Light Scattering and Absorption Spectroscopy in Three Dimensions Using Quantitative Low Coherence Interferometry for Biomedical Applications

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate Program in Medical Physics in the Graduate School of Duke University

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ABSTRACT

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Abstract

The behavior of light after interacting with a biological medium reveals a wealth of information that may be used to distinguish between normal and disease states. This may be achieved by simply imaging the morphology of tissues or individual cells, and/or by more sophisticated methods that quantify specific surrogate biomarkers of disease. To this end, the work presented in this dissertation demonstrates novel tools derived from low coherence interferometry (LCI) that quantitatively measure wavelength-dependent scattering and absorption properties of biological samples, with high spectral resolution and micrometer spatial resolution, to provide insight into disease states.

The presented work first describes a dual window (DW) method, which decomposes a signal sampled in a single domain (in this case the frequency domain) to a distribution that simultaneously contains information from both the original domain and the conjugate domain (here, the temporal or spatial domain). As the name suggests, the DW method utilizes two independently adjustable windows, each with different spatial and spectral properties to overcome limitations found in other processing methods that seek to obtain the same information. A theoretical treatment is provided, and the method is validated through simulations and experiments. With this tool, the spatially dependent spectral behavior of light after interacting with a biological medium
may be analyzed to extract parameters of interest, such as the scattering and absorption properties.

The DW method is employed to investigate scattering properties of samples using Fourier domain LCI (fLCI). In this method, induced temporal coherence effects provide insight into structural changes in dominant scatterers, such as cell nuclei within tissue, which can reveal the early stages of cancerous development. fLCI is demonstrated in complex, three-dimensional samples using a scattering phantom and an ex-vivo animal model. The results from the latter study show that fLCI is able to detect changes in the morphology of tissues undergoing precancerous development.

The DW method is also employed to enable a novel form of optical coherence tomography (OCT), an imaging modality that uses coherence gating to obtain micrometer-scale, cross-sectional information of tissues. The novel method, named molecular imaging true color spectroscopic OCT (METRiCS OCT), analyses the depth dependent absorption of light to ascertain quantitative information of chromophore concentration, such as hemoglobin. The molecular information is also processed to yield a true color representation of the sample, a unique capability of this approach. A number of experiments, including hemoglobin absorbing phantoms and in-vivo imaging of a chick embryo model and dorsal skinfold window chamber model, demonstrate the power of the method.
The final method presented in this dissertation, consists of a spectroscopic approach that interrogates the dispersive biochemical properties of samples to independently probe the scattering and absorption coefficients. To demonstrate this method, named non-linear phase dispersion spectroscopy (NLDS), a careful analysis of LCI signals is presented. The method is verified using measurements from samples that scatter and absorb light. Lastly, NLDS is combined with phase microscopy to achieve molecular imaging with sub-micron spatial resolution. Imaging of red blood cells (RBCs) shows that the method enables highly sensitive measurements that can quantify hemoglobin content from single RBCs.
To my wife, Kristy, and family who have shown 
unwavering love and support.
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1. Introduction

1.1 Motivation

When an electromagnetic field is incident on a medium, the absorption of the field as well as the intensity, angular distribution, and spectral modulation of the scattered field can reveal detailed information about the medium [1]. For example, when we look at the color or spectral modulation of the scattered light coming from the sky, we can readily deduce that there is a preferential scattering of the lower wavelengths, thus giving the sky its blue appearance. We can even go further and use a bit of physics to reach the conclusion that the scatterers in the atmosphere must be small, on the order of a few nanometers, to produce a scattering cross section that is inversely proportional to the wavelength of light (actually, it is $\propto 1/\lambda^4$) [2]. Examples of absorption are just as abundant: for example, look at the color of a glass of wine, if it is red, then we immediately know that the grape skin was used in the maceration and fermentation process. Surprisingly, however, a more detail analysis will reveal that the color of the wine does not simply depend on the color of the grape skin, but it depends more strongly on the chemical composition of the grape wall (e.g., uronic acids, cellulosic glucose, proteins, etc.) [3]. In a similar manner, biomedical optics utilizes the mechanisms of light scattering and absorption to investigate structures and functional processes in biological samples.
The allure of using optics for biomedical applications is that it can provide a wealth of quantitative information using non-ionizing radiation, probe length scales much smaller than is possible with other diagnostic modalities/techniques, and be used without altering samples (i.e., non invasively). Because of these features, it is not surprising that a great deal of effort in the field has been focused on the understanding and early detection of many diseases including cancer.

1.1.1 Early cancer detection

As is commonly known, cancer is a serious concern worldwide; and in fact, it has now become the largest killer in the world, surpassing heart disease and stroke [4]. Cancer accounted for 7.6 million deaths in 2008 (approximately 13% of all deaths), and it is expected to rise to over 11 million in 2030 [4]. In order to prevent such high mortality rates, experts agree that the most successful practice is to regularly screen people at risk, thus allowing initiation of localized therapies and prevention of metastatic disease. Detecting cancer at an early stage cannot only improve the survival rate, but it can also greatly reduce patient morbidity.

The first stages of cancerous development are demarcated by architectural and cytological changes that begin with the stochastic, malignant mutation of a single cell. This malignant mutation then develops into a small group of abnormal cells which continue to proliferate uncontrollably. In later stages, these neoplasms (new growths) continue their progression through pre-cancerous dysplasia, malignancies in-situ,
invasive carcinomas, and finally metastatic disease [5]. Unfortunately, current screening techniques are unable to detect malignant changes at the early stages of this cancerous progression, making it more favorable for the disease to grow and eventually metastasize.

As an example for the need to develop better screening techniques for cancer, consider colorectal cancer (CRC), which is the third leading cause of cancer death in men and woman in the United States [6]. This type of cancer exemplifies the problem because it is largely asymptomatic until it has reached an advanced stage. Screening for CRC is achieved via colonoscopy, whereby an expert visually inspects the colon through an endoscope to detect polyps and/or adenomas. If any are identified, the decision to remove these mucosal growths is based on size, where it is recommended that lesions >5 mm in diameter be removed [7]. However, this approach, like other screening techniques, suffers from serious weaknesses: 1. There is no reliable metric for determining whether lesions are adenomatous or metaplastic; hence, the decision to remove these lesions is left to the discretion of physicians, which may vary significantly from one individual to the next. 2. Despite the fact that small lesions (<5 mm) are not typically removed, some studies have presented evidence that these are likely to contain neoplasias, particularly for lesions proximal to the left colon [8]. 3. Flat adenomas, which are ten times more likely to contain malignancy compared to similarly sized polyps, appear similar to the surrounding tissue, and are consequently very difficult to
detect [9]. Because all detected polyps are considered adenomatous [9], many unnecessary biopsies and polypectomies are preformed, which increase the probability of complications [10]. These weaknesses clearly motivate the need for more adequate screening technologies.

Screening for cancer is not the only part of the current paradigm that needs improvement; specifically, the gold standard for diagnosing cancer, histology, also poses problems. In histology, physical biopsies of tissues are taken either randomly or after a suspicious area has been identified through screening. Then, the tissues must be fixed, sliced, and stained before being analyzed by an expert pathologist using a light microscope. In this practice, however, only a small percent (~2%) of the areas in question may be sampled due to the invasive nature of physical biopsies. Further, the fixing process requires many hours of preparation which prevents use of the pathological information during surgical procedures. This also makes it difficult to return to specific regions that have been identified as suspicions. But perhaps the most significant problem is the fact that the pathological interpretation used for tissue diagnosis is subjective, thus resulting in poor interobserver agreement and increasing the risk of false positives and even more alarming, false negatives.

The weaknesses of the technologies used to screen and diagnose cancer, magnified by the vastness of the disease, highlight the need for novel technologies that
assess tissue health quantitatively, in a minimally invasive manner, and at early stages. In this dissertation, early cancer detection using biomedical optics is a central focus.

1.1.2 Other biomedical applications

Light scattering and absorption may be applied to study many types of diseases and functional mechanisms, and while disease mechanisms other than cancer are not directly investigated in this dissertation, the tools developed here are certainly widely applicable. To highlight some of these applications it is worth mentioning two: sickle cell anemia and angiogenesis.

Sickle cell anemia (SCA) is a recessive genetic blood disorder caused by a single-point mutation in the hemoglobin (Hb) gene, which leads to production of a different type of Hb, known as hemoglobin S (HbSS) [11]. This type of Hb causes red blood cells (RBC) to be more fragile, less flexible, and more adhesive resulting in severe health consequences [12]. SCA is particularly common among people from sub-Saharan Africa, where approximately 2% of children are born with the disease and up to 30% of people in certain regions (for example, Nigeria) carry the mutated gene [13]. Unfortunately, because of its low prevalence in the United States, limited resources have been devoted to the understanding and diagnosis of SCA [14].

To gain insight into this particular disease, the internal structure of the mutated RBCs may be utilized as a surrogate biomarker. Specifically, the HbSS polymerizes when it is deoxygenated causing aggregation in an ordered phase and resulting in
birefringence [15], which describes the phenomena where different polarizations of light have different propagating speeds (i.e., different refractive indices). Thus, by probing the dispersive properties (i.e., refractive index changes as a function of wavelength) of individual cells using orthogonal polarizations, one can ascertain signs of SCA. Note that dispersion is directly related to absorption due to principles of causality. A method that paves the way for such measurements has been developed and is described in Chapter 7.

Angiogenesis is another potential application for the tools described here. Angiogenesis describes the process by which new blood vessels and capillaries are formed in order to provide nutrients and oxygen to cells in tissue [16]. This process is regulated by a complex sequence of anti- and pro-angiogenic stimulators that can be disrupted by metabolic stress, mechanical stress, inflammatory response, and genetic mutations inflicted by a number of diseases including hepatitis, pneumonia, asthma, diabetes, and also cancer [16-18]. Insight into this process requires the ability to visualize the complex tissue morphology, measure oxy- and deoxy- Hb, and detect exogenous molecular species. A novel imaging technique that achieved this is described in Chapter 6.

1.2 Dissertation overview

The work detailed in this dissertation demonstrates a number of novel methods that provide insight into the light scattering and absorption properties of biological
These methods are based on probing spectral information, spatially-resolved in three dimensions using low coherence interferometry (LCI) for a number of applications, some of which are described in Section 1.1. This work may be divided into four major sections: 1. development of a novel spatially-spectral method from LCI signals, 2. investigation of light scattering spectral signatures for early cancer diagnosis, 3. investigation of absorption spectral properties for molecular imaging, and 4. development of a novel approach that assesses scattering and absorption simultaneously and independently.

In the first section, a theoretical treatment of a novel dual window (DW) processing method is given. This method achieves simultaneously high spatial and spectral resolution, thereby overcoming the limitations of other available methods, and ultimately permitting detailed studies of the scattering and absorption properties of complex, three-dimensional structures such as tissue. In the second section, the DW method is applied to investigate scattering signals using a technique known as Fourier domain low coherence interferometry (fLCI). Here, both phantom and an animal model of carcinogenesis are used to demonstrate the utility of the method. In the third section, the DW method is applied to investigate absorption. Here, hemoglobin and various exogenous contrast agents are utilized in phantoms and in-vivo animal models. Quantitative molecular-imaging in true-color and in three dimensions is demonstrated using this approach. Lastly, the fourth section describes a novel method to
independently assess absorption and scattering using the phase information of LCI signals. To validate this approach, a phantom that scatters and absorbs light, and healthy RBCs are analyzed.

1.3 Document organization

This dissertation is organized as follows: In Chapter 2, a brief overview of the primary diseases under investigation, such as carcinoma, is given. This chapter also outlines several biomedical optics techniques used for diagnosis, where their strengths, weaknesses, and current progress in relevant tissue studies are discussed. This serves as a reference point that underscores the demand for the methods presented herein.

Chapter 3 describes the DW method, which is introduced as a means to probe the spatially dependent scattering and absorbing properties of biological samples with high spatial and spectral resolution. First, a theoretical analysis is provided, and other methods that probe non-stationary signals are compared. Then, simulations and experimental results are used to demonstrate that the utility of the DW method.

Chapters 4 and 5 employ the DW method to investigate scattering using fLCI and absorption using molecular imaging true-color spectroscopic optical coherence tomography (METRiCS OCT), respectively. In Chapter 4, first the fLCI instrumentation is described, and a phantom study is presented. Then, an ex-vivo animal model is used to demonstrate the ability to detect precancerous development. In Chapter 5, METRiCS OCT is presented as a means to quantify absorption from endogenous and exogenous
chromophores. Hemoglobin absorbing phantoms and imaging of two \textit{in-vivo} animal models demonstrate this cross-sectional, molecular imaging approach.

Chapter 6 described a method named non-linear phase dispersion spectroscopy (NLDS). This method measures the dispersive properties of samples to provide an independent assessment of absorption. A theoretical analysis is provided. Then, the method is applied to separate absorption and scattering, and also to achieve molecular imaging in phase microscopy.

Lastly, Chapter 7 gives a brief summary of the major findings and their implications, as well as a discussion of future directions.
2. Background

This chapter gives a brief background of various topics discussed throughout the document. First, Section 2.1 gives an overview of carcinoma which is the primary disease mechanism under investigation in this dissertation. Then, Section 2.2 discusses various biomedical optics techniques that have been developed for diagnosing tissue. Each method’s strengths, weaknesses, and current progress/success in tissue studies, particularly for cancer detection, are highlighted.

2.1 Carcinoma

Malignant tumors typically follow four steps: transformation, growth of transformed cells, local invasion, and metastases. Those that originate from epithelial cells are known as carcinomas, which can then be further classified depending on the tissue of origin. For the purpose of this document, it not practical to go into detail about the different types, but it is important to note that carcinomas may afflict nearly all tissues/organs due to the ubiquity of epithelial cells, which make up the body’s surfaces and cavities. However, it is worth mentioning that colorectal cancer and esophageal cancer, which are two types of cancer that will be revisited later, typically fall into two categories of carcinoma: adenocarcinoma, which originates from epithelial lining of glands and ducts, and squamous cell carcinoma, which originates from stratified squamous [5].
One of the characteristics of malignant neoplasms is that they do not resemble normal cells morphologically or functionally, or in other words, they are not well differentiated. This is known as anaplasia, which, by histological inspection, is characterized by drastic variations in cell size and shape, loss of polarity, and a large number of mitoses. Anaplasia also exhibits abnormal nuclear morphology resulting from large DNA production, which may increase the cell’s nucleus-to-cytoplasm ratio from a normal 1:6 up to 1:1 [5]. Higher production of DNA has also been linked to an increase in the cell nuclei refractive index [19,20]. Fortunately, these changes are not instantaneous; instead, they follow a subtle continuum through the four steps mentioned above and may not necessarily always develop into malignant tumors. An early step in this continuum, known as dysplasia, which mostly affects epithelia, resembles lesser degrees of anaplasia: it exhibits loss of uniformity and architectural orientation in individual cells. Most notably, dysplasia is also characterized by hyperchromatic nuclei which are larger than other nuclei in their surroundings. Moreover, dysplasia is categorized into low grade- and high grade- dysplasia (LGD and HGD, respectively) depending on the stage along this progression [5].

The hallmarks of dysplasia are important because they present potential biomarkers for identifying precancerous states. As mentioned in Chapter 1, biomedical optics has the capabilities to take advantage of the early signs of carcinoma for
diagnosis, thereby providing a pathway for improving the current screening and diagnostic paradigms.

2.2 Biomedical Optics Techniques

This section provides a brief description of various optical methods that seek to assess tissue health, and that have shown promising results particularly for early cancer detection.

2.2.1 Diffuse reflectance spectroscopy (DRS)

In DRS, broadband light, typically ranging from ultraviolet (UV; ~300 nm) up to near infrared (NIR; ~800 nm), is delivered directly onto the tissue surface using an optical fiber; then, some of the light that undergoes multiple elastic scattering migrates back to the surface some distance away, where several optical fibers are used to collect the diffused light. The signals are recorded as a function of wavelength using a spectrometer, which reveal a modulation that is dependent on the absorbers (e.g., oxy-/deoxy- Hb and beta-carotene) as well as the average reduced scattering coefficient of the interrogated tissue. To obtain these physical parameters, most DRS methods assume that tissue is a homogenous, semi-infinite, turbid medium such that the steady-state diffusion theory applies [21], thus permitting use of iterative or inverse methods [22]. Often researchers also resort to more robust, but computationally expensive, Monte Carlo simulations [23,24]. In either case, careful consideration of the design parameters (e.g., fiber source-detection distance, collection area, etc.) must be taken into account.
and/or system calibrations must be performed in order to correctly assess the tissue properties of interest [23]. Up to date, DRS has been implemented to distinguish between normal tissue and malignant lesions in the colon [22], breast [25,26], cervix [27], and skin [28].

The strengths of DRS are that it provides a quantitative method for identifying lesions, it is inexpensive, and has shown good sensitivity and specificity (ranging from ~70 to 100% depending on the specific system and method). Further, DRS has been combined with autofluorescence thereby allowing calculations of the inherent tissue fluorescence, corrected for absorption. This is known as intrinsic fluorescence spectroscopy (IFS) [29,30]. The integration of these two techniques has allowed for more parameters to be obtained (e.g., NADH and collagen), thus increasing the diagnostic power and allowing for further differentiation of the classification of tissues, including benign lesions [25]. However, due to the nature of diffused light, the method only obtains average bulk tissue properties of relatively large volumes (> 1 mm$^3$). As a result, the stage at which a suspicious region can be identified is limited to the local invasion stage (i.e., carcinoma in-situ).

