. This angle was constrained to a range of 2°-4° below the horizontal such that theoretically Doppler methodology should apply in addition to the cross-correlation method. The reason for this range constraint is that given the comparatively large beam separation value, this created difficulties regarding the appropriate placement of the dual-beam arrangement insofar as comparable signal strength in both channels diminished with increasing angle. By taking each method’s experimental and processing minutiae into account, an appropriate comparative assessment could be performed.

For these experiments, *in vitro* samples of flowing 2% Intralipid were investigated. Several different capillary sizes were used (500 $\mu m$, 300 $\mu m$, 50 $\mu m$) to test for any dependency of either technique on vessel size and a large flow rate range was used (1 mm/s, 5:5:20 mm/s). Due to the minimum flow rate limit of the syringe pump, 0.42 mm/s was used for the 50 $\mu m$ capillary instead of 1 mm/s. The calculation of mean phase change from Eq. 72 is numerically efficient, especially in fixed-point, because it relies mostly on summations and multiplications. Accordingly, Doppler processing was performed (see Figure 74) by using the resulting in-phase (real) and quadrature (imaginary) components from the complex OCT data (*k*-space calibration, DC subtraction and FFT performed in advance of this) and the Doppler frequency shift ($f_D$) was computed.
Figure 74: Outline of the Doppler processing implemented in this comparative study of modalities.

The accuracy of the velocity estimation improves via larger values for the chosen window size, i.e. $p \times q$ [609], with more spatial averaging (see Figure 75). Consequently, the choice of window size was crucial in order to accurately represent the Doppler shift data but also was required to be efficient in terms of processing time. Considering both of these factors, a $(8 \times 8)$ window was chosen as the 2-D average of the velocity values obtained approached that of the theoretical flow when compared to that of window sizes $(2 \times 2)$, $(4 \times 4)$, $(10 \times 10)$, $(16 \times 16)$, and $(32 \times 32)$. It was seen that the accuracy of the velocity estimation markedly improved for larger window sizes as noted above; however, as the $(64 \times 64)$ window took 4.5
hrs to complete, it was not a timescale which was feasible for appropriate comparison to the db-SdOCT method which completes in \( \leq 1 \) min (sample size dependent). All data processing was performed offline.

![Image of different window sizes](image)

**Figure 75:** Comparison of the resulting Doppler shift data using different window sizes for a 500 \( \mu m \) capillary with 0.1 mm/s 2% Intralipid flow.

The average percentage error between the theoretical and calculated values for flow velocity using the db-SdOCT technique was 10.98\% and 8.77\% for the intensity and phased based methods respectively in these studies. The shows once again the improvement which may be gleaned from utilising the phase as the metric in correlation based analysis, compared with the raw OCT intensity. However, the errors obtained for this experiment using the db-SdOCT technique are in general greater than those obtained via previous experiments. This may be attributed to signal loss due to the addition of the intentional angle for Doppler analysis. If the beam separation had been small, then the effects of this angle become less and less of an issue. This is illustrated in Figure 76, in which the corrected value for beam separation value, \( \delta \) is:

\[
\delta' = \frac{\delta}{\cos \theta}
\]  

(74)

Increasing the angle by as small amount as 4\(^\circ\), increases the actual dual-beam separation by 0.24\%, thereby influencing the velocity values obtained.

![Diagram of beam separation and angle](image)

**Figure 76:** Illustration of the influence of angle on the quoted value of beam separation, \( \delta \).
With reference to Doppler analysis, data was analysed in two different ways: (a) a 2-D region was selected in the resulting Doppler image and the centroid of the resulting frequency components was computed (see Figure 75); and (b) axial plot of the resulting velocity values was obtained. Regarding Doppler data acquisition, data was oversampled to ensure a requisite amount was obtained for analysis, especially for smaller capillary values. This created a laterally elongated Doppler capillary image and forward scattering effects are apparent from the resulting 2-D Doppler analysis.

The 2-D velocity profiles obtained for the 50 μm capillary (see Figure 77) displayed approximate laminar flow behaviour for all instances of flow velocity. The maximum velocity value obtained for the 50 μm capillary at 0.42 mm/s flow velocity was determined to be 0.33 mm/s (i.e. 21.14 % error) via Doppler analysis. All other values of velocity converged to a value of 0.53 mm/s irrespective of the velocity values applied.

In reference to the 300 μm capillary (see Figure 78), the maximum velocity values obtained converged to a value of 0.53 mm/s irrespective of the velocity values applied. Although the peak positions of the resulting axial plots did scale linearly with increased applied velocity, the maximal values achieved were not representative of the velocities contained within the capillary. In addition a departure from the laminar flow profiles present in the 50 μm investigation is evident.

Regarding the 500 μm capillary (see Figure 79), a complete departure from laminar behaviour is clear from the resulting axial velocity profiles. This is not due to the applied velocities as their respective Reynolds numbers do not approach that which is required to cause flow turbulence; phase-wrapping effects may be cause of this behaviour. Doppler flow velocities are calculated by [535]:

\[ v_Z = \frac{\lambda_0 \Delta \phi}{4\pi T\eta} \] (75)

where \( \Delta \phi \) is the phase difference between two consecutive A-scans and all other terms have their usual meaning. Using an acquisition rate of 16846 Hz, the maximum axial velocity range measurable without phase wrapping is ±4.06 mm/s. As such, this comparative study shows that for the angles induced and the limitations imposed on the Doppler analysis by system hardware, the Doppler method is unable to discern velocity values representative of those applied. However, the capability of the Doppler method at discerning flow direction was readily seen, without the need for supplementary processing as in the db-SdOCT auto-correlation method.
Figure 77: Doppler analysis for a 50 μm capillary for flowing 2% Intralipid solution at (a) 0.42 mm/s, (b) 5 mm/s, (c) 10 mm/s, (d) 15 mm/s, (e) 20 mm/s. Although laminar flow profiles resulted from 2-D axial averaging of the resulting Doppler profile, the Doppler flow velocities consistently underestimated the theoretical values of flow applied. (Measured \( \theta_D = 86.82^\circ \).)
Figure 78: Doppler analysis for a 300 μm capillary for flowing 2% Intralipid solution at (a) 1 mm/s, (b) 5 mm/s, (c) 10 mm/s, (d) 15 mm/s, (e) 20 mm/s. Although laminar flow profiles resulted from 2-D axial averaging of the resulting Doppler profile, the Doppler flow velocities consistently underestimated the theoretical values of flow applied. (Measured $\theta_D = 87.38^\circ$.)
Figure 79: Doppler analysis for a 500 μm capillary for flowing 2% Intralipid solution at (a) 1 mm/s, (b) 5 mm/s, (c) 10 mm/s, (d) 15 mm/s, (e) 20 mm/s. Although laminar flow profiles resulted from 2-D axial averaging of the resulting Doppler profile, the Doppler flow velocities consistently underestimated the theoretical values of flow applied. (Measured $\theta_D = 86.82^\circ$.)
6.2.3 Discussion

The frequency resolution obtained by Doppler based methods depends on the amount of time spent detecting the Doppler shifts at a given location. The longer the time between pixels, more scatterer motion is captured creating additional high spatial frequency content; thus detection of smaller velocities precludes imaging in a moving sample. It has been recently demonstrated that velocity resolution can be improved by orders of magnitude without compromising acquisition time by calculating Doppler flows across sequential scans [519].

Phase-resolved Doppler OCT (PrDOCT) measures the phase difference ($\Delta \phi(z)$) between two consecutive axial lines [558]:

$$ v_{axial}(z) = \frac{\sigma \Delta \phi(z)}{4 \pi T} $$  \hspace{1cm} (76)

where all terms have their usual meanings. The phase sensitivity of an OCT system governs the lowest observable velocity ($v_{min}$) of a moving scatterer [465], regardless whether the system is of the swept source or spectrometer based variety. As $\Delta \phi(z)$ is normally computed through the use of the inverse tangential function, it is therefore subject to the $\pi$ phase ambiguity. Therefore, the maximum detectable axial velocity is:

$$ v_{axial, max}(z) = \frac{\sigma}{4T} $$  \hspace{1cm} (77)

and

$$ v_{axial, min}(z) = \frac{\sigma \Delta \phi_{err}(z)}{4 \pi T} $$  \hspace{1cm} (78)

where $\Delta \phi_{err}$ is the phase error which can be statistically quantified as the deviation from the mean of the phase difference measured from a stationary sample.