2.2.2 Light scattering spectroscopy (LSS)

LSS is a spectroscopic method that is similar to DRS in the way light is collected, however this method seeks to isolate singly scattered light from diffused light in order to analyze the resulting spectra using Mie theory. Mie theory is an analytical solution to
the scattered field of a spherical scatterer using Maxwell’s equations (so, it is in fact not a theory), and is a function of a number of variables including wavelength, scattering angle, refractive index (RI), and polarization [2]. The means by which the diffused background is removed in LSS includes mathematically modeling the diffused light and subtracting its contributions (i.e., model-based) [31], polarization gating (see Section 2.2.2) [32], or coherence gating (see Section 2.2.5) [33]. After the diffused background is eliminated, the resulting spectra are observed to contain periodic fine structures which are hypothesized to originate from the scattering of cell nuclei in epithelial tissue. The periodic fine structures are then analyzed using the Van de Hulst approximation, which is a simplification of Mie theory that assumes 1. that the scatterers are larger than the applied wavelength of light, and 2. that the medium is weakly scattering (i.e., the Born approximation) [2]. LSS is significant because it introduced the potential of assessing pre-cancerous development by measuring the nuclear size increase associated with dysplasia.

Backman et al. demonstrated that LSS could distinguish between normal tissue, pre-cancerous development (i.e., growth of transformation stage) and pre-invasive cancer (i.e., local invasion stage) in-vivo, in the colon, bladder, esophagus, and oral cavity [34]. This study used both model-based LSS and polarization-gated LSS. Furthermore, model-based LSS was combined with DRS and IFS, and renamed as trimodal spectroscopy (TMS). Using TMS, Georgakoudi et al. showed that HGD and
LGD in human esophagus could be differentiated with 100% sensitivity and 100% specificity, and that LGD could be distinguished from normal esophageal tissue with 93% sensitivity and 100% specificity [30].

Obvious from the results quoted above, LSS and TMS seemed to have overcome some of the major weaknesses of DRS. However, two recent studies have casted serious doubt on the validity of the method. First, Choi et al. used a tomographic phase microscope to map the three dimensional distribution of the RI of inta-cellular content, suggesting that the assumptions made about the nuclear and cytoplasm RI in the Mie theory implementation in LSS, were not accurate [35]. Secondly, Lau et al. performed Monte Carlo simulations showing that the observed oscillating residual spectrum measured in model-based LSS does not correspond to the spectrum predicted by Mie theory [36]. In fact, he concluded that the model-based LSS spectrum was largely a result of erroneous assumptions made about the distribution of Hb in the diffusion model. Lau at al. accounted for the residual spectrum by including vessel-packing factors (i.e., Hb inhomogeneity). The final remarks of this manuscript stated, “all use of model-based LSS should be discontinued” [36].

2.2.3 Four dimensional elastic light fingerprinting (4D-ELF)

4D-ELF is a technique that improves upon DRS and LSS by exploiting additional dimensions of the scattered field. Specifically, 4D-ELF collects light as a function of wavelength (equivalent to DRS), scattering angle, initial polarization (0°, 45°, 90°
azimuthal angles), and co-polarization and cross-polarization of the scattered field with respect to the incident field. Similar to polarization-gated LSS, 4D-ELF isolates light that is primarily singly scattered by polarization gating, or in other words, by subtracting the cross-polarized light, which has been randomized by multiple scattering, from the co-polarized light which is the component containing the majority of the singly scattered light. This process also preferentially gates for photons that scatter from locations close to the tissue surface, including the epithelium. Furthermore, by collecting three or more azimuthal angles, a more complete description of the scattering matrix is provided. As a result, Mie Theory can be readily implemented along with use of other phenomenological markers, to analyze the spectral and angular dependence of the scattered field to yield micrometer-scaled morphological information, and provide insight into the nano-architecture of tissue [37].

Kim et al. used an ex-vivo, azoxymethane (AOM) rat carcinogenesis model to show that 4D-ELF could provide a wealth of information, including number probability density of sub-micrometer scatterers, fractal dimension (i.e., self-similarity), and spectral slope to detect pre-cancerous development [37]. Roy et al. showed similar finding for ex-vivo human colon tissue [38]. Then, in a later study, Roy et al. implemented a less complex system that forgoes angular sampling to achieve in-vivo screening of the human colon [39]. Analysis of oxy- and deoxy- hemoglobin absorption was also included to gain access to the early increase in blood supply arising from angiogenesis.
This study showed statistically significant results for patients that harbored adenomas, not only at specific areas containing malignancies, as determined by colonoscopy and confirmed by histology, but also up to 30 cm away from the lesions [39].

These studies are significant because they are the first to provide evidence that light scattering can be used to exploit the “field-effect” of carcinogenesis. This phenomenon describes observations that neoplastic development in one part of an organ is accompanied by distorted micro-/nano- tissue morphology and tissue function along the entire organ. It has been hypothesized that the field effect is a result of environmental and genetic factors that provide a viable environment for stochastic malignant mutations to occur and persist [40]. The implications of this effect have been a subject of much interest since it indicates that adequate screening and risk stratification may be achieved by only probing certain and more readily accessible sections of tissues (for example, the distal colon) [41].

2.2.4 Angle-resolved low coherence interferometry (a/LCI)

a/LCI provides an improvement over the techniques discussed thus far by resolving angular scattering features with high depth resolution. Specifically, a/LCI employs LCI either in the time domain [42] or in the Fourier domain [43] to achieve coherence gating (which gives depth resolution; see Section 2.2.5). Also, the use of interferometry inherently gates for light that is primarily singly scattered; therefore, by
collecting light as a function of angle, a/LCI can use Mie theory to obtain quantitative information regarding the size of cell nuclei in specific tissue layers.

The latest generation a/LCI clinical system consists of a single mode delivery fiber and collection of the scattered light as a function of angle using an imaging fiber bundle. The sample arm is then compactly engineered into a small hand held probe allowing for in-vivo detection. Recently, an in-vivo study was completed where dysplasia in human Barrett’s esophagus was detected with 100% sensitivity and 87% specificity [44].

### 2.2.5 Optical coherence tomography (OCT)

OCT operates under the principles of LCI to provide high-resolution tomographic images of biological samples. Since its introduction by Huang et al. in 1991 [45], OCT has gone through a number of configurations, of which Frequency domain (FD-) OCT has become the most widely implemented due to the improved acquisition speed [46] and sensitivity [47]. As the name suggests, FD-OCT samples the frequency space of samples, and is hence analogous to the signal acquired by magnetic resonance imaging (MRI) where the bandwidth of the source dictates the spatial resolution and the sampling frequency dictates the imaging field of view. Thus, a Fourier transform of the interferometric signal yields an A-scan, typically with an axial resolution of a few micrometers and imaging depths of 1-2 mm. Then, multiple A-scans are acquired to generate an OCT image (this process is more closely related to that of ultrasound).
Figure 2.1: (a) Schematic of FD-OCT system using a Michelson interferometer geometry and a source with power spectral density $S(\lambda)$. (b) Interferogram resampled into a linear wavenumber vector. (c) A-scan: absolute value of the Fourier transform of (b) after background subtraction. See text for details.

Figure 2.1(a) illustrates a typical FD-OCT system using a Michelson interferometer geometry, where a source with a wide bandwidth, $S(\lambda)$, is split into a sample field, $E_S$, and reference field, $E_R$; then $E_S$ is incident on a sample with RI $n$, and $E_R$ is reflected off a reference mirror. In this particular example, light is scattered off of three different locations in the sample ($m = 1, 2, 3$) and is then recombined with the reference field at the beamsplitter (BS) and detected using a spectrometer. The interferometric signal (i.e., interferogram) is recorded as a function of wavelength and resampled to a linear wavenumber vector, $k = \lambda/2\pi$ (the resulting signal is illustrated in Fig. 1(b)). This process enables use of a fast Fourier transform (FFT) to reveals the optical path length difference, $\Delta OPL = (z_S - z_R)\cdot n$, between the scatterers and the reference
mirror (A-scan). Figure 1(c) illustrates the A-scan after the interferogram has been corrected for back-ground signals. A more rigorous mathematical description and more detailed figures to illustrate the process are given in Chapter 3.

OCT has been particularly successful in ophthalmology due to the optical transparency of the eye for wavelengths in the near infrared region of the spectrum, thus allowing visualization of retinal layers for diagnosing diseases and assisting in surgeries [48]. OCT has also been used for many other biomedical applications, including cancer detection. For example, Pfau et al. found statistically significant results between adenomas, hyperplastic polyps, and normal colon tissue using an endoscopic OCT (EOCT) system by looking at structure and scattering intensity [49]. Using a similar method, Isenberg et al. applied EOCT to detect dysplasia in patients with Barrett’s esophagus, and had a performance of 68% sensitivity and 82% specificity [50]. Nguyen et al. used OCT to evaluate intraoperative breast tumor margins, and achieved 100% sensitivity and 82% specificity [51]. Lastly, Vakoc et al. applied OCT to intravital microscopy, where intrinsic contrast mechanisms were used to measure tumor angiogenesis, lymogioenesis, tissue viability, cellular response to therapy, and vascular response to therapy using a dorsal skin and cranial window rat model [52]. This study demonstrated a significant advantage over multiphoton microscopy, where contrast is limited mostly to exogenous agents.
2.2.6 Extensions of OCT: spectroscopic OCT (SOCT) and spectral domain phase microscopy (SDMP)

In addition to measuring scattering intensity, various extensions of OCT have allowed for other parameters to be assessed: of particular relevance to this document are SOCT and SDPM, which measure spectrum and phase, respectively.

2.2.6.1 Spectroscopic optical coherence tomography (SOCT)

![Figure 2.2](image)

Figure 2.2: (a) Relative wavenumber (or wavelength) intensity returning from three simulated scatterers, as shown in Fig 2.1. (b) SOCT processing using STFT. (c) resulting TFD.

SOCT combines the non-invasive, 3-dimensional (3-D), high resolution, imaging capabilities of OCT with the rich source of knowledge available with spectroscopy by leveraging the wide spectral bandwidth of low coherent light sources required for depth sectioning via the coherence gating process. In fact, SOCT uses the same data acquired for conventional OCT imaging, but provides spectral information at each voxel of the sampled volume, revealing the wavelength-dependent attenuation of light traversed through the biological medium. Note that attenuation includes both scattering and absorption.
Processing for SOCT typically involves using a short time Fourier transform (STFT) or continuous wavelet transform (CWT) to provide insight into the spatially-varying, spectral properties of samples. Figure 2.2 illustrates how SOCT signals are processed using a STFT. For this example, the light returning from the simulated sample from Fig. 2.1 now undergoes attenuation such that the spectral intensity returning from each scatterer is different, as shown in Fig. 2.2(a). An STFT entails using a window, in this case a Gaussian window, that truncates the spectral bandwidth of the measured interferogram; then an FFT is computed, which again reveals the location of scatterers (i.e., depth information) but now the relative magnitude at each point in depth depends on the intensity of the wavenumbers (or wavelengths) of the truncated spectral region. This window is then swept across the full bandwidth and an FFT is computed at each step to obtain a map that describes the spectra (or wavelength intensity) returning from each scattering point. This map is known as a time frequency distribution (TFD). It is important to point out that the width of the window used in this process has a significant impact on the resulting TFD: if the window is chosen to be large, then the TFD contains high spatial resolution but suffers from poor spectral resolution and vice versa. This trade-off in resolution is discussed in much more detail in Chapter 3.

SOCT was first introduced by Morgner et al., and was used only to assess the center-of-mass of depth-resolved spectra to provide an additional mechanism of contrast [53]. However, more recent studies have explored using the full spectrum to obtain
detailed functional information. For example, Faber et al. [54] and Yi et al. [55] have used SOCT to quantitatively measure the absorption coefficient of Hb.

SOCT also permits use of exogenous contrast agents that modulate the source spectrum to tag specific cell receptors and trace functional mechanisms. This has been explored by Cang et al. using plasmon-resonant gold nanocages in phantoms [56], and by Oldenberg et al. using nano rods in ex-vivo human breast carcinoma [57].

Up to date, SOCT has not been widely applied to in-vivo samples or to study disease mechanisms, and progress has been limited due to the trade-off between the spatial and spectral resolution, as previously mentioned. Note that this trade-off is inherent in STFT and CWT, but it is not necessarily present in all methods that seek to reconstruct TFDs, for example the Wigner distribution which will be discussed later in Chapter 3. The choice of wavelengths has also been a limiting factor in studies using SOCT, especially in assessing Hb concentrations and oxygen saturation levels. Here, measurements have been assessed in spectral regions with low absorption and relatively weak contrast between oxy- and deoxy- Hb, which results in low confidence intervals [58].

2.2.6.2 Spectral domain phase microscopy (SDPM)

SDPM is another extension of OCT. In this method, the phase of the interferometric signals in FD-OCT are retrieved to provide sub-coherence length information. This is achieved by looking at the angle (arc tangent of the imaginary part
divided by the real part) rather than intensity of the Fourier transformed signal. This method is analogous to holography where the complex form of the optical wave is measured; however, one important difference is that SDPM detects the phase of spectrally resolved oscillations rather than spatial fringes. SDPM has been applied as a quantitative phase imaging technique [59], as a means to measure cellular dynamics [60], and as a method to assess small but statistically significant differences in the RI of normal and malignant cells in histological specimens of breast biopsies [20,61].

2.2.7 Fourier domain low coherence interferometry (fLCI)

fLCI may be described as an extension of SOCT where depth-resolved spectra are further processed to yield information regarding the temporal coherence induced by samples, which provides insight into their micro-morphology. This method is also somewhat similar to a/LCI in that it uses coherence gating to detect singly scattered light to ascertain early signs of cancer. However, an advantage of fLCI is that it is equipped with the same imaging capabilities of OCT (and SOCT) to enable high-resolution image guidance for its analysis.

fLCI analyses depth-dependent, high frequency spectral oscillations (also referred to as local oscillations) to measure the size of dominant scatterers in tissue, which have been associated with cell nuclei [62-64]. As figure 2.2 illustrates, light that scatters from cell nuclei will contain two components—one that originates from the front surface and another from the back surface—which will interact and produce periodic
oscillations that are proportional to the optical path length difference of the two components. It is important to understand that these local oscillations are found in the vicinity of the scatterer, and hence fLCI necessitates high spectral and spatial resolution.

Figure 2.3: (a) Light is incident on cell nuclei of different sizes. (b) Localized spectra which exhibit local oscillations resulting from the induced temporal coherence of the light that is scattered from the front and back surface of the nuclei. The periodicity of the local oscillations is proportional to the size of the cell nuclei. Taken from Ref. [65].

Wax et al. first demonstrated the technique using a scattering phantom consisting of dried polystyrene beads on a coverglass [66], then Graf et al. validated fLCI’s ability to measure cell nuclei by using cells in-vitro [67]. The structures of these samples were basically two-dimensional surfaces, and hence STFT processing could be applied since high spatial resolution in the spectral analysis was not necessary. However, the trade-off in resolution from processing with STFTs precluded analysis of the local oscillations in more complex, three-dimensional structures. With the development of a novel processing method, named the dual window (described in detail in chapter 3), the
resolution trade-off could be avoided and thus enable detection of the local oscillations in tissue. Equipped with this novel processing method, Graf et al. applied fLCI to detect dysplasia using an ex-vivo hamster cheek pouch carcinogenesis model. The results from this study yielded 100% sensitivity and 100% specificity, clearly demonstrating the potential of fLCI as a powerful diagnostic method [65].

2.3 Summary

Chapter 2 provided a brief background on various topics needed to understand the foundation of the tools developed in this dissertation. Many of these topics will be revisited later in much more detail and some have been introduced to provide a complete picture of the different biomedical optics techniques. When available, results from animal and/or human studies were summarized.

In the first section, a quick overview of carcinoma was given. This section highlighted the different stages of cancerous development and stressed that the increase in cell nuclear diameter is a compelling biomarker, since it gives access to pre-cancerous development. In Section 2.2, many biomedical optical techniques for detecting cancer were described. To summarize, it is worth reiterating a few points: First, LSS was the first method to attempt measurements of singly scattered light to detect pre-cancerous development, and even though its results have been disputed, it provided a platform for other, more robust methods such as 4D-ELF, a/LCI and fLCI. 4D-ELF has shown very exciting results that provide evidence of the field effect of carcinogenesis. Further, a/LCI
and fLCI sample information of scatterers from specific tissue depths by employing LCI, thus providing information with higher spatial resolution compared to the other light scattering methods. Lastly, SOCT yields spatially-resolved spectral information, thereby enabling investigation of absorption and scattering with depth resolution, however the inherent resolution trade-off in STFT and CWT has limited the implementation of this method.
3. Theory and validation of a dual window method for processing SOCT signals with simultaneously high spatial and spectral resolution

As discussed in Section 2.2.6, current methods for analyzing SOCT signals suffer from an inherent trade-off between the spatial (or temporal) and spectral (or frequency) resolution. In this chapter, a dual window (DW) method for reconstructing time frequency distributions (TFDs) that overcomes this limitation of SOCT is introduced. First, a rigorous theoretical treatment is given, starting from LCI (or OCT) signals and continuing to TFD analysis where various types of distributions are discussed. Also, since the DW method is the foundation for the dissertation, simulations and experimental results will be presented to validate it. The results explore the improved spatial and spectral resolution of the DW method in the context of both absorption and scattering.

3.1 Introduction

The general challenge in SOCT consists of decomposing a signal sampled in a single domain (in this case the frequency domain) to a distribution that simultaneously contains information from the original domain and the conjugate domain (spatial or temporal domain). To solve this problem, both linear and bilinear approaches have been utilized. In the linear approach, short time Fourier transforms (STFTs) and continuous wavelet transforms (CWTs) have been widely implemented; however, as previously discussed in Section 2.2.6, these suffer from a well-known trade-off in resolution
between the spectral and spatial domains that significantly limits quantification of the spatially-resolved spectra [68-70]. On the other hand, bilinear distributions, including Cohen’s class bilinear functions such as the Wigner and Margenau & Hill distributions, do not suffer from a resolution trade-off, and thus they can reconstruct TFDs with high spatial \textit{and} spectral resolution. Bilinear distributions are only limited by the signal’s own bandwidth and duration rather than by an arbitrary window, as is the case for linear distributions [71]. These distributions however, suffer from artifacts that can make the interpretation of the TFDs very difficult [70-72]. Further, bilinear distributions require knowledge of the complex optical field which is typically not available in SOCT/OCT. Nonetheless, it is useful to leverage these representations due to the fact that they are well understood and have been extensively studied for applications such as radar, sonar, acoustics, and geophysics [71], as well as in the context of OCT signals [69,70].