Problematic factors which hinder functional imaging and accurate assessment of the detected Doppler signal include phase wrapping and interferometric fringe washout effects, which occur in large blood vessels with fast flow rates. Phase wrapping occurs if $|\Delta \phi| \geq \pi$ and this is the maximal axial velocity component which can be unambiguously measured within a given time interval $\Delta t_A$ [535]. In order to avoid phase wrapping in big arteries and veins, shorter time spacing is required. Precise detection and measurement of such factors are difficult in the in vivo biological environment due to SNR loss resulting from the sample scatterer motion. The commercial availability of wavelength-swept sources have made them an increasingly attractive alternative as they have been shown to be more robust against these
artifactual effects compared with spectrometer based detection schemes [610], however fringe washout effects will still be present at higher velocities.

The maximum velocity is typically characterised as the speed at which a π phase shift is induced in the Doppler signal \( v_{\text{wrap}} \). However, it is possible to detect scatterers moving at even higher velocities, though phase-wrapping effects are then evident. It has been demonstrated that the maximum detectable Doppler velocity is determinable by the fringe washout limit and not phase wrapping \( v_{\text{wash}} \). Fringe washout occurs due to motion of a sample causing approximately a π phase shift in the spectral interferogram during the acquisition of a single spectral resolution element, which can result in significant SNR degradation. However, as swept source systems have \( M \) spectral samples per A-scan and thus a much higher sampling rate, these systems are more robust to fringe washout effects as compared to their spectrometer counterparts. Values for \( v_{\text{wash}} \) determine the maximum scatterer speed detectable for all flow imaging methods before severe signal loss occurs. A distinction between the related expressions and different limiting factors of velocity quantification extrema may be seen in the following table.

<table>
<thead>
<tr>
<th>Velocity Limit</th>
<th>Expression</th>
<th>Determining Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum velocity ( (v_{\text{min}}) )</td>
<td>( \frac{\lambda_0}{2 \pi^2 \eta T \cos \theta \sqrt{\text{SNR}}} )</td>
<td>Image SNR</td>
</tr>
<tr>
<td>Wrapping velocity ( (v_{\text{wrap}}) )</td>
<td>( \frac{\lambda_0}{4 \eta T \cos \theta} )</td>
<td>Time between A-scan acquisitions</td>
</tr>
<tr>
<td>Washout velocity ( (v_{\text{wash}}) )</td>
<td>( \frac{\Omega \lambda_0}{4 \eta \cos \theta} )</td>
<td>Time between single spectral channel acquisitions</td>
</tr>
</tbody>
</table>

Determination of absolute flow requires knowledge of the angle between the flow direction and propagation direction of the illumination beam. A 5° change in vessel angle with respect to the incident beam can cause a change of more than 30% in the measured velocity [451]. The variation of the Doppler angle has been shown to be more sensitive to the variation of axial movements [613]. Therefore, numerical methods [614] of motion correction need to be employed if additional sources of error in resulting velocity values are to be avoided. This, however, increases the computational expense associated with such protocols. Methods have been developed to measure axial blood velocity in an en face plane by integrating over the vessel area, without the need for Doppler angle measurement [484]. The unnecessary computation of the Doppler angle by en face methods dramatically
simplifies blood flow measurements. Raster scanning over an *en face* area intercepting the blood vessel in this manner requires higher imaging speeds due to the larger number of A-scans necessary compared with traditional cross-sectional plane scanning methods. As such, this method requires a very high speed camera and vast oversampling of data, and has increased computational and memory requirements. Additionally, variation in segmentation of vessel cross sections could result in flow measurement differences.

In conclusion, a comparative study of Doppler and db-SdOCT analysis was performed. Limitations associated with the system hardware in terms of phase sensitivity and wrapping ultimately impeded the ability of the Doppler analysis to compute velocity values. Although the ability of the Doppler method to discern directionality was evident without specialised processing, the Doppler angle chosen restricted the computation of velocity, causing the values to plateau and converge to a common value irrespective of the flow rate applied. The db-SdOCT methods was unaffected by the hardware parameters; however, an increase in error was seen in the resulting velocity data obtained which may be attributable to the intentional application of an angle (thereby making positioning difficult) and the large beam separation value chosen.
7 Future directions

Expanding on the in vivo application of the db-SdOCT system as outlined in Chapter 6, investigations into the future potential of the cross-correlation method of dynamic assessment is presented. Given the imaging range restrictions of OCT, application of such systems to an endoscopic regime is the focus of ongoing work by various groups. As such a macroscopic model of a side-viewing endoscope is constructed and tested with a view to future cardiological applications. In addition, as biological systems are transient environments, the application of the db-SdOCT technique to the investigation of pulsatile flow detection is also outlined.

7.1 Development of a prism-pair db-OCT for endoscopic applications

Interventional cardiology presents a unique set of opportunities and challenges for in vivo imaging. Drug coated intravascular stents can be implemented in the treatment protocol of coronary artery disease without the necessity of invasive and expensive surgical procedures [615]; however, guidance and follow-up assessment by techniques such as intravascular ultrasonography (IVUS) are ultimately limited in their achievable resolution and assessment capabilities [616, 617] (see Figure 80).

![Figure 80](image)

Figure 80: Example of the restrictive nature of modalities in terms of intravascular assessment. (Left) OCT and (Right) IVUS renderings of in vivo stent placement. (Left) Tissue prolapse (filled arrow) and under deployed stent (open arrow) features are evident in OCT image; IVUS (b) is unable to resolve such features [384].

The relatively poor spatial resolution performance of IVUS methods and their requirement for acoustic coupling limits their efficacy for image guided intervention, such as for the staging of tumour growth progression [618-621]. An additional non-optical method, endoscopic MRI, has been used in the assessment of the gastrointestinal (GI) tract. However, the requirement of placing the subject inside a large magnet and with depth resolution on the order of mm
[622], the cost and inconvenience of the procedure are significant. Nevertheless, these non-optical modalities serve as an indicator to the utility of minimally invasive endoscopic imaging techniques for improving the accuracy and outcome of clinical procedures [623].

Figure 81: Examples of side-viewing OCT endoscopes. (Top) Circumferential scanning catheter endoscope utilising a microprism at the distal end to emit and collect a single spatial-mode optical beam [624-626]; (bottom) a linear scanning needle-based DOCT system using angle-polished ball-lensed fiber [627].

A typical fiber endoscope uses a flexible fiber-optic cable (i.e. light guide) composed of a bundle of glass or plastic fibers to transfer the image from the distal end to the proximal end. However, such light based endoscopes do not proffer any sort of depth resolved perception [384, 628]. In an effort to surmount this, optical imaging techniques such as fluorescence [384], confocal [629], multiphoton microscopy [630], in addition to OCT, offer advantageous improvements in terms of portability, cost, and spatial resolution metrics. In an effort to expand the applicability of the use of such endoscopic devices, the primary design consideration is to minimise the diameter of the OCT probe. To this end, single-mode fiber (used for the transmission of light in most OCT systems) is ideally suited for this purpose. Fiber-optic probes for sensing and biomedical imaging applications frequently employ sections of gradient-index (GRIN) fiber to re-focus the diverging light from the delivery fiber at the distal end of the catheter. Such GRIN fiber microlenses often possess aberrations that cause significant distortions of the focused output beam [102]; however, novel methods have been reported to extended the depth of focus of such GRIN lenses by a factor of two using phase masks [631]. Supplementary to tissue reflectance data, spectroscopic data may also be gleaned from such techniques enabling a multifaceted view of the sample in question. Given the advancements in second generation OCT system in terms of sensitivity (see §3.2.1), this can translate to a greater depth scan range at high acquisition speeds [632].
Fiber-based OCT probes [526] may be classed into two primary categories: forward-viewing [633] (imaging beam exits along the catheter direction) and side-viewing [634-636] (imaging beam exits orthogonally to the catheter direction). A majority of endoscopic probes reported in the literature are side-scanning. As endoscopic probes are inherently 1-D/2-D in nature, 3-D realisations of *in vivo* environments may be obtained by circumferential or linear scanning. Side-viewing catheters may perform such 3-D scanning by either proximal or distal scanning mechanisms. However, proximal scanning catheters typically employ a flexible rotating fiber which can create non-uniform rotational distortion in sharp bends [637], although the catheter sheath can serve as a reference surface for phase shift calibration against phase artifacts [638]. Distal scanning catheters, on the other hand, do not require a rotary junction and thus, rotational distortion can be avoided. Consequently, faster scanning speeds can be achieved; for example, a miniature motorised OCT endoscopic probe capable of 208 fps (12,500 rpm) [639] and a micro-motor based miniature endoscopic catheter capable of 1,200 fps [640], have recently been reported. In addition, polarisation and dispersion mismatch issues, and an increase in the vibration tolerance of such systems can be circumvented through common-path approaches [641]. Side-imaging endoscopes are suitable for imaging within a tubular organ such as the vascular system [642], GI [643] and urinary tracts [644]. In contrast to side-imaging endoscopy, the forward-imaging variety can be useful in providing tissue structural information for surgical guidance or device placement [645].

A MEMS-driven micro-reflector (thickness, 80 μm) capable of circumferential OCT scanning utilising a Au-coated micro-pyramidal polygon reflector mounted on chevron-beam microactuators, has been reported [646, 647]. A near-360° range 3-D tissue image may be reconstructed by either sequential or synchronous data acquisition from four channels, reflected at 45° from the slanted facets of the reflector. Reduction of artifacts such as those created by mechanical rotation and degradation of imaging performance due to mirror deformation induced by residual stresses may be alleviated through the use of such novel scan platforms. In addition, for OCT applications, the utilisation of four synchronous channels increases the potential acquisition speed four-fold. Mechanical considerations aside, it is necessary to develop appropriate designs and scanning protocols to prevent damage to the vascular endothelium, especially in the case of smaller vessel sizes.

Blood flow velocity and volumetric flow measurements are important parameters for the assessment of stenosis severity and the outcome of interventional therapies. Despite the inherent haemodynamical alterations of catheter placement, IVUS Doppler [648] and
endovascular Doppler OCT [649] have been used for coronary blood flow measurements. The inclusion of functional extensions, such as flow assessment, does not require revisions of the OCT probe design and as such their inclusion are driven by the usefulness of the functionality, as opposed to any technical implementation difficulties. Improvements in *in vivo* intravascular OCT penetration may be performed by diluting blood by saline. However, the refractive index variation of various mixing ratios of water, blood and saline may result in changes in the scanning angle between the scanning beam and the catheter axis. In addition, in most cases, the imaging catheter is not coaxial with the blood vessel and consequently, this orientation represents an inherent source of error in determining absolute flow velocity values. A 3-D reconstruction from pull-back imaging may help to deduce the angle between the catheter axis and the vessel center-line, and thus provide better estimations of the beam to the flow angle albeit at computational expense and temporal constraints. It has been shown that frame to frame phase shift calculation is not reliable with fiber rotational speeds of 100 rps during *in vivo* imaging [650].

### 7.1.1 Experimental considerations and results

To investigate the feasibility of utilising the db-SdOCT method in future endoscopy studies, a simplified db-SdOCT apparatus was constructed; a macro-model of a side-viewing endoscopic device. In order to bring the beams from each channel to the sample plane, a pair of Au-coated prism mirrors (MRA05-M01; Thorlabs GmbH) was used in a 30 mm cage arrangement. A slight angular separation of the prisms induces a separation δ in the beams and these were clamped in place; if this was not done, the minimum displacement possible would have been the twice the width of each prism at their point of reflection.

![Figure 82: (Left) Schematic and (right) experimental image of the db-SdOCT macro-model endoscope.](image)
A 30mm achromatic doublet lens was used to collimate the beams to the sample. The beam profiler was used to validate the beam separation for use in the cross-correlation analysis computation. As the design is too large to test within an enclosed cavity, the macro-model was tested on in vitro samples (of diameter 500 μm and 1000 μm) of flowing 2% Intralipid solution at various velocities (2.2:20 mm/s).

**Figure 83:** Graphical results of flow experiments using the cross-correlation prism-pair db-SdOCT macro model for potential endoscopic uses; (a) intensity and (b) phase-based correlation analysis. Model was tested on in vitro flow phantoms of size 500 μm (denoted by dots) and 1000 μm (denoted by asterisks) with flowing 2% Intralipid at varying flow rates. Error bars: ±10% of values provided by syringe pump.

As can be seen from the graphical data in Figure 83, phase-based analysis outperformed that of the intensity-based correlation analysis by a factor of 2.13 in terms of accuracy. From Table 10, comparable results may be obtained using phase-based correlation analysis for both 500 μm and 1000 μm size test capillaries.

**Table 10:** Resulting experimental values using a simplified macro-model of a side viewing db-SdOCT set-up for endoscopic applications.

<table>
<thead>
<tr>
<th></th>
<th>% error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intensity</td>
<td>Phase</td>
</tr>
<tr>
<td>500 μm</td>
<td>13.38</td>
</tr>
<tr>
<td>1000 μm</td>
<td>10.54</td>
</tr>
<tr>
<td></td>
<td>5.33</td>
</tr>
<tr>
<td></td>
<td>5.86</td>
</tr>
</tbody>
</table>
7.1.2 Discussion

Aggressive research and development efforts aim to place even better technology in the hands of interventional cardiologists, where the focus is placed more on the appropriate selection, optimal delivery and careful post-interventional assessment of coronary therapies. The GI tract is the first internal organ for which endoscopic OCT was applied [639]. The ability to study this organ non-invasively and with high precision was welcomed clinically, especially given the plethora of diseases which can arise in the GI tract alone; examples include Barrett’s oesophagus [26], ulcerative pancolitis [651, 652] and cancer staging [653].

Atherosclerosis is a process in which fatty deposits, cholesterol, cellular waste products, calcium and other substances build up (forming stenotic lesions) in the inner lining of arteries, which can significantly decrease or block completely blood flow. Lesions can rupture that may result in arterial occlusion, but can be difficult to classify in terms of their vulnerability to rupture; endoscopic OCT methods have been show to be able to reliably detect and discern plaque morphology [654, 655]. The respiratory system is another venue in which OCT endoscopy can have significant utility. The system is readily accessible and a large variety of conditions could benefit from OCT assessment, notably sleep apnoea [656, 657], laryngeal carcinoma [658] and bronchial studies [659].

Among gynaecological cancers, ovarian cancer (although rare) has the highest mortality rate [660]. The ability to image and assess cancerous formations can significantly improve the diagnostic clinical accuracy of ovarian cancer [384], in addition to other types such as prostate cancer [661], urinary cancer [662]. The cure rate of neurological tumours depends upon its complete removal, which necessitates the ability to accurately define resection boundaries. Although MRI can assess the proximity of a tumour to certain regions which control motor skill, its low resolution renders it unlikely to be able to discriminate boundaries. OCT, on the other hand, has been shown to have the capability to discriminate between healthy and pathological brain tissues in several pioneering studies [663], [664].

Endoscopic OCT offers a means of obtaining high-resolution and high-speed depth-resolved visualisation of tissue structures in vivo. Its relatively low cost and non-destructive nature are highly attractive clinical advantages. With a view to future potential application of the db-SdOCT set-up and algorithm for intravital exploratory applications, a macro-model of a side-view endoscope device was implemented to investigate the efficacy of this simplified approach relative to the general db-SdOCT system configuration. This model has been shown to be successful in this respect; implementation of this mechanism in a catheter probe
is a prospective avenue for this technique, opening up exciting avenues for exploratory research.

7.2 Extraction of pulsatile information for *in vivo* research applications

The ability to provide a clinician with real-time visualisation and guidance is particularly important for imaging purposes as *in vivo* flow can be pulsatile, intermittent and/or otherwise changeable with time [665]. Enabling and adapting a method to discern such functional information expands the remit in which the method is useful. Although the ability to discern and quantify pulsatility allows quantification and characterisation of this temporal phenomenon in its own right, it also permits its gating if it is hampering imaging applications though the introduction of transient artifacts, e.g. dynamic ECG-gated CT [25].