\textbf{3.2 Theory}

\textbf{3.2.1 OCT signals and STFT}

To understand the DW method, we return to the FD-OCT system discussed in Section 2.2, but with more in-depth mathematical rigor. Figure 3.1 illustrates the system with all parameters of interest defined. Here, a broadband source, with field \(s(\lambda)\), and power spectral density \(S(\lambda) = |s(\lambda)|^2\), is incident on a beamsplitter (BS) that separates the field into a reference and sample arm, \(E_R\) and \(E_S\), respectively. The light returned from
the reference arm may be described as 
\[ E_R(\lambda) = s(\lambda) \exp(i2z_R(2\pi/\lambda))/\sqrt{2}, \]
and the light backscattered from the sample (with \(m\) scatterers) may be described as,
\[
E_S(\lambda) = \frac{1}{\sqrt{2}} \sum_m s'(\lambda, z_s^{(m)}) \cdot e^{i2z_s(2\pi/\lambda)} \cdot e^{i2n(z_s^{(m)} - z_d)2\pi/\lambda}
\] (3.1)
where \(\lambda\) is the wavelength of light, \(s'\) is the source field modulated by the sample, \(z_s^{(m)}\) is the distance from the BS to the \(m^{th}\) scatterer, \(z_d\) is the distance from the BS to the beginning of the sample, and \(n\) is the refractive index (RI). Further, consider that the sample’s modulation of the source intensity may be described by Beer’s law:
\[
S'(\lambda, z_s^{(m)}) = S(\lambda) \cdot \exp(-2\mu_{tot}(\lambda) (z_s^{(m)} - z_d)),
\]
where \(\mu_{tot}\) is the total attenuation coefficient and the factor of two accounts for the round trip path across the sample. This notation will be useful for future discussions where the form of \(S'(\lambda, z_s^{(m)})\) will change depending on the specific situation.

![Figure 3.1: FD-OCT system using a Michelson interferometer geometry.](image-url)
Next, assume that there is only one scatterer, \( m = 1 \), and that \( z_R = z_0 \) such that the signal detected by the spectrometer may be described as,

\[
I(\lambda) = |E_R(\lambda) + E_S(\lambda)|^2
\]

\[
= |E_R(\lambda)|^2 + |E_S(\lambda)|^2 + 2E_R(\lambda)E_S^*(\lambda) \cdot \cos(2n \cdot (z_S^{(1)} - z_0) \cdot 2\pi / \lambda).
\]  

(3.2)

Then, in order to obtain an A-scan, \( I(\lambda) \) is resampled into a linear wavenumber array, \( k = 2\pi / \lambda \), such that an FFT reveals the location of the scatterers in the sample. Consider that the background can be subtracted, and that \( S'(\lambda) = 2E_R E_S^* \), such that \( \text{FFT}\{I(k)\} = \tilde{I}(z) = \tilde{S}'(z) \otimes \delta(z \pm d) \), where the tilde denotes a Fourier transform pair, \( \otimes \) is a convolution, \( \delta \) is the delta function, and \( d = 2n \cdot (z_S^{(1)} - z_R) \) is the optical path length difference. Note that \( \tilde{S}'(z) \) dictates the spatial resolution: for a source with bandwidth \( \Delta \lambda \) and center wavelength \( \lambda_0 \), the resolution is given by the coherence length of the source, \( l_c = 2 \ln(2) / \pi \cdot \left( \lambda_0^2 / \Delta \lambda \right) = 0.44 \cdot \lambda_0^2 / \Delta \lambda \) [73]. For the remainder of the analysis, only the cross-correlation term of Eq. 3.2 will be used since the first two terms (‘DC’ terms) give a constant background signal that may be readily eliminated (as was the case for the example given in Fig. 2.1.) Also, note that if \( m > 1 \), \( \tilde{I}(z) \) is composed of a linear sum over \( m \) delta functions convolved with the Fourier transform of the source intensity. By laterally scanning the sample arm, one can reconstruct a B-mode or volumetric OCT image.

Consider the STFT of the cross-correlation term in Eq. 3.2,
\[ STFT(k, z) = \int 2E_k(\kappa')E_k^*(\kappa') \cdot \cos(\kappa' d) \cdot e^{-\frac{(\kappa-k)^2}{2u^2}} \cdot e^{-i\kappa' \cdot z} d\kappa'. \] (3.3)

The standard deviation, \( u \), of the Gaussian window artificially changes the bandwidth of the source and hence it also changes the effective coherence length, i.e. the resulting spatial resolution. This parameter must be chosen carefully in order to obtain acceptable spectral or spatial resolution. If, for example, \( u \) is chosen to be the same order of magnitude as the bandwidth of the source, then the STFT produces a TFD with good spatial resolution, but poor spectral resolution. On the other hand, if \( u \) is chosen to be much smaller than the bandwidth of the source, then the STFT generates a TFD with good spectral resolution, but poor spatial resolution.

### 3.2.2 DW method

The DW method is based on calculating two separate STFTs and then combining the results as a product. The origin of this operation is traced back to works in signal processing and quantum physics where detailed momentum/frequency and position/time information is crucial in order to better understand properties of non-stationary fields [74-76]. For example, Lee et al. demonstrated a novel two-window heterodyne scheme using simultaneous focused and collimated light beams to permit independent control of the position and momentum resolution [76]. The DW method proposed here builds upon the concepts proposed by Lee et al. to the processing of SOCT signals.
Consider the TFD resulting from two STFTs, \( \text{STFT}_1 \) and \( \text{STFT}_2 \), generated by a narrow spectral window and a wide spectral window, respectively. Assuming that the reference field in Eq. 3.2 is slowly varying over the wavelengths of interest, the processed DW signal is given by,

\[
\text{DW}(k,z) = \text{STFT}_1(k,z) \cdot \text{STFT}_2^\prime(k,z)
\]

\[
= \int \left( 4E_s^\ast(k_1)E_s(k_2) \cdot \cos(k_1 \cdot d) \cos(k_2 \cdot d) \cdot e^{-\frac{(k_1-k_2)^2}{2a^2}} \cdot e^{-\frac{(k_2-k_1)^2}{2b^2}} \cdot e^{-ik_1q_2} \cdot dk_1dk_2 \right)
\]

where \( a \) and \( b \) are independent parameters that set the widths of the windows. In order to obtain a more insightful form of the processed signal, consider a coordinate change such that,

\[
\Omega = \frac{k_1 + k_2}{2}, \quad q = k_1 - k_2, \quad k_1 = \Omega + \frac{q}{2}, \quad \text{and} \quad k_2 = \Omega - \frac{q}{2}
\]

(3.5)

where the Jacobian of the transform is unity. Thus, the DW signal can be written as,

\[
\text{DW}(k,z) = \int \left( 4E_s^\ast(\Omega + \frac{q}{2})E_s(\Omega - \frac{q}{2}) \cdot \cos\left(\Omega + \frac{q}{2} \cdot d\right) \cos\left(\Omega - \frac{q}{2} \cdot d\right) \right)
\]

\[
\times e^{-\frac{(\Omega+q/2-k)^2}{2a^2}} \cdot e^{-\frac{(\Omega-q/2-k)^2}{2b^2}} \cdot e^{-iq_2} \cdot d\Omega dq
\]

(3.6)

The term \( E_s^\ast(\Omega + q/2)E_s(\Omega - q/2) \) from Eq. 3.6 can be expressed in terms of a bilinear distribution, for example the Wigner TFD, by utilizing the ambiguity function [69,71]:

\[
E_s^\ast\left(\Omega + \frac{q}{2}\right)E_s\left(\Omega - \frac{q}{2}\right) = \int W_s(\Omega, \zeta) \cdot e^{-iq_2} d\zeta
\]

(3.7)

where \( W_s(\Omega, \zeta) \) is the Wigner TFD of the sample field in the new coordinate system. After substituting Eq. 3.7 into Eq. 3.6 and simplifying, the processed DW signal yields,
By integrating Eq. 3.8 with respect to $q$ and assuming $a$ is small compared to $b$, such that $a^2/b^2 \ll 1$, the DW TFD simplifies to,

$$DW(k, z) = 4b\sqrt{\pi} \int \int W_S(\Omega, \zeta) \cdot e^{-2(\Omega-k)^2/b^2} \cdot e^{-2(d+\zeta+z)^2/a^2} \cdot \cos(2\Omega \cdot d) \, d\Omega \, dz.$$  \hspace{1cm} (3.9)
differences of scatterers within LCI signals [69]. The utility of this oscillatory term is further explored below in the discussion of the local oscillations and fLCI.

As a last note on the theoretical basis of the DW method, the limits as $a$ approaches zero and as $b$ spans a bandwidth greater than that of the source, $\Delta k$, are investigated. In this case, the window with standard deviation $a \rightarrow 0$ approaches the delta function, while the second window whose standard deviation is $b \gg \Delta k$, becomes a constant across the spectrum. If the signal $F(k) = 2E_\mu E_z^* \cos(k \cdot d)$, and $\tilde{F}(z) \leftrightarrow F(k)$ is a Fourier transform pair, Eq. 3.4 yields,

$$DW(k,z)\big|_{a \rightarrow 0,b \gg \Delta k} = STFT_1(k,z)\big|_{a \rightarrow 0} \cdot STFT_2(k,z)\big|_{b \gg \Delta k} = \frac{1}{\sqrt{2\pi}} \tilde{F}(z)F(k)e^{-ikz}.$$  (3.10)

Equation 3.10 is equivalent to the Kirkwood & Rihaczek (KR) bilinear TFD, and if the real part is taken, it is equal to the Margenau & Hill (MH) bilinear TFD [71]. Either of these two bilinear distributions can be simply transformed to produce any of Cohen’s class functions, which includes the Wigner TFD [71]. However, operating under these limits is subject to the same undesirable artifacts associated with the KR and MH time-frequency representations, which are known as ‘reflections in time.’ This effect is important because it sets a limit on the window sizes that can be used for processing with the DW method without producing undesirable artifacts.

### 3.3 Simulations of non-stationary signals

To illustrate the power of the DW method, two different simulations with non-stationary signals (i.e., signals whose spectral content varies with space/time) are
presented. In the first simulation, a complex signal is considered so that the performance of bilinear distributions may be directly compared with the resulting distribution from the DW method. Specifically, a signal consisting of two optical fields separated in space and center frequency is simulated. The total sample field is given by

\[ E_s = E_1 + E_2, \]

where \( E_1 = E_0 \exp(-z^2) \exp(i \cdot k_1 \cdot z) \), \( E_2 = E_0 \exp\left(-(z-z_0)^2\right) \exp(i \cdot k_2 \cdot z) \), and \( k_1 > k_2 \), as shown in Fig. 3.2. The Wigner distribution of the total sample field is given by

\[ W(k, z) = \frac{1}{2\pi} \int E_s^*(z - \frac{\zeta}{2}) E_s(z + \frac{\zeta}{2}) \cdot e^{i k \zeta} d\zeta, \quad (3.11) \]

and the MH distribution of the total sample field is given by

\[ MH(k, z) = \text{Re} \frac{1}{\sqrt{2\pi}} \tilde{E}_s(k) E_s(z) \cdot e^{i k z}, \quad (3.12) \]

where \( \tilde{E}_s(k) \leftrightarrow E_s(z) \) is a FT pair. Figure 3.3 illustrates the resulting TFDs.

Figure 3.2: Real part of simulated sample field in the spatial domain containing two components.
The ideal TFD, shown in Fig. 3.3(a), is produced by treating each pulse as an individual field and superimposing their respective TFDs onto one map. However, this can only be obtained with *a priori* knowledge of the individual fields, which is unknown in practice. The ideal TFD in Fig. 3.3(a) contains two pulses with Gaussian shapes in both the spatial and spectral dimensions. The pulses are well separated in each dimension. Figures 3.3(b)-(d) show different TFDs that can be generated from a single mixed-field sampled in the spatial domain. The Wigner distribution, shown in Fig. 3.3(b), reveals the two Gaussian pulses along with an additional cross term that appears between them. The cross term contains modulations in each dimension which, in some cases, reveal important information about the temporal phase differences [69]. More often, however, these cross terms are viewed as undesirable artifacts as they yield non-zero values at depths/times and frequencies that do not exist in the field. Moreover, as more components are added to the field, the cross terms may interfere with the local signals, making it increasingly difficult to interpret the results. The MH distribution, shown in Fig. 3.3(c), contains four pulses: in addition to the two pulses comprising the signal field, the MH TFD also contains two artifact pulses known as ‘reflections in time’ [71]. As is the case with the Wigner distribution, these artifacts yield non-zero intensities at depths and frequencies that contain no actual signals.

The TFD generated using the DW method is presented in Fig. 3.3(d), and it clearly shows that the cross term present in the Wigner distribution is completely
eliminated as a result of the use of two orthogonal windows. The artifacts of the MH distribution are also avoided, and no appreciable loss in resolution in either dimension is observed.

Figure 3.3: (a) Ideal TFD with $E_1$ centered at $z_0 = 5$ and $k_1 = 13$ and $E_2$ centered at $z_0 = 0$ and $k_2 = 26$. (b) Wigner TFD. (c) MH TFD. (d) DW method TFD. Taken from Ref. [72].

The second simulation models an SOCT signal from an OCT system using a Michelson interferometer geometry (see Fig 3.1) with an experimental sample containing two distinct reflecting surfaces. The first sample surface reflects the entire Gaussian spectrum of the source while the second sample surface absorbs the high frequency
portion (upper half) of the source spectrum. This simulation is analogous to the absorbing phantom experiment discussed below in Section 3.5. In the scenario of this simulation, i.e. a SOCT system, neither the Wigner nor the MH distributions can be constructed because the detected signal is the intensity of the field, and therefore, the phase information is lost. Thus, we reconstruct the TFDs via the STFT and the DW method.

Figure 3.4: (a) Ideal TFD with simulated source bandwidth of $\Delta k = 35$ length$^{-1}$ units. (b) Narrow spectral window STFT with standard deviation $= 2$ length$^{-1}$ units. (c) Wide spectral window STFT with standard deviation $= 45$ length$^{-1}$ units. (d) DW method using the two windows used in (b) and (c). (e) Time marginals (depth profile) of (a), (b), and (d). (f) Spectral profile at $z = 4.5$ in (a), (c), and (d). Taken from Ref. [72].
Figure 3.4(a) shows the ideal TFD of the simulated signal, while Figs. 3.4(b) and (c) show the TFDs generated by the STFT using narrow and wide spectral windows, respectively. In each case, the effects of the spatial-spectral resolution trade-off are obvious. The TFD generated with the wide spectral window suffers from degraded spatial resolution while the TFD generated with the narrow spectral window suffers from degraded spectral resolution. As Xu et al. showed [70], the STFT window can be optimized for specific applications, but regardless of the window size, a resolution trade-off must be made. Fig. 3.4(d) shows the TFD generated using the DW method which computes the product of the TFDs shown in Figs. 3.4(b) and (c). Figure 3.4(e) shows the time marginals (or depth profiles) computed from Figs. 3.4(b)-(d) which demonstrate that the DW method resolves the two sample surfaces with a spatial resolution comparable to that of the ideal case, whereas the narrow spectral window STFT does not. Figure 3.4(f) shows the spectral profile of the rear surface reflection in Figs. 3.4(b)-(d) illustrating that the DW method maintains higher spectral fidelity than the wide spectral window STFT. Note that the DW method is able to accurately portray the absorbed spectrum, while the wide spectral window STFT reveals no absorption information. The DW spectral profile also reveals the same high frequency spectral modulation that is seen in the narrow window STFT and that is characteristic of the Wigner TFD. This modulation results from cross correlations between field components that overlap in depth and is analyzed further in Section 3.4.
3.4 Local oscillations

In the Wigner representation of sample fields, the cross term artifacts exhibit temporal coherence features that have been shown to contain useful structural information of scatterers in the sample [69]. In the DW representation, the cross terms are completely suppressed due to the orthogonal windows used to probe the Wigner distribution, but the coherent effect still occurs locally, i.e. at the exact position of the scatterers. This is described in Eq. 3.9 by the modulation from the cosine term whose frequency depends on the constant path difference, \( d \), between the sample and reference arms.

![Figure 3.5](image)

**Figure 3.5**: (a) TFD of simulation 2 generated by the DW processing method. (b) Spectral profile corresponding to the dashed red line in (a). (c) Correlation plot with peak corresponding to sample spacing distance of 1.5 units. Taken from Ref. [72].

This effect can be observed in Fig. 3.5(b) which shows the spectral profile from the front reflecting surface of the sample in simulation 2 (Fig. 3.4). This frequency spectrum is taken from depth 3 of the TFD shown in Fig. 3.5(a) which was generated by the DW method. Fourier transforming the spectrum generates the correlation plot

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shown in Fig. 3.5(c) which exhibits a clear correlation peak corresponding to a physical distance of 1.5. This distance agrees with the 1.5 unit spacing of the surfaces in the simulated sample, thus providing additional information about the structure. This induced temporal coherence is the operating principles behind fLCI which will be utilized to detect early cancer development in Chapter 4.