As velocities are generally pulsatile in nature, there is an added restriction for imaging modalities that the time over which the sequence of exposures is acquired be small compared to the period of the pulse [666]. Blood flow dynamics such as pulsatility and autoregulation have been shown to change throughout the progression of ocular diseases such as diabetic retinopathy and glaucoma [519]. In addition, the administering of anaesthesia can effect total blood flow [451], e.g. isoflurane is a known vasodilator at high concentrations, and so the monitoring of the effects of such are of importance. Irregularity regarding known pulsatility values also provides valuable clinical knowledge; for example, pseudoaneurysms or false aneurysms are tender pulsatile arterial masses which can occur due a previous invasive medical procedure that necessitated intrusion into an artery [667], e.g. stent placement. Correct characterisation of such as a pseudoaneurysm (which can be misinterpreted as an abscess) is vital as incision or drainage could lead to extensive haemorrhage. Assessment of the effects on flow caused by vascular abnormalities, e.g. stenosis (see §5.5), can provide a direct overview of the stresses imposed on the plaque by the flow field and thus provide a means of estimating the likelihood of possible rupture. Investigations into irregularities of related pulsatile waveforms can provide such stress information, thereby allowing a self-diagnosis of the associated dangers of particular stenotic lesions [668].

7.2.1 Experimental considerations and results

The non-linear, transient pulsations in pressure and flow of the cardiovascular flow mechanism results in variations in applied shear stress to vessel walls. Although such flow characteristics may be modelled using Navier-Stokes equations [669, 670], the Womersley
number (see §1.1.2) is a convenient dimensionless measure of the pulsatile flow frequency in relation to viscous effects rather than a model of the actual flow. Womersley numbers were calculated using Eq. 3 for 500 μm, 300 μm, 50 μm in vitro phantoms, with flowing 2% Intralipid solution. As some typical values for the Womersley number in the cardiovascular system for a canine at a heart rate of 2 Hz are 0.04, 0.035 and 0.005 for arterioles, venules and capillaries respectively [671-673], the calculated experimental values correlate well so as to provide an accurate representation of in vivo pulsatile flow.

When values for $\alpha$ are small ($\leq 1$), this implies that the frequency of pulsations is sufficiently low that a parabolic velocity profile has time to develop during each cycle.

### Table 11: Experimental Womersley numbers, $\alpha$, using 1060 kg/m$^3$ and 0.006 (Ns/m$^2$) for the density and dynamic viscosity parameters.

<table>
<thead>
<tr>
<th>Beats per min</th>
<th>500 μm</th>
<th>300 μm</th>
<th>50 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>0.527</td>
<td>0.316</td>
<td>0.053</td>
</tr>
<tr>
<td>70</td>
<td>0.569</td>
<td>0.341</td>
<td>0.057</td>
</tr>
<tr>
<td>80</td>
<td>0.608</td>
<td>0.365</td>
<td>0.061</td>
</tr>
<tr>
<td>90</td>
<td>0.645</td>
<td>0.387</td>
<td>0.065</td>
</tr>
<tr>
<td>100</td>
<td>0.680</td>
<td>0.408</td>
<td>0.068</td>
</tr>
</tbody>
</table>

In order to induce pulsations in the flow of the in vitro model experimental setup, a section was cut in the plastic tubing connecting the syringe pump to the flow phantom, which was...
placed in the focal plane of the db-SdOCT system. This section was replaced with a larger diameter section of tubing which had greater malleability (see Figure 85); this ‘valve’ regulated and controlled the pulse applied to the phantom and ensured pulse propagation throughout the entire length of the closed system. The pulse was applied using a motive force generator (MFG) (model: mini-shaker 4810; Brüel & Kjær, Denmark), onto which the valve was clamped. A signal generator (Unilab, UK) supplied voltage to the MFG and a square waveform was applied, monitored with an oscilloscope (model TDS 210; Tektronix, USA). The amplitude of the voltage supplied dictated the extent of the displacement performed by the MFG (maximum displacement, ± 3 mm). It was evident that if the chosen voltage amplitude (peak to peak) was too high, the pressure variations caused by the pulse created great turbulence within the phantom. Although Intralipid was still flowing under these circumstances, the large amplitude of the pulse voltage impeded the flow, which was not representative of dynamics which would be present in an in vivo biological environment. Consequently, the amplitude was reduced such that the flow course was pulsed but not inhibited by the simulated pulse.

![Figure 85: Illustration of the experimental setup to investigate pulsatile flow phenomena in in vitro flow models.](image)

Pulsatile information may be gleaned through either physical measurements or imaging methods. With regards Doppler based modalities, a common trend exists in their respective processing of pulsatile information, even though they may be markedly different in the parameters they consider for processing or the methods of processing adopted [674], e.g. phase, variance, etc. Generally, a region may be cropped and transient behaviour analysed by integrating the phase of the demarcated region over time. Power spectral densities may then be obtained by implementing non-parametric methods [675]. Alternately, sequences of flow profiles may be extracted; this serves a dual purpose in that both pulsatility and turbulence
may be analysed if flow profile departs from the laminar condition (see Figure 86). However, with regards the db-SdOCT method, as the method of extracting and analysing velocity data is dissimilar to the aforementioned methods, an alternative means of extracting pulsatile information is considered.

![Figure 86](image)

**Figure 86:** Example of methods used to yield pulsatile information. (a) Longitudinal retinal (a) and corneal (b) displacements were measured and FFTs were performed on the resulting data (c and d) revealing the spectral components present [676, 677]. (b) Extraction of retinal flow dynamics by colour DOCT. (b)(i) A sequence of images of a selected retinal blood vessel. (b)(ii) A sequence of extracted flow profiles [678].

As described in §4.4, velocity values are obtained by computation of cross-correlation of intensity and/or phase data. In short, the differences in time which correspond to the A-scans which yield the highest values of correlation between Channel A and Channel B are then averaged and upon division of this into the calibrated value for the beam separation, values for the intraluminal velocity may be computed. Although it has been shown that obtaining axial velocity profiles utilising the db-SdOCT technique is achievable (see §5.2), the computation of such for each instant in time and tracking the maximum velocity obtained through appropriate fitting, although possible, would greatly increase the computational expense involved and would be limiting factor if applied to real-time applications. An alternative method of approaching this issue is to utilise these maximal correlation differences in time between channels and to examine this using FFT for any spectral components of interest; this processing method is outlined in Figure 87.
Various different flow rates were considered for this pulsatility investigation and five different pulse rates were artificially applied to *in vitro* samples to study whether the pulse affects the db-SdOCT methods’ ability at discerning velocity data with forced haemodynamical changes present. In order to increase the resolution of the frequency axis, a large dataset of values was obtained. This comprised 5000 A-scans with switching time $\tau = 1.59$ ms. To remove artifact ghost lines from each frame, all spectra comprising the image are averaged, generating a single background spectrum. Each individual raw spectrum has this background divided out, removing all fixed pattern noise in the image [451]. OCT and cross-correlation analysis was applied to the data, after which corresponded to a total time available for FFT analysis of 3.98 s. The criterion for normal sinus rhythm includes a heart rate range of 60-100 beats per min (i.e. a simulated pulse range 1-1.67 Hz). As such, given the total analysis time available, this corresponds to a possibility of detecting between 4–6 pulses during any single acquisition.
Experimental investigations involved capillaries of size 500 μm, 300 μm and 50 μm, performing both cross-correlation (for velocity quantification) and FFT analysis (for spectral analysis) using both intensity and phase data (see Figure 88). With regards the cross-correlation velocity data for all capillary sizes, deviation from the theoretical velocity values supplied by the syringe pump were, 3.57% and 3.18% for intensity and phase-based analysis, respectively, indicating (a) the application of the simulated pulse did not impede the ability to yield velocity values via cross-correlation, and (b) the advantageous use of the phase as the cross-correlation metric in dynamic assessment (see Figure 89).

**Figure 88**: FFT analysis of cross-correlation gleaned velocity data for simulated in vitro pulses in a 300 μm capillary with flowing 2% Intralipid solution at 11 mm/s. The pulse values were 60:10:100 bpm (beats per min), corresponding to cardiac sinus rhythm. The pulse frequency values detected correspond to <0.8% deviation from the simulated pulse frequencies applied.
Figure 89: FFT analysis of (top) 500 μm and (bottom) 50 μm capillary with flowing 2% Intralipid solution at 7 mm/s with a simulated pulse of 60 bpm. Both (left) intensity and (right) phase-based cross-correlation velocity values were used. Phase-based analysis demonstrated decreased error in its replication of simulated pulse values. In the above examples the relative errors were (Intensity, left) 500 μm = 8.40%, 50 μm = 5.13%; and (Phase, right) 500 μm = 0.8%, 50 μm = 0.77%.

Insufficient OCT speckle data within the probing coherent volume at faster velocities makes discernment of instances of high statistical correlation more difficult. As such, an increased deviation from theoretically applied flow rates occurs for decreasing capillary size using cross-correlation analysis [679]. In terms of justifying the trend of increasing accuracy of velocity values gleaned with increasing capillary diameter, this may be understood using the rationale of speckle OCT. Regions of movement within a real-time OCT image capture are apparent by the intermittent flickering of the speckle present. Resultantly, in reference to db-SdOCT cross-correlation analysis, a sufficient amount of transient speckle OCT data (although this is arbitrary) must be present and detectable within the coherent probe volume (see §5.4.3, Figure 59) of the impinging light beams, such that clear computation of like events (i.e. instances of high correlation) may be realised. However, for lower values of velocity in e.g. 50 μm sizes, the reduction in speckle data available from the resultant reduced coherent volume is compensated for by the slower applied flow rates (i.e. flow rates in 50 μm diameter capillaries are $10^2$ smaller than in 500 μm sizes). In reference to FFT analysis of the simulated in vitro pulses applied, smaller capillary diameter sizes result in better discernment and consistency of the pulsatile values computed. This stems from the fact that although slower flow rates are applied, any temporal change in the volume of flowing media in such smaller capillary sizes is more appreciable, thereby enabling pulsatile detection with greater
efficacy. In general, however, it was clear from the resulting analysis that the errors relating to the values of the FFT maximum frequency obtained increased as the applied velocity values decreased; this is illustrated in Figure 90. The mean error of the detected simulated pulsations for the three quoted capillary sizes over the velocity range of 1:2:11 mm/s is 9.43% (Figure 90(a)) and 6.52% (Figure 90(b)) based on FFT analysis resulting from, respectively, intensity and phase-based cross-correlation velocity data. This point is also highlighted in Figure 91, in which for the same applied flow rate, the errors relating to the position of the resulting maximal frequency obtained increased with decreased simulated applied pulse.

![Figure 90](image1.png)  
![Figure 91](image2.png)

**Figure 90**  
In pulsatile FFT analysis, an evident error increase in the location of the FFT maximum occurred with decreasing velocity values. The above plots reveal the % error obtained for the maximal correlation frequency and the simulated applied pulse for (a) intensity and (b) phase-based correlation analysis. The errors obtained were more prominent for larger capillary sizes at lower flow rates. (A 2nd order polynomial fit is shown for clarity.)

As velocities decrease, the time to travel between channels of the db-SdOCT system increases with decreasing velocity and this is reflected in the cross-correlation analysis, requiring more data for computation of velocity values. This reduction in flow velocity speed may also impede the ability to discern pulses resulting in inevitable errors in the FFT analysis. This perhaps indicates a limitation involved with applying FFT to cross-correlation yielded velocity data, but this may also be attributed to the unrealistic inelastic expansion of the *in vitro* flow model implemented. Regular expansion of biological vessels assists in the regulation of pulsatile flow patterns; the rigidity of the silica capillaries, therefore, may not be
a realistic analogue. Thus, although this is a possible limiting aspect to the db-SdOCT method and algorithm for \textit{in vitro} investigations, the naturally occurring elasticity of the \textit{in vivo} biological environment lends credibility to its use.

Figure 91: \textit{In pulsatile FFT analysis, an increased error in the location of the FFT maximum occurred with decreasing velocity values. Data shows a 500 \mu m capillary with flowing 2\% Intralipid solution at 9 mm/s at (top) 80 bpm and (bottom) 90 bpm, representing a respective deviation of 4.31\% and 2.14\% from the simulated pulse frequency (intensity-based cross-correlation velocity data).}

Higher harmonics are evident in the resulting FFT plots, which is unsurprising as the cross-correlation derived velocity values exhibit sharp corners and steep slopes in the time domain (see Figure 87). Although the resulting maximal pulsations rise well above the noise floor, this may prove problematic in distinguishing the maximum dynamic frequency present with decreasing velocity values. A smoothing filter prior to FFT processing performed on the resulting correlation data could mitigate these effects; however, the influence of the use of such filtering on correlation data would require further investigation.

The computed temporal maps of the cross-correlation velocity data revealed some turbulent changes to the flow investigated if increased pulse frequencies were applied. Figure 92 demonstrates the increased turbulent behaviour present due to higher pulse frequencies at the same applied flow rate in the resulting temporal cross-correlation maps. However, these ‘disturbances’ had only minor effect on the ability of the db-SdOCT algorithm at discerning velocity data, with an overall difference between an applied pulse of 60bpm and 100 bpm for all velocity values and all different capillary sizes of 0.19\%. This may be juxtaposed to the
previously described analysis of Figure 90, in which the applied flow rates impacted on the
discernment of applied pulse values. This reveals that the converse is untrue. Similar effects
in the rendered temporal correlation maps result if increasing velocities are considered at a set
simulated pulse rate (see Figure 93).

**Figure 92**: Temporal cross-correlation maps resulting from analysis of a 50 μm capillary with flowing 2% Intralipid at 5 mm/s with simulated pulse at (left) 60 bpm and (right) 100 bpm. The increased pulse frequency resulted in a more turbulent correlation map.

**Figure 93**: 50 μm capillary with a simulated pulse at 60 bpm with flowing 2% Intralipid solution at (left) 9 mm/s and (right) 11 mm/s. Although both images reveal temporally segmented regions of high correlation, this illustrates the effect of increasing velocity on correlation plots. The slow flow rates applied to the 50 μm capillary enable the capture of this type of phenomena in comparison to 500 μm capillaries in which to establish 9 mm/s or 11 mm/s, require flow rates 10^2 larger.
7.2.2 Discussion

There exists different means of extracting pulsing phenomena from \textit{in vitro} and \textit{in vivo} samples, as evident from the relevant literature. Aside from imaging methods, techniques which monitor physical changes due to blood flow have also been highly successful in characterising transient processes. For example, pulsatile ocular blood flow assessment has been calculated from the ocular pulse wave produced by the bolus of blood entering the eye during cardiac systole. The volumetric blood changes caused by this imbalance are transferred into a pressure gradient that is recorded by a pneumotonometer [387]. In addition, ocular fundus pulsation can be assessed by measuring distance changes between cornea and retina during the cardiac cycle [680].

Sequences of images of selected vessels obtained by Doppler means allow correlation to the phases of the cardiac cycle obtained synchronously by a pulse plethysmograph [678, 681, 682]. Doppler ultrasound spectrum mode is a method of clinical importance as the spectral display is usually calculated at a particular location within a blood vessel using a St-FFT method (see §6.2.1). The velocity (or Doppler frequency) distribution is illustrated as a function of time, making it especially useful for imaging pulsatile flow [451]. Methods utilising the optical interferometric principle have been employed to investigate pulsatility: averaged Doppler shift, normalised velocity variance and integrated phase difference all plotted over each instant in time, revealing a pulsing pattern [606]. In addition, the pulsatility of blood flow in a vessel can be observed through time-varying changes in phase variance, demonstrating the ability to study blood flow dynamics \textit{in vivo} [380, 387, 606]. \textit{En face} Doppler OCT methods have been utilised to measure pulsatile blood flow by gating methods [498] and by rapid repeated scanning of a small area to characterise pulsatility [683]. Phase analysis methods allow the assessment of murine coronary artery dynamics by utilising cardiac cycle averaging and phase wrapping [535].