### 3.5 Experimental validation of DW method

Experiments were performed using a white light parallel frequency domain OCT (pfdOCT) system, which will be described in detail in Chapter 4. To evaluate the ability of the DW processing method to generate TFDs with simultaneously high spectral and spatial resolution, we constructed an absorption phantom consisting of a glass wedge filled with an absorbing dye as shown in Fig. 3.6(a). The inner surfaces of the wedge were abraded in order to produce more scattered light rather than light from specular reflections. Figure 3.6(b) shows a pfdOCT scan of the absorption phantom with the two inner glass surfaces clearly visible. Note that the signal from the rear surface is significantly attenuated at the thicker end of the wedge due to considerable signal absorption from the increased volume of absorbing dye present. Because the experimental system operates in the visible wavelength band, a visible absorbing dye consisting of a red food-coloring gel and water solution was used. Figure 3.6(c) shows the transmission spectrum of the absorbing dye exhibiting strong absorption in the high wavenumber range of the detected spectrum, as was the case for the second simulation.
presented in the previous section (see Fig. 3.4). The source spectrum is also plotted for reference. We expect that signals returning from the front surface of the phantom will exhibit a relatively flat spectrum, while signals scattered by the back surface of the phantom will exhibit spectra with significant attenuation of the higher wavenumbers, mirroring the absorption spectrum of the dye through which it passed.

![Diagram](image)

**Figure 3.6:** (a) Illustration of absorption phantom. (b) pfdOCT image of absorption phantom. (c) Transmission spectrum of absorbing dye used in absorption phantom. Taken from Ref. [72].

The raw data corresponding to the position of the dashed red line in Fig. 3.6(b) were processed with four different methods to yield the four TFDs shown in Fig. 3.7. Figure 3.7(a) was generated using the STFT processing method with a narrow spectral window of 0.0405 µm⁻¹. The resulting TFD has excellent spectral resolution. For example, the spectrum acquired at the depth corresponding to the front surface of the phantom (220 µm) shows good agreement with the source spectrum, while the sharp spectral cut-off at high wavenumbers, characteristic of the dye absorption, is evident at deeper depths (245 µm). However, the narrow spectral window used to generate this
TFD yields very poor spatial resolution, resulting in an inability to resolve the two surfaces of the phantom. Figure 3.7 (b) was also processed using the STFT method, but in this case a wide spectral window of 0.665 \( \mu \text{m}^{-1} \) was used. The resulting TFD has excellent spatial resolution, clearly resolving the two surfaces of the phantom. However, the spectral resolution of the resulting TFD is too poor to resolve the spectral modulation expected from the rear surface spectrum. Figure 3.7(c) shows the TFD generated using the STFT method with a window of moderate spectral width, 0.048\( \mu \text{m}^{-1} \). As expected, the spectral and spatial resolutions of the resulting TFD fall between those of Figs. 3.7 (a) and (b), illustrating the spatial-spectral resolution trade-off associated with the STFT processing method. While the spectral characteristics of the absorbing dye are apparent in this TFD, the two phantom surfaces still cannot be resolved.

The TFD in Fig. 3.7(d) was generated using the DW method. By processing the raw data with both a narrow and a wide spectral window, the TFD simultaneously achieves high spatial and spectral resolution. The spectrum from the front phantom surface exhibits excellent agreement with the spectrum of the source, while the rear surface spectrum clearly reveals a spectral cutoff at high wavenumbers due to the absorbing dye through which the signal field has passed. Additionally, the front and back surfaces of the phantom are clearly resolved in depth.
Figure 3.7: TFD of absorption phantom reconstructed with (a) Narrow spectral window STFT, (b) Wide spectral window STFT, (c) Moderate spectral window STFT, and (d) DW method. Taken from Ref. [72].

The utility of the DW processing method is further demonstrated by examining the spectral cross-sections and time marginal (or depth profile) of the generated TFDs. Figure 3.8(a) shows spectral cross-sections from depths corresponding to the phantom’s front surface in the moderate-window STFT and DW TFDs (Figs. 3.7(c) and (d)), along with the source’s reflectance spectrum for reference. Figure 3.8(b) displays spectral profiles from depths corresponding to the absorption phantom’s rear surface. For reference, the absorbing dye transmission spectrum is displayed as well. The time marginals of each TFD are displayed in Fig. 3.8(c) along with the corresponding A-scan.
from Fig. 3.6(b). Note that because the DW is the product of two STFTs, the noise floor for the DW time marginals is lower than that of the A-scan (labeled Ideal in Fig. 3.8(c)). The square-root of the DW (not shown) yields a noise floor equal to that of the A-scan with slightly broader peaks. It is important to point out that we choose to process with the DW and not the square-root of the DW because the DW is directly related to Cohen’s class functions, as described in Section 3.2. From Fig. 3.8, it is evident that the TFD generated by the DW method maintains the ability to resolve the two peaks of the absorption phantom, while the TFD generated by the STFT method does not.

Figure 3.8: (a) Spectral cross-section at depth 220 μm in 3.7(c) and (d), along with source spectrum. (b) Spectral cross-section at depth 245 μm in 3.7(c) and (d), along with dye transmission spectrum. (c) Time marginals from 3.7(c) and (d), along with corresponding A-scan. Taken from Ref. [72].
Figure 3.9: (a) Spectral profiles of Fig. 6(a) with high frequency modulations removed. (b) Spectral profiles of Fig. 6(b) with high frequency modulations removed. Taken from Ref. [72].

Table 3.1: Chi-squared calculations. Taken from Ref. [72].

<table>
<thead>
<tr>
<th></th>
<th>DW</th>
<th>STFT</th>
<th>STFT$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Front surface spectrum</td>
<td>0.0248</td>
<td>0.0317</td>
<td>0.0305</td>
</tr>
<tr>
<td>Rear surface spectrum</td>
<td>0.0980</td>
<td>0.1329</td>
<td>0.1245</td>
</tr>
</tbody>
</table>

The spectral fidelity of each processing method are now compared quantitatively. The normalized spectra from Figs. 3.8(a) and (b) are plotted in Fig. 3.9.
with the high frequency modulation removed by an ideal low-pass filter with a cut off frequency of 3 µm. By separating the low frequency content from the high frequency local oscillations, induced temporal coherence effects from scatterers are eliminated, thus allowing analysis of only the absorbing contributions. Chi-squared values for each processing method were calculated to assess goodness-of-fit. Table 3.1 summarizes the chi-squared values. For both the front surface spectra in Fig. 3.9(a) and the rear surface spectra in Fig. 3.9(b), the chi-squared values associated with DW method are lower than those of the STFT indicating that the DW processing method recreates the ideal signal with greater spectral fidelity. In addition, the goodness of fit for the square of the STFT is calculated to account for the fact that the DW method produces a bilinear distribution. The DW method is also seen to produce superior spectral fidelity than the squared STFT.

As with the simulated SOCT signals, the local oscillations (high frequency component) seen in the TFD obtained from probing the absorption phantom (Fig. 3.6) can also be analyzed to gain structural information about the experimental sample. This gives the fLCI signal. Fig. 3.10(b) shows the DW method TFD’s spectral profile from the front surface of the absorption phantom indicated by the dashed red line in Fig. 3.10(a). Fourier transforming this spectrum produces a correlation plot as shown in Fig. 3.10(c) with a clear correlation peak corresponding to a physical distance of 20.60 µm ± 0.57 µm. This measurement represents the spacing between the phantom surfaces and is
in excellent agreement with the spacing measured in the OCT image of the phantom, 20.60 µm ± 5.97 µm.

![Figure 3.10: (a) Absorption phantom TFD generated with the DW method. (b) Spectrum corresponding to the dashed red line in (a). (c) Correlation plot with peak corresponding to phantom spacing distance of 20.60 µm ± 0.57 µm, in good agreement with the OCT thickness measurement. Taken from Ref. [72].](image)

### 3.6 Summary

The DW method has been introduced for processing SOCT signals that can simultaneously maintain high spectral and spatial resolution. The theoretical treatment shows that the DW method probes the Wigner TFD of the signal field with two orthogonal windows that independently determine the spectral and spatial resolution and thus the DW method avoids the resolution trade-off that hinders traditional SOCT processing methods. In addition, local oscillations present in the TFDs generated by the DW method have been shown to contain valuable information about the structure of experimental samples. These local oscillations give the signal that is analyzed to determine structure in fLCI. By comparing the performance of the DW and STFT
processing methods in analyzing SOCT signals from an absorbing phantom, it was demonstrated that the DW method recovers TFDs with superior fidelity while simultaneously maintaining high spatial resolution.

In the remainder of this dissertation, the DW processing method is used in novel applications which provide access to absorption and scattering properties of samples that were previously unattainable.
4. Investigation of light scattering spectral signatures using fLCI for early cancer detection

4.1 Introduction

In this chapter, fLCI and the DW method are applied to gain access to morphological features from complex, three-dimensional structures, such as tissues, by analyzing spatially resolved, light scattering spectral oscillations. As described in Section 2.2, light scattering methods benefit from high spatial resolution because information can be obtained from specific regions of interest without having large background signals or overlaying structures that may obscure the features of interest. In fact, high spatial resolution is paramount in fLCI because otherwise the local oscillations would be unresolved due to phase and frequency differences in the spectra of neighboring regions. The goal of this chapter is to demonstrate fLCI’s ability to assess early cancer development by measuring an average cell nuclear diameter increase from dysplastic regions in tissue.

In the section following this introduction, Section 4.2, the fLCI instrumentation is described in detail. This consists of a parallel frequency domain OCT (pfdOCT) system using a thermal light source that enables use of a wide spectral bandwidth centered in the visible region of the spectrum. In section 4.3, a multi-layer scattering phantom is used to demonstrate the method’s ability to measure morphological features from samples with three-dimensional structure using the spatially resolved spectra of scatterers. With encouraging results from the phantom study, section 4.4 describes a
study using an ex-vivo rat carcinogenesis model. Here, data show that fLCI is able to
detect precancerous development. Further, the results also suggest that micro-
morphological features are indicators of the field effect of carcinogenesis.

4.2 Instrumentation: parallel frequency domain OCT (pfdOCT) with a thermal light source

fLCI utilizes the interferometric information from FD-OCT, however fLCI requires a much wider spectral bandwidth compared to typical OCT imaging systems in order to gain access to the local oscillations produced from induced temporal coherence effects from small scatterers. Specifically, the bandwidth needs to be wide enough to yield a spatial resolution of a few micrometers, which enables detection of the local oscillations from cell nuclei. To satisfy this criterion, fLCI employs a thermal light source consisting of a Xenon (Xe) arc-lamp. The nature of this source presents some interesting challenges which lead up to a unique OCT system design, named pfdOCT, which is described below.

Light emitted by a Xe-arc lamp can be thought of as consisting of multiple point sources each emitting light with a random phase. Thus the light is said to have multiple modes, where each mode spans an area given by the square of the transverse coherence length. To gain insight into the effects of multimode illumination, Graf et al. used time-frequency analysis to show that signals sampled in the Fourier domain add coherently, meaning that if multiple modes are present, they will all contribute to the signal, each with a random phasor [69,77]. The result of this coherent summation is reduced
heterodyne efficiency in the detected interferograms, which consequently reduces the signal-to-noise ratio (SNR) of OCT images and hence fLCI signals. An interesting note is that this effect is not present in time-domain (TD) OCT, where signals from different modes are added incoherently. As a result, TD-OCT is less vulnerable to multimode source illumination.

To overcome this limitation in FD-OCT, the number of contributing modes is limited by collimating the light source onto the sample and spatially resolving the detected signal by coherence area at the detection plane. This means that a relatively large area of the sample is illuminated. Then, detection is achieved by using an imaging spectrograph where multiple interferograms (or A-scans) are recorded simultaneously, each corresponding to a different lateral/transverse point on the sample. This allows for the detected signal to be spatially resolved by coherence area, and also allows for an incoherent summation of multiple lateral lines for averaging. Thus, because multiple lateral regions are sampled in parallel, this system is known as a parallel frequency domain OCT system [77].
Figure 4.1: pdfOCT system consists of a modified 4f Michelson interferometer geometry. See text for details.

Figure 4.1 illustrates the pdfOCT system. First, a Xe arc lamp (150 W, Newport Oriel, Stratford, Connecticut) is coupled into a multimode fiber, 200 µm in diameter. This acts as a spatial filter which also reduces the number of modes entering the interferometer. Light is then collimated using lens (L) L1 and sent into a 4f interferometer [78]. Using L2-L3 and L2-L4, light is collimated onto the sample and reference mirror, respectively. In the sample arm, the samples are tilted to avoid saturation from specular reflection by the sample-air or glass-air interface, thus allowing detection of only the scattered light. This is known as scatter mode imaging. Using L3-L5, light scattered from a sample is imaged onto the entrance slit of an imaging spectrograph. Light reflected from the reference arm is mixed with the sample field at the BS and collimated onto the entrance slit of the imaging spectrograph using lenses L4-L5. (For the studies presented in this chapter, two types of spectrographs were used:
Shamrock 303i, Andor Technology, South Windsor, CT; and SP-2156, Princeton Instruments, Trenton, NJ.) After light is dispersed into its wavelength components by the spectrograph, the interference between the sample and reference fields across the spatial dimension of the slit is recorded using a CCD camera. To reduce background noise from autocorrelation terms, the background is subtracted by taking a separate acquisition of the sample field intensity $I_S$, reference field intensity $I_R$, and dark signal $I_{BG}$, and using: $I_c = I_S - I_R + I_{BG}$, where $I_c$ is the corrected interferometric signal. Note that after interpolating the data from wavelength to wavenumber, a 1D FFT yields a B-mode, pfdOCT image.

For the study described in Section 4.3, the Shamrock 303i spectrograph is utilized, where detection is centered at 565 nm with a bandwidth of 135 nm and spectral resolution of 0.1 nm. The resulting theoretical axial resolution is 1.0 µm and the penetration depth is 0.8 mm in air. All lenses in this system have a focal length of 100 mm which set the lateral resolution equal to the size of the CCD pixels, 26 µm.

For the study described in Section 4.4, the SP-2156 spectrograph is used which detects light centered about 575 nm with a bandwidth of 240 nm and spectral resolution of 0.2 nm, resulting in a theoretical resolution of 0.6 µm and penetration depth of 0.4 mm. Note that this second system is able to sample the full visible region of the spectrum (this is particularly important for Chapter 5); however, this also results in reduced penetration depth compared to the system described above and other OCT
systems. In this system set up, lenses L3 and L4 are set to a focal length of 50 mm, which achieve a 2X magnification of the sample and reference fields at the spectrometer slit. With a CCD pixel size of 20 µm, this resulted in a lateral resolution of 10 µm. The use of shorter focal length lenses also allows for the total footprint of the system to be reduced, ultimately permitting the system to be made portable. Portability is achieved by placing the system inside a 8”X18”X24” custom made aluminum alloy box atop a heavy-duty stainless steel utility cart for transportation to on-site analysis of tissue samples.

The experimental axial resolution for both systems is approximately 1.2 µm, where the small discrepancy from the theoretical resolution results from chromatic dispersion. While the system dispersion can be compensated for digitally to recover the theoretical resolution, dispersion originating from within samples that scatter continuously are much more difficult to correct. Dispersion effects are discussed further in Chapter 6, where they are exploited to gain additional information from sparse samples.

### 4.3 Measuring morphological features of a multi-layer phantom using fLCI and LSS

This section presents measurements of morphological features from a thick turbid sample using fLCI and LSS by processing with the DW method in order to confirm the method’s ability to measure the size of scatterers in complex, tissue-like structures [79]. The tissue phantom consists of two layers containing polystyrene beads
(RI \( n_b = 1.59 \)) of different sizes suspended in a mixture of Agar (2% by weight) and water, with RI \( n_a = 1.35 \). The bead diameter in the top layer is \( d = 4.00 \pm 0.033 \mu m \), and in the bottom layer \( d = 6.98 \pm 0.055 \mu m \) with both bead distributions possessing a standard deviation of 1% in size. The scatterer concentration is chosen to yield a mean free scattering path length of \( l_s = 1 \) mm, which is slightly higher than that of tissue to ensure sufficient SNR at deeper depths. Figure 4.2 shows a pfdOCT image of the phantom acquired in a single 0.3 second exposure, after background subtraction and dispersion correction of the system [80]. (A detailed description of dispersion correction is given in Chapter 6.)

![Illustration and pfdOCT image of a two-layer phantom](image)

**Figure 4.2:** (a) Illustration and (b) pfdOCT image of a two-layer phantom consisting of 4 \( \mu m \) and 6.98 \( \mu m \) polystyrene beads in the front and back layers, respectively. Taken from Ref. [79].

As discussed in Chapter 3, each interferogram (or A-scan) may be decomposed into a distribution that contains spatial and spectral information with high resolution in both dimensions by using the DW method. Further, a depth-resolved spectrum, from
depth $z_0$ for example, contains two components that relay information, which may be analyzed independently. The first component, contained in the low frequencies of the local spectrum, corresponds to the spectral dependence of the optical signal at $z_0$, which arises from absorption and scattering in the sample. Since this is a scattering phantom with effectively no absorption, this component reveals the spectral dependence of the scattering cross section of the polystyrene beads. These samples are spherical scatterers that satisfy the assumption of the Van de Hulst approximation [2], thus this component may be analyzed with (coherence-gated) LSS [31,33]. Note that this analysis requires knowledge of the RI of the scatterers and surrounding medium. Further, in order to accurately recover this signal, the spectral dependence of the source needs to be eliminated. This is achieved by normalizing the interferograms by the reference field intensity before processing with the DW method.

The second component of the DW local spectrum, at depth $z_0$, describes the morphological features in the vicinity of this point, arising from temporal coherence effects of the scattered light. This phenomenon is contained in the local oscillations (high frequencies) of the depth-resolved spectral signal, and is analyzed with fLCI. Figure 4.3 shows a flow chart that summarizes the steps to compute the pfdOCT image, and fLCI and LSS signals.
Figure 4.3: Flow chart of the data processing used to process the pfdOCT image and determine scatterer size using fLCI and LSS.