Presented here, is the first application of correlation based dynamic assessment for the extraction of pulsatile behaviour in an \textit{in vitro} environment. Although errors relating to maximal pulse position are increased for slower flow rates, this may be attributed to the unrealistic inelastic expansion of the \textit{in vitro} flow model implemented. However, despite this limitation, the db-SdOCT method has been shown to be capable of discerning both velocity flow metrics concurrent to the identification of intermittent pulsatile behaviour.
7.3 Conclusions

The macrocirculation of the cardiovascular system enjoys a special place in medicine and is well catered for with the imaging modalities ever present in today’s hospitals. X-ray, CT, ultrasound, MRI, PET and others (see §2.2 and §2.3) all play an instrumental role in the diagnosis, treatment and management of the disorders of the large vessels. However, diseases which most threaten the quality of life of the individual have their origins in the microcirculation and therefore new techniques with appropriate resolution are required for the visualisation of such. Optical imaging techniques such as confocal, fluorescence and multiphoton microscopy enable high axial and transverse (<1 μm) resolution imaging, but have limited penetration in biological tissue. Excisional biopsy and histopathology remain the gold standard for cancer diagnostics. However, as information is not available in real time, biopsy diagnoses can suffer from unacceptable false-negative rates due to sampling errors. Newly developed methods can contribute to a better understanding of disease pathogenesis and therefore advance the development of novel therapies. The evolution of such methods largely depends on the rapid developments in photonics. For example, new technologies based on photon interaction with tissues will expand the biophotonics market from $23B in 2012 to $36B by 2017 [684].

Many modern medical technologies employ laser radiation and fiber-optic devices. Since the application of lasers in medicine has both fundamental and technical purposes, coherence is very important for the analysis of the interaction of light with tissues and cell ensembles. The coherence of light is of fundamental importance for the selection of photons that have experienced a small number of scattering events (or none), as well as for single and multiple scattering instances. Such approaches are important for coherent tomography, diffractometry, holography, photon-correlation spectroscopy and speckle interferometry of tissues and fluxes of biological fluids. The use of optical sources with a short coherence length opens up new opportunities in coherent interferometry and tomography of tissues, organs and blood flows.

Optical coherence tomography has established itself a firm foothold in the realm of non-invasive optical medical diagnostic imaging, enabling in vivo cross-sectional tomographic visualisation of the internal microstructure of biological systems, and is now considered an optical analogue to CT or MRI, but with microscopic resolution. The original concept of OCT was to enable non-invasive real time in situ imaging of tissue microstructure with a resolution approaching that of histology but without the need for tissue excision and
processing; i.e. an optical biopsy. As OCT has developed throughout time, functional extensions have enabled non-invasive depth resolved imaging, in addition to providing spectroscopic and polarisation-sensitive (see §3.3), blood flow and physiologic information. The rate of growth of OCT publications is dramatic (~200 papers/year), dominated by ophthalmology, general technology advances and cardiology. The driving force for these publications is a combination of fertile and promising research frontiers, the availability of government funding, clinical needs and trials, and the availability of commercial regulatory approved OCT products.

Non-invasive methods for imaging and quantifying blood flow dynamics are of great value for biomedical research and clinical diagnostics where many diseases have a vascular etiology or component. The work described in this and in the previous chapters of this thesis has validated the principles, technology and associated algorithms for rheological research by means of cross-correlation assessment using a dual-beam Spectral-domain Optical Coherence Tomography (db-SdOCT) system.

Great attention has been paid in the past two decades to the measurement of flow velocity on the micrometer scale and many techniques for assessing the blood supply have been investigated. This is borne out of recognition of the vitally important role that blood flow plays in the health of the individual. Blood perfusion through the capillaries serves several key functions within the body including regulation of blood pressure, supplying oxygen and nutrients to tissue, and removal of carbon dioxide and waste metabolites. Currently, videocapillaroscopy is the clinical ‘gold standard’ for capillary imaging and analysis. However, the depth of tissue penetration is a limiting factor and restricts capillaroscopic techniques to the nutritive portions of the skin.

The use of the Doppler functionality has been the predominant force for the quantification of moving particles within media. However, despite the advancements of DOCT techniques, phase shift assessment of velocity values requires that the angle between the incident light source and the vessel in question be known a priori. A precise estimation of the Doppler angle is difficult particularly when the flow is embedded in the highly scattering medium, e.g., in vivo blood flow monitoring. Due to the extensive tortuosity of the microvasculature, the Doppler angular dependency may lead to incomplete vascular maps. It has been shown that Doppler techniques are only appropriate for larger blood vessels with faster flow rates, in which Doppler shifts are easier to detect. Furthermore, as a Fourier transformation is usually performed by sliding short-time windows, tradeoffs between axial and velocity resolutions exist in the flow estimation.
Correlation mapping OCT (cm-OCT) is a non-invasive high sensitivity imaging technique which demarcates static from transient and altering aspects of the microvasculature by calculating correlation values between adjacent B-scans of structural images. Although the method was successful in a qualitative sense, it was not, however, capable of providing a quantitative view of the flow involved and was therefore limited.

In an effort to surmount the restrictions imposed by angular uncertainties, the in-house cross-correlation dual-beam SdOCT (db-SdOCT) system operates by quasi-simultaneous illumination and measurement of two distinct planes; this forms a miniature time-gate. By analysis of light intensity fluctuations at two points a known distance apart, transit times may be deduced via temporal cross-correlation, thereby yielding velocity values irrespective of vessel tortuosity. As DOCT methods are sensitive to motion normal to the incident beam, this technique eliminates the need for phase sensitive detection and instead utilises the temporally evolving phase itself as a metric for quantifying velocity by statistical means.

A comparative study of Doppler and db-SdOCT analysis was performed (see §6.2). Limitations associated with the system hardware in terms of phase sensitivity and wrapping ultimately impeded the ability of the Doppler analysis to compute adequate velocity values. The db-SdOCT method was unaffected by such parameters.

Sensitivity to flow is inversely proportional to the acquisition speed. To improve sensitivity, acquisition speeds have to be decreased or multiple scans taken of the sample position; neither is an attractive prospect as both reduce the scanning speed of the system. However, by utilising a dual-beam configuration, the same area of illumination may be scanned in half the time. A detailed outline of the construction, application and processing procedures associated with the described db-SdOCT system was provided in §4.4. An optical switch is employed as a means of discriminating the interferometric data of each channel for detection by a digital line scan camera and in this way did not necessitate the requirement of having separate equipment for each side of the dual-beam system, thus substantially reducing costs and bulk system size.

Statistical analysis of dynamics in OCT images offers an advantage over DOCT methods because subwavelength motion of a scatterer along the axial dimension is necessary to influence the phase of a signal. However, with regards statistical methods, it is necessary for a scatterer to enter and leave a focal volume in the lateral dimension to induce fluctuations in intensity and/or phase. This is advantageous as statistical based analysis is also capable of yielding transverse flow data, normal to the incident beams’ optical axis. It has been shown
that this is approximately one order of magnitude more sensitive to motion parallel to the optical axis.

FdOCT devices (of either the spectral-domain or swept source variety) construct the sample structure via spectral analysis of the interference fringe signal. The advantageous possibility of having direct access to the spectral fringe pattern with FdOCT methods is that it enables a wide range of novel applications such as measurement of tissue absorption [685, 686] and contrast enhancement [687, 688]. Coupled with this high speed, the capability to simultaneously register all spectral components provides stable fringe phase information which, for example, is extremely sensitive to dispersion mismatching between sample and reference arm [689]. SsOCT systems are inherently less sensitive phase-wise than that of their spectral-domain counterparts; however, it has been shown that increased acquisition speeds lead to increased accuracy regarding emulating velocity values in db-SdOCT (see §5.3). As such, adoption of the correlation mechanism in SsOCT systems is feasible. Despite the challenge of achieving high phase stability, SsOCT systems exhibit less fringe wash-out and faster imaging speeds compared to SdOCT detection. However, the application of vertical cavity surface-emitting laser light sources (VCSELs) to SsOCT operation has recently been reported [690], enabling the measurement of pulsatile total retinal blood flow with high sensitivity and phase-stability.