Figure 4.4: (a) TFD generated from a single representative lateral channel from the pfdOCT image using the DW method (dashed red line in Fig. 4.2(a)). (b) Corresponding A-scan. Points 1 and 2 identify representative points of interest to be analyzed with LSS and fLCI. Taken from Ref. [79].
The parameters used to calculate the DW method TFD are $a = 0.0454 \, \mu m^{-1}$ and $b = 0.6670 \, \mu m^{-1}$. Figure 4.4(a) shows the processed DW TFD of a representative lateral line (red dashed line in figure 4.2(b)), with the corresponding A-scan (Fig 4.4(b)).

![Figure 4.4(a)](image1)

Figure 4.5: (a)-(b) DW spectral profiles (blue lines) and low pass-filtered spectra (dotted green lines) from point 1 and 2 in Fig. 4.4(b), respectively. Dashed red lines are the theoretical scattering cross sections for a 3.97\(\mu m\) and 6.91\(\mu m\) spherical scatterers for points 1 and 2, respectively, obtained by least-squares fitting the low passed-filtered spectrum. (c)-(d) Correlation function from points 1 and 2, with correlation distance ($d_c$) of 4.25\(\mu m\) and 6.87\(\mu m\), respectively. Taken from Ref. [79].

To further illustrate the process of calculating the scatterer size using fLCI and LSS, two representative points from the A-scan shown in Fig 4.4(b) are selected and the spectrum from each point is analyzed. Figs. 4.5(a) and (b) give the spectral profiles (blue lines) from points 1 and 2, respectively. To analyze the first component (low frequency
oscillations corresponding to the scattering cross section), the DW spectral profiles are low-pass filtered with a hard cut off frequency of 3.5 µm (three cycles); then, to obtain the scatterer diameter, the spectra are fit to the theoretical spectra given by the van de Hulst approximation using a least-squares method. In Figs. 4.5 (a) and (b), the dotted green lines show the low pass filtered data used for fitting, which yield $d_1 = 3.97$ µm and $d_2 = 6.91$ µm for points 1 and 2, respectively, in good agreement with the true bead sizes. The dashed red line gives the theoretical scattering cross section corresponding to the best fits: note that these are in excellent agreement with the processed signals.

The high frequency components of the local spectra at each point ($DW(k,z_0)$) give the fLCI measurement. First, the spectral dependence is removed by subtracting the line of best fit from the LSS analysis above (which is approximately equivalent to high pass filtering). Then, the residuals are Fourier transformed to yield a correlation function. The maxima of this function give the differences in optical path length between dominant scattering features in the analyzed region. For the bead phantom, the local oscillations predominately result from scattering by the front and back surfaces of a single bead (as illustrated in Fig 2.3), and thus the correlation function maximum indicates the round trip optical path length through the scatterer. Simulated OCT images by Yi et al. [68] show that light scattered by a single microsphere gives rise to multiple peaks at longer correlations which correspond to a whispering gallery mode—these are also seen in the data. Figures 4.5(c) and (d) plot the correlation function for points 1 and

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respectively, giving correlation peaks at \( d = \Delta OPL/(2n_v) = 4.25 \, \mu m \) and \( 6.87 \, \mu m \), in good agreement with both the LSS measurements and expected bead sizes.

Figure 4.6: Overlay of the fLCI measurements with the OCT image. Scatterers without color-coding correspond to points that produced non-unique size measurements. Taken from Ref. [79].

The procedure was repeated for all points in the OCT image, where an automated algorithm selected peaks that were above a threshold (int. > 10) and 10% higher than any other maxima in the correlation function. Further, only points where the LSS and fLCI measurements were in agreement within the system’s resolution (\(\pm 1.22 \, \mu m\)) were included in the analysis with the remaining points omitted. Prior to the last criteria, the LSS and fLCI measurements showed 82% agreement in the top layer and a lower 35% agreement in the bottom layer due to lower SNR at the deeper sample depths. Figure 4.6 shows an overlay of the pfdOCT image with the fLCI measurements.
that pass all the algorithm criteria. The LSS map yields similar results since the LSS and fLCI measurements were required to be in agreement for inclusion.

The average results of this tissue-phantom study are summarized in Table 4.1: In the top layer, the average scatterer size was 3.82±0.67 μm and 3.68±0.41 μm for the fLCI and LSS measurements, respectively (112 points). In the bottom layer, the average scatterer size was 6.55±0.47 μm and 6.75±0.42 μm for fLCI and LSS, respectively (113 points). These results show that by utilizing two independent methods to analyze scattering structure (fLCI and LSS), accurate and precise measurements throughout the entire pfdOCT image may be obtained. Sources of error for the fLCI measurement can arise due to partial volume effects where multiple beads lie within a single voxel region (26^2 μm^2 x 1.22 μm) giving multiple maxima in the correlation function.

**Table 4.1: Scatterer size. Taken from Ref. [79].**

<table>
<thead>
<tr>
<th>Layer</th>
<th>Accepted</th>
<th>fLCI</th>
<th>LSS</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top layer</td>
<td>4.00</td>
<td>3.82±0.67</td>
<td>3.68±0.41</td>
<td>112</td>
</tr>
<tr>
<td>Bottom layer</td>
<td>6.98</td>
<td>6.55±0.47</td>
<td>6.75±0.42</td>
<td>113</td>
</tr>
</tbody>
</table>

In summary, by processing with the DW method, the LSS and fLCI measurements yield results consistent with the morphological features of the multi-layer scattering sample. The results presented here confirm the potential to measure the
enlargement of epithelial cell nuclei, which are non-absorbing, to detect precancerous
development within intact tissues.

4.4 Detection of early colorectal cancer development in the azoxymethane rat carcinogenesis model with fLCI

This section describes an application of fLCI to measure early colorectal cancer (CRC) changes using an analysis of ex-vivo tissues drawn from the azoxymethane (AOM) rat carcinogenesis model. Specifically, fLCI is used with the DW method and pfdOCT imaging to provide a spatially resolved, functional analysis of the ex-vivo tissue samples at three depths and along two different sections of the left colon to demonstrate fLCI’s ability to detect early CRC development.

4.4.1 Animal models

This study used the AOM rat carcinogenesis model, a well characterized and established model for colon cancer research and drug development [81]. The cancerous progression of this model is similar to that seen in humans and is a good surrogate for human colon cancer development. In addition, the short induction period and high incidence of aberrant crypt foci (ACF), which are pre-neoplastic lesions [82], make this model a practical choice for testing the ability of fLCI to detect precancerous development in the colon.

All animal experimental protocols were approved by Institutional Animal Care and Use Committee of The Hamner Institute and Duke University. Forty F344 rats (six-week old, male; Charles River Laboratories Inc., Kingston, NY) were housed in the
Hamner’s animal facility for a 10-day acclimation period prior to any testing. All the animals were provided with a regular National Institutes of Health-07 diet (Ziegler Brothers, Gardners, PA) for the first 4 days of acclimation. Thereafter, the diet was switched to the pellet form of American Institute of Nutrition (AIN)-76A (Dyets Inc., Bethlehem, PA) and continued for the rest of study period. Two animals per cage were housed in polycarbonate, solid-bottom cages with Alpha-dry bedding in an animal room with a 12-hr light/dark cycle. Cages were changed twice a week. Pelleted, semipurified AIN-76A diet and water were available ad libitum. Weekly body weights were collected during the whole study period, and clinical observations were performed to monitor the health of the animals.

After 10 days of acclimation, the 40 rats were randomized into groups of 10. Thirty animals received intraperitoneal (IP) injections of AOM >90% pure with a molar concentration of 13.4 M (Sigma, St. Louis MO) at a dose level of 15 mg/kg body weight, once per week, for 2 consecutive weeks (2 doses per animal). The remaining ten animals received saline by IP and served as the control group. At 4, 8, and 12 weeks after the completion of the dosing regimen, the animals (10 AOM-treated and 3 or 4 saline-treated rats per time point) were sacrificed by CO2 asphyxiation. The colon tissues were harvested, opened longitudinally, and washed with saline. Then, the tissues were divided into 4-5 different segments, each with a length of 3-4 cm. Only the two most distal segments of the colon were analyzed for these experiments: the distal left colon
(LC) and proximal LC. Then, the samples were placed on a #0 cover glass for examination with the pfdOCT system (see Section 4.2). After imaging, the tissue samples were fixed in formalin and stained with methylene blue in order to be scored based on the number of ACF, which are defined as foci containing more than two aberrant crypts. Figure 4.7 shows an image of a stained tissue sample, 4 weeks post treatment with three ACF that contain 2, 3, and 4 aberrant crypts.

![Stained tissue sample, 4 weeks post treatment with three ACF containing 2, 3, and 4 aberrant crypts. Taken from Ref. [83].](image)

**Figure 4.7:** Stained tissue sample, 4 weeks post treatment with three ACF containing 2, 3, and 4 aberrant crypts. Taken from Ref. [83].

**4.4.2 Data processing**

The fLCI process for assessing cell nuclei diameter follows the same procedure as outlined in Section 4.3 and Fig. 4.3 with a few modifications. In short, data are acquired using the pfdOCT system, autocorrelation signals are removed by background subtraction, then interferograms are resampled from wavelength to a linear wavenumber vector, corrected for chromatic dispersion, and Fourier transformed to obtain pfdOCT images. A RI of $n = 1.38$ is used to convert the optical path length to
physical axial distance in tissue [84]. Figure 4.8 illustrates a representative image of an \textit{ex-vivo} rat colon sample. Then, the DW method is used to obtain depth-resolved spectroscopic information [72]. Here, Gaussian windows with standard deviations $a = 0.029 \ \mu\text{m}^{-1}$ and $b = 0.804 \ \mu\text{m}^{-1}$ were used, resulting in TFDs with an axial resolution of 3.45 $\mu\text{m}$ and spectral resolution of 1.66 nm.

![Figure 4.8: pfdOCT image of an ex-vivo rat colon sample. The red line delineates an example region that is averaged across to determine the nuclear diameter. Taken from Ref. [83].](image)

The last step to obtaining spectral information from specific tissue depths (i.e., local oscillations) is to co-register the pfdOCT images with the DW TFDs. This process involves using the images to identify the contour of the tissue surfaces and calibrate the analysis relative to this ‘zero’ depth. Note that if a surface is not clearly discernable at any particular A-scan, no further analysis is conducted there. With this information, the DW TFDs can be properly aligned and thus consistently provide spectral information from specific tissue depths.

Once the spectra are properly aligned, regions of interest, both laterally and axially, are identified and averaged in order to provide sufficient SNR for the spectral
analysis that follows. In the lateral direction, twenty DW TFDs are averaged to yield 10
different lateral segments in each pfdOCT image. In the axial direction, the spectral
averages of 25 µm depth segments from three different sections are calculated: at the
surface (surface section 0-25 µm), centered about 35 µm in depth (mid section 22.5-47.5
µm), and centered about 50 µm in depth (low section 37.5-62.5 µm). The area inside the
red dotted line in Fig. 4.8 gives an example of a resulting mid section from which the
spectra are averaged to determine the nuclear diameter.

As described previously, the spectra from the averaged regions contain two
components. The first component is the low frequency oscillations which may be
analyzed with LSS [2,79]. However, in tissue, lack of knowledge of the precise RI of the
scatterers and the surrounding medium limits the amount of useful information that can
be extracted from the LSS method [35], which necessitates an estimate of both
parameters. In addition, this component is now coupled with absorption from
hemoglobin in tissue, which was not the case in the phantom study described
previously. Consequently, the low frequency oscillations are ignored and removed from
the local spectra using a smoothing function in MATLAB (Mathworks, Natick,
Massachusetts). This process isolates the second component: the high frequency
oscillations of the spectra, which correspond to the local oscillations resulting from
coherent fields induced by the cell nuclei in the averaged regions. Unlike the periodic
fine structures in LSS, the local oscillations in fLCI only depend on the size and RI of the
scatterer, and are also not confounded by absorption, since absorption is contained in the lower frequencies of the spectra. Figure 4.9(a) illustrates the average spectrum (solid blue line) along with the isolated low frequency component (dotted black line) for the averaged region shown in Fig. 4.8. Figure 4.9(b) shows the resulting local oscillations.

A Fourier transform of the local spectral oscillations is taken to produce a correlation function, where the peak in this function indicates the average cell nuclear diameter in the region of analysis [65]. Other scatterers, such as other cellular organelles and nuclear content, may also produce peaks in this function, but due to their random orientation, size, and spacing with one another, the resulting signal is unlikely to produce a peak greater in magnitude than that of the average cell nuclear diameter. The correlation function for the local oscillations in Fig. 4.9(b) is shown in Fig. 4.9(c), where the correlation distance \(d_c\) has been properly scaled to account for the round trip optical path length and the RI of the cell nuclei. A constant nuclear RI of \(n_n = 1.395\) is assumed for this analysis [84]. As a last step, the peak detection process is automated to enable analysis of large data sets. To achieve this, the correlation function is subject to further processing, where the \(1/f\) noise is removed using a smoothing function. Then, only maxima that are 3.5 standard deviations above the mean of the correlation function are considered to be clear peaks. If this criterion is not met at any particular region, the measurement is discarded.
Figure 4.9: (a) Average spectrum (blue line) from the delineated region in Fig. 4.8, along with low frequency component (black dotted line). (b) Local oscillations. A Fourier transform yields a correlation function (c), where the peak corresponds to an average cell nuclear diameter of 7.88 µm in the region of analysis. Taken from Ref. [83].

4.4.3 Results

4.4.3.1 Depth sections

The nuclear diameters from the three different tissue depth sections and for all time points are summarized in Fig. 4.10 and Table 4.2. Note that the control group measurements of all the time points were combined, since no statistically significant differences were found between them. Statistical tests were conducted using a two-sided student t-test and significance was shown when \( p-value < 0.05 \).

As shown in Fig. 4.10, the mid section (35 µm depth) provided the most statistically significant results, where the treated groups at all three time points yielded \( p-values < 10^{-4} \)** when compared to the control group. The fLCI measurement for the control group at the mid section yielded an average cell nuclear diameter of 5.15+/−0.05 µm, while for the treated groups it was found to be 5.91+/−0.15 µm, 6.02+/−0.18 µm, and 6.49+/−0.49 µm at 4, 8, and 12 weeks after treatment, respectively. For the deepest (low,
50 μm depth) section, mildly statistically significant results were observed, with \(p\)-values<0.05 *. At the surface, however, no statistical significance was found.

![Figure 4](image.png)

**Figure 4.10**: Nuclear diameter by depth sections. The mid section (35 μm in depth) provided the most significant results, with \(p\)-values<10\(^{-4}\) ** for the treated samples at all time points when compared to the control group. No statistical significance was found at the surface, and mildly significant differences (\(p\)-values <0.05 *) were found at the low (50μm) section. Taken from Ref. [83].

**Table 4.2**: Measured cell nuclear diameter by depth sections. Taken from Ref. [83].

<table>
<thead>
<tr>
<th></th>
<th>Surface (mean+/- SEM)</th>
<th>Mid (mean+/- SEM)</th>
<th>Low (mean+/- SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.88 +/- 0.20</td>
<td>5.15 +/- 0.05</td>
<td>5.25 +/- 0.05</td>
</tr>
<tr>
<td>Week 4</td>
<td>5.96 +/- 0.18</td>
<td>5.91 +/- 0.15**</td>
<td>5.84 +/- 0.16*</td>
</tr>
<tr>
<td>Week 8</td>
<td>6.36 +/- 0.21</td>
<td>6.02 +/- 0.18**</td>
<td>5.97 +/- 0.18*</td>
</tr>
<tr>
<td>Week 12</td>
<td>5.48 +/- 0.33</td>
<td>6.49 +/- 0.49**</td>
<td>5.95 +/- 0.47*</td>
</tr>
</tbody>
</table>

All measurements in μm; \(p\)-values<10\(^{-4}\) **; \(p\)-values<0.05 *; N=10.

4.4.3.2 Length segments

The two tissue segments (proximal and distal left colon) were further analyzed separately for the mid depth section. The measured cell nuclear diameters and number
of ACF are summarized in Table 4.3. The data show that for all the time points, and for both segments, the measured nuclear diameters for the treated groups were significantly different from the control group ($p$-values$<10^{-4}$).

The results are also summarized in Fig. 4.11. Note that significant differences were observed for both segments after only 4 weeks post treatment. The measured increase in the nuclear diameter, however, remained relatively constant after this initial enlargement, with the exception of the last time point in the proximal LC. Here, the nuclear diameter increased dramatically from \( \sim 6.0 \, \mu m \) to \( \sim 7.2 \, \mu m \). To investigate this further, Fig. 4.12 plots the nuclear diameter as a function of the average number of ACF, which are pre-neoplastic lesions. For clarity, each point is identified with its corresponding time period. Note that the formation of ACF was faster in the proximal LC compared to the distal LC, and that the plot shows a region of little nuclear morphological change after the initial formation of ACF. This plateau region is present in both sections and is initially independent of the number of ACF. However, once the number of ACF increased to the maximum value observed in this study (\( \sim 70 \)), the measured increase of the nuclear diameter was specific to the region manifesting more advanced neoplastic development, in contrast to the ubiquitous and relatively constant cell nuclear diameter measurements of the plateau region.
Table 4.3: Measured cell nuclear diameter (fLCI measurement) and number of ACF by length segments. Taken from Ref. [83].

<table>
<thead>
<tr>
<th></th>
<th>Proximal LC (mean+/−SEM)</th>
<th>Distal LC (mean+/−SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fLCI (µm)</td>
<td>ACF</td>
</tr>
<tr>
<td>Control</td>
<td>5.15+/−0.05</td>
<td>0</td>
</tr>
<tr>
<td>Week 4</td>
<td>5.87+/−0.19</td>
<td>13.4+/−3.64</td>
</tr>
<tr>
<td>Week 8</td>
<td>5.88+/−0.27</td>
<td>55.1+/−5.60</td>
</tr>
<tr>
<td>Week 12</td>
<td>7.23+/−1.21</td>
<td>68.6+/−4.74</td>
</tr>
</tbody>
</table>

all p-values<10⁻⁴**; N=10.

Figure 4.11: Results by colon length segments. Highly statistical differences (p-value <10⁻⁴**) were observed between the control group and treated groups for the proximal LC (a) and distal LC (b). Taken from Ref. [83].
Figure 4.12: Measured cell nuclear diameter as a function of the number of ACF for each colon length segment. For clarity, the time of measurement is noted next to each point (wk = week). Taken from Ref. [83].

4.4.4 Discussion

These results presented in Section 4.4.3.1, highlight the importance of obtaining spatially resolved information for assessing tissue health. Other optical methods have also demonstrated the need for depth selectivity, but the specific depth that provides the most diagnostic information has varied. Using 4D ELF (see section 2.2.3), it was found that a penetration depth of 100 µm yielded the most significant results [39]; whereas using a different light scattering technique, named low-coherence enhanced backscattering (LEBS) which assesses changes in tissue nano-architecture, a penetration depth of 70 µm provided the most significant results [85]. With these optical methods, however, useful information is obtained by integrating to a particular depth, rather than sampling specific locations, which may explain the differences. In contrast, fLCI is an
interferometric technique that uses a broadband source, and thus enables the coherence gating imaging capabilities of OCT and allows sampling of specific points in three-dimensional space. Image guidance was also vital in this study in order to identify the tissue surface and probe specific tissue depths.

In the results presented in section 4.4.3.2, fLCI detected significant changes in both colon segments at time points that presented early evidence of preneoplastic development, underscoring the sensitivity of the method. Further, the measured early nuclear morphological change was observed in both segments and independently of the number of ACF, which suggests a ubiquitous micro-morphological change of the colonic epithelium cells. This, however, was not the case when neoplastic development became more advanced (demarcated by the high number ACF); at which point, a sharper nuclear diameter increase was seen specific to the affected region. These sets of results present significant findings: First, these results suggest that fLCI may be able to detect the “field effect” of carcinogenesis. As discussed in the Section 2.2.3, this phenomenon describes observations that neoplastic development in one part of the colon distorts nano- and micro- tissue morphology, as well as tissue function, along the entire organ. This has been a subject of much interest since it indicates that adequate screening may be achieved by only probing certain (and more readily accessible) sections of the colon [39,41,86]. These results also indicate that fLCI can identify specific regions where more
advanced neoplastic development has occurred, which is paramount for detecting CRC
development and initiating a localized therapy.

While the results presented here are very promising, there are certain limitations
that still need to be explored in order to take advantage of all the information provided
by the method. As described in section 4.4.2, the procedure for obtaining fLCI
measurements assumes a constant RI value for the cell nuclei, and a different constant
value for the bulk tissue; however, it is known that the RI can vary depending on tissue
type and tissue health. Thus, these variations, which are currently not assessed with this
method, may be introducing an additional degree of uncertainty in the calculated
nuclear diameters. Further, these variations in RI have hindered use the low frequency
oscillations with LSS, as done previously using tissue phantoms described in Section 4.3
[79].

4.5 Summary

In this chapter, light scattering signatures from induced temporal coherence
effects were measured using the DW method and fLCI to gain insight into the
morphological features of samples. First, Section 4.2 introduced the fLCI
instrumentation which consists of a pfdOCT system. It was shown that illumination
with a collimated beam and detection by coherence area, limited the number of modes
from the Xe arc lamp light source, thereby enabling OCT imaging with the full visible
spectrum. Then in Section 4.3, the pfdOCT system was used to acquire data from a
tissue phantom. Here, the DW method’s ability to obtain high spatial and spectral resolution yielded access to the local oscillations for fLCI processing, thus confirming the method’s ability to measure depth-resolved scattering signatures from three-dimensional structures. Further, since the sample was purely scattering and the RI of the scatterers and surrounding medium were known, LSS was also used to assess structure.

In Section 4.4, ex-vivo measurements from a rat carcinogenesis model were used to demonstrate fLCI’s ability to detect early signs of cancer development. Here, the size of cell nuclei was used as a surrogate biomarker of disease. The results showed a statistically significant increase in the cell nuclear diameter of treated rats compared to a control group, even at the earliest stages of precancerous development. The study showed that depth resolution is crucial, as observed by the fact that not all depths provided statistically significant results. The results of this animal study also suggested that fLCI may be able to detect changes due to the field effect of carcinogenesis, and that areas where more advanced neoplastic development occurred could be identified.
5. Investigation of absorption spectral signatures using a novel form of SOCT for molecular imaging

5.1 Introduction

Molecular imaging holds a pivotal role in medicine owing to its ability to provide invaluable insight into disease mechanisms at the molecular and cellular level. To this end, various techniques have been developed for molecular imaging, each with its own advantages and disadvantages [87-90]. For example, fluorescence imaging achieves micrometer scale resolution, but has low penetration depths and is mostly limited to exogenous agents. In this chapter, the DW method and a super continuum laser source are employed to enable a novel form of SOCT that achieves molecular imaging by analyzing depth-resolved, absorption spectral signatures of both endogenous and exogenous chromophores. Further, structural/morphological information is provided with micrometer scale resolution in three dimensions.

Section 5.2 describes a modified pfdOCT system using a super continuum laser source, with detection centered in the visible region of the spectrum. In Section 5.3, Hb absorbing phantoms are analyzed to assess the feasibility of measuring Hb concentrations and oxygen saturation levels in tissue using SOCT. Section 5.4, describes two phantom studies to demonstrate true-color SOCT imaging using any absorber in the visible region of the spectrum. Then, two animal models are used to demonstrate molecular imaging, true color SOCT (METRiCS OCT) in vivo, using both endogenous
and exogenous contrast agents. The implications of this novel MERTiCS OCT approach are discussed.

### 5.2 Instrumentation: pfdOCT with a super continuum laser source

Section 4.2 described a pfdOCT system using a thermal light source that grants access to the visible region of the spectrum. The motivation behind this set up was to avoid signal washout or loss of SNR resulting from the coherent addition of multiple modes emitted by the thermal light source, while still having access to the wide visible spectrum. However, by collimating the light onto the sample, a significant amount of light was discarded. And while imaging of biological samples was achieved, the SNR of this system (89 dB [77]) was lower than most OCT systems (typically around 120 dB [47]). An alternative source, with a wavelength range that still spans the visible spectrum, is a super continuum laser source. This source is based on pumping a photonic crystal fiber with a shot pulse, where, through a number of nonlinear processes, a super continuum is generated in the fundamental mode of the optical fiber (i.e., the output is single mode). The advantage of using this type of light source for OCT is that it has a single spatial mode, which means that light may be focused to a diffraction limited point (or line), ultimately allowing for more photons to be collected and thus resulting in an increase in SNR (see below for details). An additional benefit is that system alignment is greatly simplified.
The specific source used for the experiments described hereforth is a commercially available Fianium SC450-4. The output of this source contains a super continuum ranging from \(~400\) nm to \(~2000\) nm with \(~4\) W of power and an average power spectral density of \(~2\) mW/nm. Detection is still centered about \(575\) nm with a bandwidth of \(240\) nm, resulting in an experimental axial resolution of \(1.2\ \mu\text{m}\) and penetration depth of \(0.4\) mm (same as with the previous system).

**Figure 5.1:** pfdOCT system with a super continuum laser source. The cylindrical lens delivers a line of illumination on the sample. The light path traced with the black lines shaded in gray is in the y-z plane, where the light is focused onto the sample. The dotted red line traces the light path in the x-z plane, where light is collimated onto the sample.

For the study presented in section 5.3, the only modification made to the pfdOCT system, previously described in section 4.2, is the light source. Here, a series of filters are used to shape the source spectrum. In this particular study the samples are ideal reflective surfaces, hence focusing the light is not paramount. For the studies described
in Section 5.4, where imaging through scattering media becomes more important, the light is focused on one dimension using a cylindrical lens, which replaces L2 from the previous system. Figure 5.1 illustrates the new system, where light in the y-z plane (black lines, shaded in gray) is not affected by the cylindrical lens and is thus focused onto the sample and reference mirror using L3 and L4, respectively. In the orthogonal plane (x-z), light is focused by the cylindrical lens and then collimated using L3 and L4 (dotted red line). The line of illumination produced at the sample and reference mirror is scattered and reflected, respectively, mixed at the beamsplitter, and imaged onto the entrance slit of the imaging spectrograph. The lateral resolution is 6 µm, achieved by setting the focal lengths of L3 and L4 to 100 mm and L5 to 300 mm. Here, the use of longer focal length lenses minimizes the effect of chromatic aberrations introduced by the 100 mm singlet, cylindrical lens.

The simple change of light source yields a SNR increase from 89 dB to 95 dB. The discrepancy from other typical OCT systems may be a result of lack of confocality in one dimension, cross talk, significant reflections from the BS, and long integration times that may cause phase washout (≥20 ms; limited by the spectrograph). These types of draw backs have also been observed in other parallel OCT systems [91]. However, it is worth noting that by using a single mode source, an optical fiber based system may be readily implemented, which will likely result in a significant increase in SNR. Chapter 7 will discuss more on future directions.
5.3 Assessing hemoglobin concentrations using SOCT for feasibility of tissue diagnostics

As discussed in Section 1.1, multiple disorders affect tissue blood content and oxygen levels, including infectious processes, polyps, and cancer [16]; hence, knowledge of these parameters can be instrumental in diagnosing diseases. To obtain this information, optical techniques can take advantage of the very distinct absorption features of oxygenated (oxy-) and deoxygenated (deoxy-) Hb and consequently have the ability to monitor these parameters noninvasively. In this section, SOCT is used to demonstrate the ability to quantitatively measure depth-resolved absorption spectra from oxy- and deoxy- Hb using absorbing phantoms. Further, the lower limit of Hb concentration and percent oxygen saturation that can be obtained using this method is investigated to determine the feasibility of characterizing Hb absorption in tissue.

5.3.1 Theory

To understand how absorbing signatures may be quantified with SOCT, consider an (S)OCT signal from one scatterer/reflector as described in Section 3.1, Eq. 3.2:

\[ I(\lambda) = 2\sqrt{I_r(\lambda)I_s(\lambda)} \cos\left(\frac{2\pi}{\lambda d}\right), \]

where \( I_r = |E_r|^2 \) is the reference field intensity, \( I_s = |E_s|^2 \) is the sample field intensity, and \( d \) is the round trip optical path length. In the case of a strongly absorbing sample, such that the contributions of scattering do not influence the spectra significantly, the modulation of the source field intensity may be described by \( S'(\lambda, z^{(1)}) = S(\lambda) \exp(- \)
2μa(λ)(zS−zd)), where μa(λ) is the absorption coefficient. Thus, the sample field intensity may be described as \( I_s = R S(λ) \cdot \exp(-2μa(λ) \cdot L) \), where \( L = (zS−zd) \) is the distance from the start of the absorbing sample to the scattering/reflecting point (see Fig 3.1), and \( R \) is the power reflected by the sample. Lastly, consider that that reference field intensity is equivalent to the source’s field intensity, \( I_r = S(λ) \), such that the OCT signal can be described as,

\[
I(λ) = 2S(λ) \cdot re^{−μa(λ)L} \cos \left( \frac{2π}{λ} \cdot d \right),
\]

(5.2)

where \( r \) is the reflectivity of the sample, \( r = \sqrt{R} \). Note that the intensity depends on \( L \) and not \( 2L \) resulting from the square root term in Eq. 5.1.

By processing with the DW method, the spectra at an optical path length \( d = 2Ln \) is expected to be modulated directly by Beer’s law, thus the Hb concentration \( C_{\text{Hb}} \) may be calculated by using,

\[
\ln \left( \frac{I(λ,L)}{I_o} \right) = C_{\text{Hb}} L \varepsilon(λ) − \ln(r),
\]

(5.3)

where \( \varepsilon(λ) \) is the extinction coefficient which is independent of \( C_{\text{Hb}} \): \( μa(λ) = C_{\text{Hb}} \cdot \varepsilon(λ) \). Here, all constants have been included in \( \varepsilon \), where the Hb molecular weight used is \( MW = 64500 \text{ g/mole} \). Figure 5.2 plots the molar extinction coefficients of oxy- and deoxy- Hb. Note that in the visible spectrum, where the pfdOCT system operates, Hb absorption is stronger compared to the wavelength range of typical (S)OCT systems, ~800nm and above. As a result, this method is more sensitive to low concentrations and small
changes of Hb. From Fig. 5.2(b), one can also observe that in this range, the oxy- and deoxy- Hb absorption coefficients are significantly different, thereby allowing a facile assessment of Hb oxygen saturation states (SO₂).

Figure 5.2: Molar extinction coefficients of oxy-/deoxy- Hb over a large spectral range (a), and across the visible region of the spectrum (b). The dotted black lines in (b) delineate the region where the oxy- and deoxy- Hb coefficients exhibit the greatest dissimilarity (correlation R~0). Data compiled by Prahl.[92] Taken from Ref. [93].

5.3.2 Materials and methods

The samples for this study consisted of oxy- and deoxy- Hb phantoms prepared at varying concentrations ranging from ~5 g/L to 70 g/L (~78 µM to 1085 µM). For the oxy-Hb samples, human ferrous stable Hb in lyophilized powder form was diluted in purified water until the desired concentration was achieved. To produce deoxy-Hb, a trace amount of sodium dithionite, which removes the oxygen in oxy-Hb, was introduced to a solution of the Hb powder and phosphate buffered saline (PBS). The samples were placed in a container composed of two glass slides separated by spacers of thickness \( L \approx 400 \, \mu m \).
The data were acquired from the Hb samples using the pfdOCT system and processed with the DW method. The spectrum from the front of the rear cover glass of the sample was analyzed. Thus, the spectrum corresponded to an absorber thickness of ~400 µm. A sample consisting of water or PBS was used as reference for these ideal samples.

5.3.3 Results

Figures 5.3(a) and (b) illustrate representative experimental, normalized oxy- and deoxy- Hb intensity profiles, respectively, obtained after processing. The theoretical absorption curves (dotted black lines) are also plotted. Figures 5.3(c) and (d) are the corresponding attenuation coefficients. Note that the ideal and the measured profiles are in excellent agreement, where the absorption peaks at 540 nm and 575 nm for oxy-Hb, and at 555 nm for deoxy-Hb are clearly evident.

For each of the prepared samples, the absorption profiles of 25 distinct spatial locations were analyzed, and the spectra were processed as described in Sections 5.3.2. Using Eq. 5.3, and known thickness $L$ (which may be obtained from imaging), the Hb concentration is calculated by fitting the data to a line of the form $y = mx + b$, using a linear least squares method. Here, $x$ is either the oxy- or deoxy- Hb extinction coefficient and the computed $m = C_{Hb}L$. This procedure was repeated for 6 different concentrations. The results are summarized in Fig. 5.4.
Figure 5.3: Oxy-Hb (a) and deoxy-Hb (b) normalized absorption spectra, with Hb concentrations of 50 g/L and 68 g/L, respectively. The solid lines are experimentally measured, and the dotted black lines are the ideal spectra. Oxy-Hb (c) and deoxy-Hb (d) measured and theoretical attenuation coefficients. Taken from Ref. [93].

Figure 5.4: Measured Hb concentration for oxy- (a) and deoxy- (b) Hb samples. Error bars represent a standard deviation from the mean of 25 measurements. The dotted black lines indicate the ideal trend with unity slope and zero intercept. Taken from Ref. [93].
The data show that the measured and expected concentration values are in excellent agreement, with $R^2_{\text{oxy}} = 0.9957$ and $R^2_{\text{deoxy}} = 0.9600$. The average standard deviation for all measurements is 3.10 g/L (no statistical significance was observed between the oxy- and deoxy- Hb values, $p\text{-val} = 0.82$). This value represents the lower limit of Hb concentration that may be detected at this depth. A more heuristic parameter, however, is obtained by multiplying the minimum measurable concentration by the depth, which gives a depth-independent value—this gives $C_{\text{Hb-min}} = 1.2$ g/L at 1 mm (herein noted as g/L-mm). It is important to note that the total Hb concentration in normal tissue has been reported to be ~1.8 g/L, and as much as 3.2 times higher for cancerous tissue [94].

In biological samples, absorption will also depend on the oxygen saturation state of Hb. Thus, a more accurate model of the expected signal in biological samples will have an attenuation coefficient of $\mu(\lambda) = C_{\text{HBO2}} \cdot \varepsilon_{\text{HBO2}}(\lambda) + C_{\text{Hb}} \cdot \varepsilon_{\text{Hb}}(\lambda)$, where $\varepsilon_{\text{HBO2}}, \varepsilon_{\text{Hb}}, C_{\text{HBO2}}$ and $C_{\text{Hb}}$ are the molar extinction coefficients and concentrations for oxy-/deoxy- Hb, respectively. Using the same formalism as with Eq. 5.3, an overdetermined set of linear equations to solve for $C_{\text{HBO2}}$ and $C_{\text{Hb}}$ may be written as,

$$\frac{-1}{L} \ln \begin{bmatrix} \frac{I}{I_0}(\lambda_1) \\ \frac{I}{I_0}(\lambda_2) \\ \vdots \\ \frac{I}{I_0}(\lambda_n) \end{bmatrix} = \begin{bmatrix} \varepsilon_{\text{HBO2}}(\lambda_1) & \varepsilon_{\text{Hb}}(\lambda_1) & -1/L \\ \varepsilon_{\text{HBO2}}(\lambda_2) & \varepsilon_{\text{Hb}}(\lambda_2) & -1/L \\ \vdots & \vdots & \vdots \\ \varepsilon_{\text{HBO2}}(\lambda_n) & \varepsilon_{\text{Hb}}(\lambda_n) & -1/L \end{bmatrix} \begin{bmatrix} C_{\text{HBO2}} \\ C_{\text{Hb}} \\ \ln(r) \end{bmatrix}$$

(5.4)
where \( \lambda_i \) for \( 1 \leq i \leq n \) are the discretely measured wavelengths within the operating region. Finally, partial oxygen saturation \((S_{O2})\) can be calculated using Eq. 5.5,

\[
S_{O2} = \frac{C_{HbO_2}}{C_{HbO_2} + C_{Hb}}.
\]

(5.5)

Based on this analysis, it is clear that the ability to correctly measured oxygen saturation depends on the extent to which the method, given by Eq. 5.4, can correlate the measured data to the correct chromophore species, i.e., oxy- or deoxy- Hb. For example, if \( y \) is the measured data and \( x \) is the model in question, the correlation of \( y = x \), using a linear least squares fit, determines how well the model fits the data. For this example, a high correlation value signifies that the model describes the observations well; more importantly, for this study, high correlation values indicate that the method will clearly distinguish between the contributions to attenuation from the oxy- species and the deoxy- species. Therefore, a high correlation value suggests that oxygen saturation levels can be measured correctly.