Comparison of the phases of successive depth profiles at the same sample location allows for the detection of small axial structural changes with nanometre precision [691]. Utilisation of the phase metric in the dynamic assessment of transient phenomena has recently increased, for example, computation of retinal and choroidal perfusion maps with phase-variance OCT. In addition, mapping of phase has been used for fast chemical analysis of glucose mixture processes [692].

As a precursor to system and cross-correlation algorithm optimisation, the feasibility of application of the db-SdOCT system and algorithm to various in vitro studies was conducted. As the experimentation performed in these studies primarily concentrated on the averaged intraluminal data within the capillary to yield velocity values, the demarcation of flow from static regions is also highlighted (see §5.1). It is evident from the investigations of correlation vs. beam separation value that smaller values of dual-beam separation deviate greatly from the imposed flow rates, irrespective of capillary diameter size. This may be attributed to the amount of data available for collection which is less for shorter separation distances given a certain particulate transit time. Applying a higher frame rate camera may allow shorter minimum separation distances to be implemented. Cross-correlation db-
SdOCT analysis, creates a variable velocity measurement range and can be set based on the flexibility of its parameters i.e. acquisition time and beam separation distance. In general, the preliminary *in vitro* results obtained indicated a tentative first step in developing a robust tool for flow velocity quantification by means of cross-correlation. However, in order to investigate the applicability of the method for *in vivo* studies, a full characterisation of the system was required in terms of beam separation, functional extensions, and optimisation of the optics involved.

A resulting trend from cross-correlation analysis revealed an increase in velocity value accuracy with increasing capillary diameter; this may be understood using the rationale of speckle OCT. Regions of movement within a real-time OCT image capture are apparent by the intermittent flickering of the speckle present. As such, sufficient transient speckle OCT data must be present and detectable within the coherent probe volume of the impinging light beams such that clear computation of like events of high correlation may be realised. As such, increasing the volume of data available for correlation analysis is therefore scaled with the capillary size, as a larger volume of intraluminal data is considered for larger diameters. Flowing media consisting of solutions of different optical properties influences light differently in terms of absorption and scattering. Multiple scattering effects which may be caused by solutions with high anisotropy values have been shown to be neither influential nor impeding in the assessment of velocity values by the db-SdOCT method.

The influence of the acquisition time as investigated in §5.4, found that increasing the line scan rate positively influenced the ability of the cross-correlation algorithm to yield increasingly accurate velocity values. In clinical terms, reducing acquisition times may improve patient throughput, increase efficiency, and reduce costs; however, reducing acquisition time also increases image noise, and therefore the ability of the intensity-based methods; thus, the utility of the db-SdOCT method in such instances may prove advantageous. The use of the phase of the OCT signal as the input parameter to the correlation computations outperformed that of intensity in terms of robustness, sensitivity and accuracy. However, the intensity information is also of interest as it can be applied, for example, in cellular systems which can take advantage of the fact that it is sensitive to the aggregation of biomolecules.

The computation of cross-sectional axial profiles of velocity enables the analysis of dynamic and temporal changes which can occur due to e.g. an obstruction. In addition, axial profile computation has also played an instrumental role in gleaning knowledge and understanding of biological developmental models. A means of discerning axial values in
order to visually represent the spatial dynamics taking place was achieved by cross-correlating windowed segments of the intraluminal data (see §5.2). Multiple scattering effects caused by the anisotropic values of the solutions investigated were not shown to impact on their respective axial velocity profiles using this method.

The ability to discern flow direction in addition to providing values of velocity has potential applications in non-invasive analysis of microscopic blood flow in several situations. In an effort to provide a fully characterised assessment of flow using the db-SdOCT method, an auto-correlation method for quantitative mapping of transverse particle-flow velocity was implemented (see §5.3). Utilisation of the auto-correlation function of each sample beam provided a simple means of discerning directional data without the need for excessive computation and may be performed simply on the raw OCT data, concurrent to the cross-correlation means of discerning velocity metrics.

As seen in §3.1, to improve axial resolution, the spectral bandwidth must be either increased or the central wavelength decreased; the light source not only determines the axial resolution, it also influences both the sample penetration and the transverse resolution. Different light sources were tested with varying values of probing coherent volume and it was found that cross-correlation analysis was best achieved by using an inexpensive light source, without the requirement of additional hardware or computational additions to compensate for dispersion effects, etc. As such, adaptation of this method into existing OCT set-ups is relatively straightforward and a cost-effective means of dynamic assessment, free from angularly induced artifacts. Although it has been shown that a degradation in transverse resolution yields more easily discernible directional data, this is true if either intensity or phase-based analysis is implemented. However, by utilising dynamic focus tracking the coherence gate may move synchronously with the confocal gate peak, ensuring signal strength and preservation of transverse resolution from all depths. Possible focus effects can be mitigated in hardware by the addition of axicon lenses [693, 694] or dynamic focus tracking [695]. In addition, a novel method to extend the depth-of-focus of OCT by using a depth-encoded synthetic aperture detection scheme has been reported [696]; lateral resolution may be maintained over a five-fold larger depth range and as such directionality studies could be implemented in multiple vessel sizes at different axial positions.

The study of the mutual effect and interactions of the blood flow with the surrounding vessel geometry is of great interest in biorheology and cardiology. The db-SdOCT technique could become a valid support for cardiologists as a means of simultaneous imaging of the vessel morphology, plaque formation, and related blood flow velocity information, in
addition to providing an insight into the extent of turbulence involved. As such, the db-SdOCT technique served to provide a means of characterisation of turbulent flow by comparison of *in vitro* atherosclerotic plaque and control flow models (see §5.5). Stemming from Taylor’s approximation, it was hypothesised that the length scale of turbulent motions has a strong influence on the capability of discerning relevant velocity metrics in a turbulent and ever-changing environment. The resulting analysis has shown that turbulence may be discerned at larger values of beam separation. In addition, using smaller values of beam separation results in the ability to quantify the underlying velocities present, irrespective of the turbulences created. Thus, it may be possible to implement both scenarios *in vivo*: confirming localised turbulent phenomena in addition to providing a representative value of the underlying flow velocity by using different length scales.

Aggressive research and development efforts aim to place even better technology in the hands of interventional cardiologists, where the focus is placed more on the appropriate selection, optimal delivery and careful post-interventional assessment of coronary therapies. Endoscopic OCT offers a means of obtaining high-resolution and high-speed depth-resolved visualisation of tissue structures *in vivo*. With a view to future potential application of the db-SdOCT set-up and algorithm for intravital exploratory applications, a macro-model of a side-view endoscope device was implemented to investigate the efficacy of this simplified approach relative to the general db-SdOCT system configuration (see §7.1). This model has been shown to be successful in this respect; implementation of this in a catheter probe is a prospective avenue for this technique, opening up exciting opportunities for exploratory *in vivo* research. The capabilities of *in vivo* assessment were examined in the analysis of the nailfold plexus (see §6.1). The resulting velocity, directionality and axial profiling computation of the constituent capillaries have demonstrated the versatility of the db-SdOCT method in such environments.

The ability to provide a clinician with real-time visualisation and guidance is particularly important for imaging purposes as *in vivo* flow can be pulsatile, intermittent, rapidly changing or otherwise changeable with time. As velocities are generally pulsatile in nature, there is an added restriction for imaging modalities that the time over which the sequence of exposures is acquired be small compared to the period of the pulse. Presented here, is the first application of correlation based dynamic assessment for the successful extraction of pulsatile behaviour in an *in vitro* environment (see §7.2).
Appendices

A: Knife-edge detection: a preliminary means of dual-beam calibration

The TEM\(_{00}\) transverse mode is a commonly used mode describing the optical output of an optical resonant cavity as the flux density ideally Gaussian over the beam’s cross section, i.e. the strength of the beam-like wave falls off transversely following a bell-shaped curve symmetric with respect to the central axis. Most of the beam energy resides within a certain imaginary radius (\(\omega\)) from the central axis. This distance is defined as the beam half width, i.e. the distance from the central axis at which the electric field drops to \(E_0/e\) or \(I_0/e^2\) (i.e. 0.14\(I_0\)) [697]. This Gaussian distribution may be described by the following [698]:

\[
G(x, y) = I_0 e^{-\frac{x^2+y^2}{\omega^2}} \tag{1A}
\]

where \(G(x, y)\) is the cross-section spatial profile of a Gaussian beam; \(I_0\) is the maximum value of \(G(x, y)\) at \((x_0, y_0)\); \(r(x, y)\) denotes position relative to the beam central axis \((x_0, y_0)\); \(\omega\) denotes the half width radius. The distribution given above is radially symmetric.