To determine the lowest concentration at which oxygen saturation states may be correctly measured, the correlation coefficient of the linear regression used to determine concentration in the previous analysis was calculated. To execute this analysis, a limited bandwidth ranging from 520 nm to 585 nm was used, which was chosen to highlight the disparity between \( E_{HbO_2} \) and \( E_{Hb} \). The correlation coefficient between the theoretical \( E_{HbO_2} \) and \( E_{Hb} \) in this region, delineated by the dotted black lines in Fig. 5.2(b), is ~0. The results are summarized in Fig. 5.5, where Fig. 5.5(a) shows the correlations of the oxy-Hb data
when compared to the oxy- and deoxy- Hb extinction coefficients, and Fig. 5.5(b) shows the results for the deoxy-Hb data.

Good agreement (R>0.65) is observed for concentration values higher than 10 g/L if the data are compared to the correct corresponding species (i.e., oxy-Hb data to oxy-Hb model, etc.); further, there exists a sharp cut off below this point. In contrast, a low correlation value (R<0.2) is observed for all concentrations above 10 g/L if the data are compared to the wrong model. This suggests that, while concentration values may be determined as low as 1.2 g/L-mm, oxygenation states can only be accurately determined for concentrations above 4 g/L-mm ( = 10 g/L × 0.4 mm).

![Figure 5.5](image)

**Figure 5.5:** (a) Correlation coefficients between the oxy-Hb data and the oxy-/deoxy-Hb extinction coefficients at varying concentrations. (b) Correlation coefficients between the deoxy-Hb data and the oxy-/deoxy- Hb extinction coefficients at varying concentrations. Taken from Ref. [93].

### 5.3.4 Discussion

The results in this section demonstrate the ability to measure Hb concentrations as low as 1.2 g/L-mm, and over a wide range of concentrations, with excellent agreement
with the expected values (R² > 0.9). As stated previously, normal tissue Hb concentrations have been reported to be ~1.8 g/L [94]; this indicates that normal Hb concentrations are well within the range of SOCT detection at depths greater than ~0.67 mm (= 1.2 g/L-mm / 1.8 g/L). Furthermore, abnormal (or cancerous) Hb concentration values have been observed to be ~3.2 times greater than normal (~5.7 g/L) [94], hence these are also detectable using this method. Oxygen saturation states may only be correctly measured using this method given concentrations higher than 4 g/L-mm. These values fall outside the range of detection for normal tissue using this approach, where penetration depths would need to be greater than L = 2 mm; however, for cancerous tissue, the expected concentration values are within feasible detection range (L<1 mm). Further, the expected concentration of Hb within blood (150 g/L) would allow oxygen saturation in blood vessels to be determined with this method.

5.4 Molecular imaging true color Spectroscopic OCT

Molecular imaging true color spectroscopic (METRiCS) OCT incorporates the imaging capabilities of OCT with the spectral analysis that has been described in this work thus far. In this section, a larger emphasis is placed on imaging, where the depth-resolved spectral analysis is used to not only quantify parameters of interest, but also to achieve molecular imaging of any absorber in the visible region of the spectrum, including endogenous and exogenous chromophores. In addition, because the system
operates in the visible region, the sample’s true colors are used to provide an intuitive display of the spectroscopic information.

5.4.1 True color imaging using SOCT

Imaging of a sample consisting of a piece of paper with three lines: red, green, and blue, is presented to demonstrate that absorbers in the visible spectrum may be detected and displayed with its true colors using METRiCS OCT. The significance of this approach is that all the spectroscopic information may be readily interpreted by using an intuitive form of display.

In order to achieve true-color tomographic imaging, first a pfDOCT image is acquired and processed as previously outlined in Chapter 4. Figure 5.6(a) shows a photograph and pfDOCT image of the piece of paper. As previously noted, pfDOCT imaging reveal the structures of the samples, which in this case includes the indentation on the paper from the pens used to draw the lines; in addition, some absorption can be observed by the loss of intensity, particularly for the red and blue lines. To obtain the spectral information, each A-scan is processed with the DW method. Figure 5.6(b) shows the spectra from four points, labeled R, G, B, and W with arrows in Fig. 5.6(a). Here, the spectra were low pass filtered using a 7th order Butterworth filter with a cutoff of 15 cycles. The spectrum obtained for a point below the red line (point labeled R), clearly shows a peak intensity at ~630 nm, corresponding to what the human eye would perceive as red. Similarly, spectra obtained for points below the green and blue lines on
the paper, show peaks intensities at \~524 \ nm and \~460 \ nm, respectively; again, in good agreement with what the eye perceives for these colors. For completeness, a point corresponding to a white region is also plotted, and as expected, the spectrum spans all wavelengths.

Figure 5.6: (a) pfdOCT image of a piece of paper with three lines, from left to right: red, green, and blue. Inset shows a photograph of the paper. (b) Spectra from four points in the image from below the red (R), green (G), and blue (B) line, as well as from a white (W) region.
Figure 5.7: Commission Internationale d’Eclairage (CIE) color functions and source spectrum.

Having the ability to extract a spectrum from each point of an image for quantitative analysis is necessary for calculating parameters of interest, such as Hb, as was done in the previous section. However, in order to more readily appreciate all of the spectroscopic information by using the sample’s true colors, each spectrum is divided into three channels using the Commission Internationale d’Eclairage (CIE) color functions [95], which give the chromatic response of the human eye. The functions are plotted in Fig. 5.7 along with the source spectrum. For image display of the spectral data, the interferograms are not normalized before processing with the DW method, as was done previously for quantifying the spectra. Omitting this step ensures that the RGB values are not polluted by noise, particularly in regions of low source intensity. Instead, image white balance is determined as follows [96]: First the RGB values for each pixel are normalized. Then the reciprocal average RGB values of a region with no expected spectral modulation are used to remove the influence (or color) of the source. Thus, a region with no absorption will have RGB values equal to 1. This gives a color-
balanced hue map, which is then multiplied by the normalized pfdOCT image (saturation map) to yield a hue, saturation, and value (HSV) image; this results in a true color representation of the sample. Figure 5.8 gives a flowchart of the steps needed to compute the true color image, and Fig. 5.9 illustrates the true color image of the piece of paper. Note that the image gives the same structural information as with pfdOCT, but now the colors provides access to the spectral information with an intuitive form of display.

Figure 5.8: Flow chart for processing true-color OCT images.

Figure 5.9: True-color OCT image of a paper with a red, green, and blue line.
Next, a tissue phantom is used to further illustrate the imaging capabilities of METRiCS OCT. This sample consists of glass capillaries, with \( \sim 100 \, \mu m \) maximum outer diameter and \( \sim 80 \, \mu m \) maximum inner diameter (Charles Supper, Natick, Ma), filled with red, green, and blue food-coloring dyes and immersed in agar and 2\% intralipid (IL). IL is a fat emulsion that is often used as a model for scattering in tissue. Figure 5.10(a) shows a photograph of the sample. Data are acquired using the pfdOCT system and processed as outlined in Fig 5.8. Figure 5.10(b) shows the results, where the absorption from the dyes in the capillaries is evident, as seen by the color modulation. However, as with OCT, METRiCS OCT is only sensitive to the light returned from scatterers/reflectors. Thus, the spectral modulation is only observed from below the capillaries, where light is returned from scattering due to IL. Note that no signal is present from within the capillaries, since the dyes do not scatter light. Further, a spectral modulation is also observed arising the IL itself. This is due to its scattering cross section which is proportional to \( \lambda^{2.4} \), as described by van Staveren et al. [97], thus resulting in the yellow color at deeper sample depths. It is important to point out that METRiCS OCT is sensitive to the total attenuation coefficient which includes absorption and scattering. However, for regions immediately following a significant amount of absorption, for example below the capillaries, the color (and hence spectrum) is dictated primarily by the absorption coefficient.
5.4.2 METRiCS OCT using an *in-vivo* chick embryo model

The results presented above demonstrated true-color, tomographic imaging of samples. In this section, the same method is applied to an *in-vivo* chick embryo model using endogenous contrast from Hb.

Four-day old chick embryos were procured from the North Carolina State University Poultry Department and transported to The Hamner Institute. Here, a small window was made on the eggshells, and then covered with tape. The embryos were kept in an incubator at ~38 °C and ~80% humidity for seven days. During this time, the embryos’ vasculature network grows considerably and thus serves as a good initial
model to demonstrate METRiCS OCT’s ability to provide molecular information from Hb absorption in-vivo.

The eleven-day old chick embryos were transported to Duke University for analysis. First, the embryos were carefully removed from the shell (see Fig. 5.11(c)) and placed atop a motorized translational stage for volumetric imaging. Remember that the samples only need to be translated along the y-axis since the pfdOCT system provides x-z, B-mode images from a single exposure. Figure 5.11(a) shows a B-mode, METRiCS OCT image, and Fig. 5.11 (b) shows a 3D volume rendition of the entire data set. As observed, these samples contain a simple structure, consisting of the vasculature network around the yolk and the chorioallantoic membrane. Further, as with the capillary phantom study presented in section 5.4.1, the results show that the red blood cells (RBCs) in the lumen of the vessels do not produce strong signals; however, the RBCs impart a clear spectral modulation that is observed by the red shift in hue in locations below the vessels. Light passing through small vessels (< 8 µm), for example in the upper left hand corner of Fig. 5.11(a), also undergoes sufficient absorption to be detected visually.
Figure 5.11: METRiCS OCT, B-mode image (a) and 3D volume (b) of a chick embryo. White x and z scale bars, 100 µm. Photograph of the sample (c).

Figure 5.12: (a) En-face METRiCS OCT image. White x and y scale bars, 100 µm. (b)-(e) Measured and theoretical spectral profiles from points b-e, along with the computed Hb oxygen saturation.
The full potential impact of this imaging method can be seen in the en-face image, presented in Fig. 5.12(a). Here, several x-y planes are averaged to incorporate vessels found at different depths. This image (Fig. 5.12(a)) clearly depicts the architecture of the chick embryo’s vasculature network, which shows one major vessel, along with several branching vessels. Smaller capillaries can also be observed, for example in the upper left hand corner of Fig. 5.12. A significant advantage of METRiCS OCT is that full detailed spectral profiles may be quantitatively analyzed. Here, Figs. 5.12(b)-(e) shows the spectra from four selected points (solid black lines). For this analysis, it is assumed that, for regions below the blood vessels, absorption is the dominant effect such that the method described in Section 5.3 may be applied to quantify the Hb oxygen levels. The calculated SO₂ levels are also displayed in Fig 5.12, along with the ideal molar extinction coefficient (red lines) for each given oxygen level. Note that the dotted lines represent the limited bandwidth used for calculating SO₂. The results suggest that the major vessel in this image is an artery, where points (c) - (d) contain varying levels of oxygenated Hb. On the other hand, the result from the smaller vessel, indicated by point (b), shows that it contains deoxygenated Hb.

5.4.3 METRiCS OCT using an in-vivo mouse dorsal skinfold window chamber model

As a final demonstration of the capabilities of METRiCS OCT, an in-vivo CD1 nu/nu normal mouse dorsal skinfold window chamber model[98] is imaged using both
endogenous contrast and exogenous contrast. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Duke University.

For imaging of endogenous contrast, the mouse was anesthetized and the window chamber was removed. Conventional OCT imaging of the data reveals tissue structures, as seen in Fig. 5.13(a). In the case of the dorsal window [99], several histological structures are evident, including the muscle layer, the lumen of blood vessels—including small capillaries—and the subcutaneous layer. However, functional information regarding the sample is not available in this format. Using METRiCS OCT (Fig. 5.13(b)), the spectral information reveals several interesting features: for example, the muscle layer at the surface appears relatively colorless due to low concentration of Hb, but once light traverses through the vasculature network beneath, a red shift is clearly observed due to higher concentrations of Hb. Also note that small color variations are observed on the top layer due to scattering from muscle and fibrous structures. With large blood vessels (>100 µm), most of the light is attenuated, thereby preventing detection of signals from below and thus creating a ‘shadow’ effect. However, in regions without apparent blood vessels, signals are obtained throughout the full penetration depth enabled by the system.
Figure 5.13: Tomographic (x-z) images with endogenous contrast. (a) Conventional OCT image and (b) METRICS OCT image, located below point (d) as shown in the en-face (x-y) image in Fig. 5.14. The white x and z scale bars are 100 μm. Taken from Ref. [100].

Similar to the chick embryo results, the en-face image, presented in Fig. 5.14(a), provides a more global prospective of the vasculature network. This image shows two major vessels, where the larger one on the right corresponds to a vein and the other corresponds to an artery. Several branching vessels can also be observed, along with the capillary plexus. Again, four points of interest are selected to analyze the spectra quantitatively; these points are found along a branching vessel from the vein (Fig. 5.14(b)), two from the artery (Figs. 5.14(c) and 5.14(d)), and from the capillary network (Fig. 5.14(e)). As Figs. 5.14(b)-(e) show, the measured spectra are well fit by the theoretical model and the calculated SO₂ levels, shown in the figure, correspond to expected values for these tissue locations [101].
Figure 5.14: En-face (x-y) image using endogenous contrast, and spectral profiles. (a) En-face METRiCS OCT image with arrows indicating points where the spectra are quantified. The white x and y scale bars are 100 µm. (b)-(e), Spectral profiles from points as noted by the arrows in (a). The measured spectral profiles (black) are superposed with the theoretical Hb molar extinction coefficients (red). The dotted portion of the curves outlines the region used to determine SO\textsubscript{2} levels. All spectra were selected from depths immediately beneath each corresponding vessel. Taken from Ref. [100].

Next, a B-mode METRiCS OCT image from the same data set is analyzed to show the variations across individual vessels. This cross-sectional image is located on point (b) as shown in the en-face image in Fig. 5.14. Figs. 5.15(b)-(d) present spectra sampled across the diameter of a deoxygenated vessel, whereas (e)-(f) show spectra sampled longitudinally along a vessel with higher oxygen saturation levels. In both vessels, relatively small variations of only a few percent in saturation are observed.
Figure 5.15: Tomographic (x-z) METRiCS OCT image with a close up of a vessel, and spectral profiles. (a) Cross-sectional image, located on point (b) as shown in the en-face (x-y) image in Fig 5.14. The white x and z scale bars are 100 µm. (b)-(d), Spectral profiles from points (b)-(d), sampled across the diameter of a deoxygenated vessel. (e)-(f), Spectral profiles from points (e)-(f), sampled longitudinally along a vessel with higher oxygen saturation levels. Taken from Ref. [100].

Finally, imaging using an exogenous contrast agent, an area that has seen limited success in OCT to date, is demonstrated. Here, 100 µL of sodium fluorescein (NaFS), diluted in sterile saline to 1% by weight, was introduced via a retro orbital injection, an intravenous injection which serves as an alternative to a tail vein injection where the agent is introduced into the retrobulbar sinus (i.e., behind the globe of the eye).
resulting images (Fig. 5.16 and 5.17) clearly depict the presence of NaFS by a severe red shift in hue, which arises from stronger absorption at the lower wavelengths compared to that observed with endogenous contrast. Note that the injection results in the agent remaining confined to the vessels since it does not leak out appreciably. Surprisingly, the agent also shows a weak increase in scattering. As a result, vessels are now characterized by the red hue of NaFS in the *en-face* image; however, large vessels still exhibit a strong ‘shadow’.

![Tomographic (x-z) images with exogenous contrast.](image)

**Figure 5.16:** Tomographic (x-z) images with exogenous contrast. (a) Conventional OCT image and (b) METRiCS OCT image, located above point (e) as shown in the *en-face* (x-y) image in Fig 5.17. The white x and z scale bars are 100 μm. Taken from Ref. [100].

Spectra from four points of interest are selected (Fig. 5.17(b)-(e)); where the contributions of the three absorbing species (oxy-Hb, deoxy-Hb, and NaFS) are seen to affect the shape of the localized spectra to varying degrees. As shown in Fig. 5.16(b)-(e), the presence of NaFS only contributes to the attenuation spectra in the lower
wavelengths, thus assessment of SO₂ may still be accomplished as described previously. Further, the relative amount of absorption due to NaFS with respect to total Hb (ε ≡ NaFS/Hb) is also computed. The results show that this ratio is relatively constant (~1), which is expected since both NaFS and Hb are contained within the vessels and their relative contributions with respect to one another should not vary appreciably.

Figure 5.17: En-face (x-y) image using exogenous contrast, and spectral profiles. (a) En-face METRiCS OCT image with arrows indicating points where the spectra are extracted. The white x and y scale bars are 100 µm. (b)-(e) Spectral profiles from points as noted by the arrows in (a). The measured spectral profiles (black) are superposed with the theoretical oxy- (dotted red) and deoxy- (dotted blue) Hb normalized extinction coefficients, and normalized absorption of NaFS (dotted green). Also shown are the SO₂ levels and the relative absorption of NaFS with respect to total Hb (ε ≡ NaFS/Hb). All spectra were selected from depths immediately beneath each corresponding vessel. Taken from Ref. [100].
As a last observation of this data, the apparent effects of detector saturation due to fluorescent light from the NaFS are discussed. This phenomenon is seen in the green areas of the en-face image (Fig. 5.17). The contrast agent (NaFS) absorbs light with maximum efficiency around 494 nm, producing the red hue of the vessels in the METRiCS OCT images. However, it also emits incoherent fluorescent light at a peak wavelength of 521 nm (green light). When this signal is particularly strong, green spots are seen due to detector saturation, which prohibits separation of the green incoherent signal.