![Figure 1A: Simulated Gaussian distribution of 3-D irradiance profile of TEM\(_{00}\) transverse mode.](image)

The transformation condition which converts Eq. 1A into the normal distribution may be described by the following [698]:

\[
N(z) = \sqrt{2\pi} \int_{-\infty}^{\infty} e^{-\frac{n^2}{2}} dn \tag{2A}
\]

where \(z\) is the direction of propagation. Letting \(\beta^2 = \frac{2}{\omega^2}\) and considering the present procedure involves a linear 1-D translation of the knife edge (with 2-D profiles necessary for obtaining information on beam ellipticity), the two arguments may be made equal if the following condition is imposed:

\[
-\beta^2(x - x_0)^2 = \frac{n^2}{2} \tag{3A}
\]
Considering the distribution is symmetric, two values may be obtained for \( n \) for a certain percentage value \( (m) \) of the maximum beam irradiance \( I_0 \), i.e. \( \pm n \). Solving Eq. 3A for \( x \), yields two simultaneous equations:

\[
\begin{align*}
  x_m &= \frac{n}{\sqrt{2\beta}} + x_0 \\
  x_{1-m} &= -\frac{n}{\sqrt{2\beta}} + x_0
\end{align*}
\]  

(4A)

which, when solved, yields:

\[
\begin{align*}
  x_0 &= \frac{(x_m + x_{1-m})}{2} \\
  \omega &= \frac{(x_m - x_{1-m})}{n}
\end{align*}
\]  

(5A)

The beam diameter \( d \) with respect to the \( e^{-2} \) distance from the center axis is determined by \( 2\omega \).

As outlined in previous work [699], the zeroing of the sample arms was achieved by axial movement of one sample arm with respect to the other (stationary) and subsequently denoting at which position a maximal intensity between the respective beams occurs. Although this was an adequate means of approximating the zero position for preliminary testing, the possibility of errors involved with the translation stage or a spurious reflection off a metallic surface cannot be ignored. Additionally, due to the aforementioned physical limitations of the set-up, a configuration of parallel beams would increase the applicability of the technique for practical purposes.

A revised system design would require another means of zeroing approximation. The best course of action to adopt would be to analyse the irradiance profiles of these incident beams \textit{in situ} upon their intended target. By plotting the profiles of these light sources, a common position in each beam based on percentile approximation may be deduced and in doing so, a relative distance between each beam may be computed.

Many optical instruments utilise the property of beam spot size; for example, the resolution power of a focused scanning microscope is limited by the diameter of the laser spot [700]. In order to develop a meaningful measure of ‘beam quality’, a practical, and readily measurable definition for beam ‘width’ is required, given a time-averaged irradiance profile \( I(x, y) \) at any given plane \( z \). Numerous possible definitions of beam width exist [701]: the variance \( (\sigma_x) \) of the transverse irradiance profile; “D86” diameter, containing 86% of the total beam energy; width of a best fit Gaussian to a measured profile; among others. As no single universally applicable and meaningful definition of laser beam quality exists, the chosen method depends upon the application for which it is intended.

The knife-edge method is a commonly used technique to characterise laser beam profiles, focused optical spots and optical surfaces [702] and has been successfully applied to the measurement of micron sized laser and soft x-ray beams [699, 703-705]. In this, a sharp knife edge plate intersects the optical field distribution of the beam and the irradiance of the unmasked portion is measured. The measured irradiance \( I(x) \) as a function of the knife edge position \( x \) is given by [706, 707]:

\[
I(x) \propto \int_{-\infty}^{x} P(x)dx
\]  

(6A)

where \( P(x) \) is the field distribution.
The resulting irradiance distribution may be modelled by the error function:

\[ \text{erf}(x) = \frac{2}{\sqrt{\pi}} \int_{0}^{x} e^{-u^2} \]

i.e., the integral of the Gaussian function curve; see below.

![Graph of erf(x)](image)

**Figure 2A:** Simulated \( \text{erf}(x) \) function; obtained using experimental stage displacement values.

The knife-edge translator was used to probe the beam irradiance as illustrated in the figure below. This translator consisted of a single axis motorised translation stage (PT1/M-Z8, Thorlabs), advanced through the beam in equal steps of 5 \( \mu \)m.

![Knife-edge translation](image)

**Figure 3A:** (a) Simulated 2-D irradiance profile for Gaussian distribution; (b) Illustration of knife-edge technique, intersecting the optical field distribution.

The knife-edge translation was performed on a single beam initially (see Figure 4A) and then the technique was applied to a dual beam set-up (see Figure 5A) in an effort to quantitatively determine the relative beam separation distance.

Upon obtaining this dual beam data, values for \( x_0 \) and the beam waist were calculated using Eqs. 5A by obtaining percentage values of the irradiance, e.g. \( I_{10} \) and \( I_{90}, I_{22} \) and \( I_{88} \), etc. In order to obtain accurate values, different values for \( x_0 \) and \( \omega \) were obtained using appropriate \( n \) values from the normal distribution table.
Figure 4A: Experimental irradiance values obtained via intersection of the optical distribution for a single beam by a straight knife-edge.

Figure 5A: Experimental irradiance values obtained via intersection of the optical distribution for two sample arm beams at an arbitrary separation distance.

The values obtained for the beam waist diameter for the different beams (i.e. ChA and ChB) were 0.115 mm and 0.137 mm respectively. Additionally, the averaged values for $x_0$
(although not the central point on the beam axis) were taken as a relative point on each beam from which a separation value \( \delta \) can be estimated; this value was determined to be 0.345 mm.

**Figure 6A:** Tracked movement of sample arm S1 via GM1. An evident decrease in intensity is noted with angular increase of GM1 with respect to the original position. (Inset: Similar data obtained for S2 which remained stationary.)

**Figure 7A:** Calibration of sample arm movement with voltage from GM1. (Inset: Input voltage was also monitored on a separate voltmeter for accuracy.)
**Figure 8A**: Experimental test of beam separation values with calibrated GM1 movement of S1 (Channel 1, red) position and its subsequent movement with respect to a stationary S2 arm (Channel 2, black); computed beam separation values for their respective input voltages applied to GM1 are included for clarity.
Table 12: Experimental values for a bidirectionality investigation of a 400 μm in vitro sample with flowing 2% Intralipid solution. In this case, Channel A initiated data capture.

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<th>Autocorr&lt;sub&gt;Phase&lt;/sub&gt;</th>
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Table 13: Experimental values for a bidirectionality investigation of a 300 μm in vitro sample with flowing 2% Intralipid solution. In this case, Channel B initiated data capture.

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Table 14: Experimental values for a bidirectionality investigation of a 150 μm in vitro sample with flowing 2% Intralipid solution. In this case, Channel B initiated data capture.

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<th>Autocorr&lt;sub&gt;Phase&lt;/sub&gt;</th>
<th>Intensity ChA</th>
<th>Intensity ChB</th>
<th>Autocorr&lt;sub&gt;Phase&lt;/sub&gt; ChB to ChA flow direction?</th>
<th>Autocorr&lt;sub&gt;Phase&lt;/sub&gt; ChB to ChA flow direction?</th>
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Table 15: Experimental values for a bidirectionality investigation of a 300 μm in vitro sample with flowing blood in a solid agar based skin mimicking phantom. In this case, Channel B initiated data capture.

<table>
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<th>Velocity (m/s)</th>
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<th>Autocorr\textsubscript{phase}</th>
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<th>Phase</th>
<th>ChB to ChA flow direction?</th>
<th>ChB to ChA flow direction?</th>
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Table 16: Experimental values for a bidirectionality investigation of a 50 μm in vitro sample with flowing blood in a solid agar based skin mimicking phantom. In this case, Channel B initiated data capture.

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<th>Autocorr_phase</th>
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<th>Phase</th>
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</table>
C: Ethics Application – Use of blood products
D: Published Works
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