5.4.3 Discussion

The results presented in this section demonstrate a significant advance in the biomedical optics field due to the method’s ability to provide molecular information of endogenous and exogenous chromophores, while also providing structural information. The unique features of METRiCS OCT, which combines the DW method with a light source that spans the visible region of the spectrum, enables micrometer-scale spatial resolution in three dimensions, and high spectral resolution for a quantitative analysis. Further, the visible region of the spectrum affords highly sensitive measurements to biologically relevant chromophores, such as Hb and many exogenous absorbers in the visible region of the spectrum. This is important because a vast number of previously developed fluorophore, such as FDA approved NaFS, may be leveraged for functional, tomographic imaging. With further developments targeting diagnosis of specific
diseases, METRiCS OCT has the potential to have a significant impact on the understanding of tumor development (e.g., angiogenesis and hypoxia), ophthalmologic pathologies (retinal microvasculature perfusion and oxygenation), early cancer detection, and delivery and monitoring of therapeutic agents.

5.5 Summary

Molecular imaging techniques are important to medicine for their ability to non-invasively monitor cellular morphology and functional processes. This chapter presented true-color, quantitative, tomographic, \textit{in-vivo} images of endogenous and exogenous contrast agents with micrometer scale spatial resolution in three dimensions. In Section 5.3, the advantages of utilizing the visible region of the spectrum to quantify Hb absorption were illustrated. It is important to highlight that previous efforts to achieve this using SOCT, provided unreliable Hb measurements due to the processing method and applied wavelength regions [58]. In contrast, METRiCS OCT has been shown to able to quantify SO\textsubscript{2} levels \textit{in-vivo}. Lastly, by having access the full visible spectrum, an intuitive form of display using sample’s true colors was achieved.
6. Non-linear phase dispersion spectroscopy

6.1 Introduction

Most tissue spectroscopy approaches measure the total attenuation of light traversed through biological media. By making appropriate assumptions regarding the samples under investigation, one may quantify spectral features using scattering or absorptive models; however for most biological samples, the measured spectra will always contain contributions from both effects. In order to assess scattering and absorption independently, additional parameters must be obtained. In this chapter, a novel method, named non-linear phase dispersion spectroscopy (NLDS), is presented as a means to retrieve the wavelength-dependent, real part of the RI, leading to the separation of the scattering and absorption coefficients of turbid samples. The method is based on detecting dispersion effects imparted to a light field and detected using the phase information as a function of wavelength in LCI signals. The same sampled information is also processed to yield quantitative phase maps and spectral information regarding the total attenuation coefficient using spectral domain phase microscopy (SDPM) and METRiCS OCT, respectively.

A derivation relating the real part of the RI to the non-linear phase term in LCI signals is provided in Section 6.2. Then, Section 6.3 presents measurements from scattering and non-scattering samples that exhibit absorption due to hemoglobin. This study demonstrates the method’s ability to independently assess the scattering and
absorption coefficients. Lastly, in section 6.4, experiments using fluorescent and non-fluorescent polystyrene beads, and another using red blood cells are used to demonstrate NLDS’s ability to quantify various absorptive and dispersive features, in addition to providing quantitative phase maps. The increased sensitivity of this novel method is compared to intensity-based spectroscopy (e.g., SOCT) and potential applications are discussed.

6.2 Theory and methods

NLDS leverages the causal relationship between the real and imaginary parts of the RI. Note that the imaginary part of the RI describes absorption, while the real part describes the speed of light in a medium relative to the speed of light in vacuum. As previously discussed, assessment of the imaginary part via the total attenuation coefficient is most often coupled with scattering. On the other hand, the real part of the RI causes phase delays in propagating light fields, an effect that may be assessed independently from the scattering contributions. Thus, by using a Kramer-Kroning (KK) relation, which provides a mathematical relationship between the real and imaginary parts of causal systems, the absorption coefficient may be calculated from the real part of the RI. This section provides a mathematical treatment describing how dispersive properties (i.e., real part of the RI as a function of wavelength) may be ascertained using LCI.
Consider an LCI signal given by a single reflector/scatterer after the light has traversed through an absorptive and scattering medium,

\[ I(\omega) = 2S(\omega) \cdot e^{-\left(\mu_a(\omega) + \mu_s(\omega)\right)L} \cdot e^{i(\omega/c_0)2(z' - L\Delta n(\omega))}, \quad (6.1) \]

where \( S \) is the power spectral density of the source, \( \mu_a \) is the absorption coefficient, \( \mu_s \) is the scattering coefficient, \( L \) is the sample thickness, \( z' \) is the difference in distance between the reference and sample surface, and \( n \) is the real part of th RI. Equation 6.1 is equivalent to Eq. 5.2, with the following modifications: First, the signal is described in terms of the optical frequency, \( \omega \), which is independent of the RI, unlike the wavelength of light; and secondly, the exponential attenuation term includes both scattering and absorption. Further, the complex form of \( I(\omega) \) is used to facilitate the analysis that follows. This may be obtained by taking the Hilbert transform of the measured, real-valued LCI signal.

Next, a Taylor series expansion of \( n(\omega) \) is employed,

\[ n(\omega) = n(\omega_0) + \frac{dn(\omega)}{d\omega}\bigg|_{\omega_0} \left(\frac{\omega - \omega_0}{c_0}\right) + \frac{1}{2!} \frac{d^2n(\omega)}{d\omega^2}\bigg|_{\omega_0} \left(\frac{\omega - \omega_0}{c_0}\right)^2 + ... \]

\[ = n(\omega_0) + \Delta n(\omega), \quad (6.2) \]

where \( n(\omega_0) \) may be evaluated at an arbitrary frequency or wavelength (e.g., at \( \lambda = 800 \text{ nm}, n_0 = 1.392 \) for Hb [102]), and \( \Delta n \) incorporates all the terms dependent on \( \omega \) from the Taylor series expansion. Thus, Eq. 6.1 may be rewritten as,

\[ I(\omega) = 2S(\omega) \cdot e^{-\left(\mu_a(\omega) + \mu_s(\omega)\right)L} \cdot e^{i(\omega/c_0)2(z' - L\Delta n(\omega_0))} \cdot e^{-i(\omega/c_0)2(L\Delta n(\omega))}. \quad (6.3) \]
Eq. 6.3 shows that the measured LCI signal from a sample that scatters and absorbs light may be represented as the product of three parts. The first part modulates the intensity, which provides spectroscopic information, and hence access to $\mu_{\text{tot}}$. This term has been analyzed extensively using the DW method and SOCT/METRiCS OCT in Chapter 5. The second term of $I(\omega)$ describes the linear phase, which can be processed to yield depth resolved information as is commonly done in OCT, and if the samples are sparse, one can also retrieve sub-coherence length information using SDPM [60] (see Section 2.2.6.2). In SDPM, the sample thickness, $L_c$, may be expanded as multiples of the source’s coherence length, $l_c$, and a sub-coherence length deviation, $\delta$, given as $L = ml_c + \delta l_c$, where $m$ is an integer. Thus, a fast Fourier transform (FFT) of Eq. 6.3, ignoring the third term for now, reveals the optical path length information with a peak located at $2n_0ml_c$ and phase at the same point $\phi = (2\pi/\lambda_0) \cdot 2n_0\delta l_c$, where $\lambda_0$ is the center wavelength of the sampled spectrum. Thus, with a reasonable assumption regarding the average RI, the linear phase term yields the value of $L_c$ thereby providing quantitative structural information with sub-coherence length, axial resolution.

The third term of Eq. 6.3 contains the changes in the RI with respect to optical frequency, $\Delta n(\omega)$, which results in a non-linear phase term that serves as the source of information in NLDS. To gain access to $\Delta n(\omega)$, a dispersion correction algorithm developed for LCI signals is employed [80]. In this method, the phase of the signal, $\phi = 2L \cdot (\omega/\omega_0) \cdot (n_0 + \Delta n(\omega))$, is unwrapped and fit to a line of the form $\phi_{\text{lin}} = (\omega/\omega_0)D - 2\pi m$, where
where \( m \) is a positive integer which retains the signal’s initial phase, and \( D \) is the best estimate of the constant \( 2L_{n0} \) in the unwrapped phase. Therefore, the residual phase, \( \Delta \phi \), which is the difference between the unwrapped phase, \( \phi \), and the line of best fit, \( \phi_{lin} \), is related to the real part of the RI by \( \Delta \phi = (\omega/\omega_0)2d\Delta n(\omega) \). Finally, by following the approach of Ahrenkiel et al. [103], a subtractive KK relation is used, which exhibits fast convergence rates for limited a bandwidth and discretely sampled values, to obtain \( \Delta n \) from the absorption coefficient:

\[
\Delta n(\omega) = \frac{c_0}{\pi} \frac{1}{(\omega^2 - \omega_0^2)^2} \int \frac{\mu_a(\omega')}{(\omega^2 - \omega')^2} d\omega',
\]

where \( P \) is the Cauchy principal value of the integral. Note that \( \Delta n \) and \( \mu_a \) are concentration-dependent quantities. \( \mu_a \) and \( \Delta n \), for whole blood concentrations, are plotted in Fig. 6.1.

![Absorption coefficient and changes in the real part of the refractive index](image)

Figure 6.1: Absorption coefficient (a), and changes in the real part of the refractive index (b) of Hb in blood (150 g/L). \( \Delta n = 0 \) at \( \lambda_0 = 800nm \), where \( n(\omega_0) = 2\pi c_0/\lambda_0 = 1.392 \).

This derivation shows that the non-linear phase term in LCI signals yields access the real part of the RI, and by employing principles of causality, this may be used to
independently assess absorption, free from the contributions of scattering. Further, information from the total attenuation coefficient and the absorption coefficient can be used to isolate the scattering properties of samples. It is important to note that even though multiple parameters are embedded in the sampled signal—specifically, $\mu_{\text{tot}}$ in the intensity modulated term, $L$ in the linear phase term, and $\Delta n$ in the non-linear phase term—each is uniquely contained in only one of the three terms of Eq. 6.3, ultimately allowing independent assessment of all these parameters of interest.

### 6.3 Separating the scattering and absorption coefficients using NLDS

To demonstrate the ability to separate the scattering and absorption coefficients using NLDS, two cases are considered. For case A, fully oxygenated Hb was diluted to 40 g/L in water, using an identical sample preparation to that described in Section 5.3. For case B, 10.75% by volume of the aqueous solution in the Hb phantom was made up of 10%-Intralipid (IL), which possesses a scattering coefficient that is four orders of magnitude greater than its absorption coefficient. The system used for these experiments is the same pfdOCT system described in Section 5.2.

Depth resolved spectral profiles were computed from pfdOCT data using the DW method, and Hb concentrations were quantified using the method described in section 5.3. Note that this method uses a linear approach to fit the spectra to the Hb absorption coefficients, without accounting for scattering. The average spectra of 10 measurements are shown in Fig 6.2. The quantitative analysis for case A yields $C_{\text{Hb}} =$
40.48 +/- 2.30 g/L, in good agreement with the expected concentration. However, for case B, the analysis yields $C_{\text{Hb}} = 58.13 +/- 4.35$ g/L, which overestimates the sample’s known Hb concentration due to the contribution from scattering, which is again, not accounted for in this model.

**Figure 6.2:** Fig. 1. Measured cumulative absorption (a) and total attenuation coefficient (b) of the Hb phantoms without scattering (case A) and with scattering (case B). The theoretical absorption with $d = 400\mu$m and $C = 40$ g/L (a) and corresponding absorption coefficient (b), are also plotted. Taken from Ref. [104].

Next, the non-linear phase term is analyzed to determine the real part of the RI. After correcting for dispersion effects inherent in the system and resulting from a water phantom, the residual phase, $\Delta \phi$, was obtained from the Hb phantoms. Then, $\Delta n(\omega)$ values, which depend linearly on concentration, were determined using the subtractive KK relation [102]; thus allowing assessment of Hb concentrations via a linear least squares fitting method. Here, a limited bandwidth was used to avoid regions of low intensity due to the source, as seen in the inset of Fig. 6.3, and due to regions of substantial Hb attenuation at the lower wavelengths, as seen in Fig. 6.1. Based on this
analysis, Hb concentrations of $C_{Hb} = 39.80 +/− 1.00$ g/L and $C_{Hb} = 41.09 +/− 1.93$ g/L for case A and B, respectively, were obtained, both in excellent agreement with the known Hb concentration. The measured and theoretical $\Delta n(\omega)$ values are shown in Fig. 6.3(a) for case A and in Fig. 6.3(b) for case B. Note that the theoretical and measured real RI profiles are in good agreement for both cases. From these results, the imaginary part of the RI, and hence $\mu_a$, may be determined independently of scattering.

![Figure 6.3: Measured change in the real part of the RI of the Hb phantoms without scattering (case A) (a), and with scattering (case B) (b). The theoretical change in the real part of the RI of Hb, with a concentration of 40 g/L, is also plotted. The inset illustrates the source’s spectrum, S, with the dotted lines denoting the bandwidth used for estimating concentration using the real part of the RI. Taken from Ref. [104].](image)

The scattering coefficient of the scattering and absorbing sample (case B) may now be determined from the total attenuation coefficient and the imaginary part of the RI, by $\mu_s = \mu_{tot} - \mu_a$. The computed scattering coefficient as a function of wavelength is plotted in Fig 6.4. These results are then compared to a model of the form $\mu_s = \alpha C_{Hb} \lambda^{2.4}$, which has been shown to be an adequate model of 10%-IL scattering for a wavelength...
range of 400 nm-1100 nm and for concentrations between 4%-17% [97]. Here, the concentration of 10%-IL, \( C_{\text{IL}} \), is defined as the ratio between the volume of 10%-IL and the total aqueous solution. Further, \( \alpha \) is a concentration-independent factor, which, in part, accounts for inconsistencies in the manufacturing process and must therefore be experimentally measured for each batch of 10%-IL. To obtain \( \alpha \), a HeNe laser was used in a transmission experiment, where 10%-IL was diluted to different concentrations in 5 mm-thick glass cuvettes. The resulting average value \( \alpha = 9.62 +/- 2.8 \) was used to determine a best-fit 10%-IL concentration from the measured \( \mu_s \) for case B. This analysis gave \( C_{\text{IL}} = 10.40 +/- 1.40\% \), in very good agreement with the actual 10%-IL concentration (10.75\%). Note that by using the average \( \alpha \) for \( C_{\text{IL}} = 10.75\% \), an ideal \( \mu_s = 3.10 +/- 0.9 \text{ mm}^{-1} \) at \( \lambda = 632.8\text{nm} \) is obtained. This value is used as a calibration point to assess the theoretical \( \mu_s \) illustrated in Fig. 6.4 by the dotted red line.

![Graph](image)

**Figure 6.4:** Measured and theoretical scattering coefficient of 10% IL with a 10.75% concentration. Taken from Ref. [104].
The results presented in this section demonstrate the ability to independently measure the absorption and scattering coefficients using a combination of NLDS and the DW method. It is important to note that NLDS requires that the samples be sparse so that the phase from a single scatterer/reflector may be unwrapped. While this limits the number of applications, this method can provide invaluable insight into the interactions of light with many biologically relevant samples, which was previously unattainable without a priori information.

6.4 NLDS and METRiCS OCT for molecular phase imaging

In this section NLDS and METRiCS OCT are combined with spectral domain phase microscopy (SDPM) to provide molecular information with submicron spatial resolution in three dimensions. Experiments using fluorescent and non-fluorescent polystyrene beads, and red blood cells are presented to demonstrate the approach.

The experimental system for this study is similar to that described in section 5.2, but lenses L3 and L4, in the sample and reference arms respectively, are replaced with microscope objectives in order to achieve submicron lateral resolution. Specifically, two 40X, 0.66 NA, infinity-corrected microscope objectives (L-40X, Newport) are used, resulting in a lateral resolution of 0.46 µm. Moreover, samples are illuminated using a collimated beam such that a plane wave is incident on the sample. This simplifies the system alignment and is achieved by setting L2 as a spherical lens rather than the cylindrical lens as was the case for METRiCS OCT in Chapter 5.
6.4.1 Fluorescent and non-fluorescent polystyrene beads

The first experiment to demonstrate SDPM with molecular information from NLDS and METRiCS OCT uses a sample consisting of fluorescent (Invitrogen F8833) and non-fluorescent (Thermo Scientific), 10 µm polystyrene beads. The beads were dried on a silvered coverglass and immersed in glycerol, to minimize the number of phase wraps. Data were acquired using the pfDOCT system, where the sample is translated along the y-axis, as described in Chapter 5, to obtain three-dimensional spatial information.

First, the linear phase of the signals is processed using SDPM to acquire structural information. To achieve this, an FFT of the interferograms is computed after dispersion effects are eliminated using the method described above in Section 6.2. Figure 6.5(a) shows the unwrapped phase of the signal for a point located at the center of the fluorescent bead, along with the corresponding linear fit, $\phi_{lin}$. Recall that the residual phase between the unwrapped phase and its linear fit is the source of information for NLDS. Once the FFT of the dispersion-compensated interferogram is computed, the location of the peak and the angle (phase) at the same point, corresponding to the depth of the reflected light after it has traversed through the sample, is used to determine the optical path length, $n_0L$, with nanometer sensitivity. For this particular sample, an average RI of $n_0 = n_{bead} - n_{glycerol} = 1.59 - 1.47 = 0.12$ is used to convert the phase information to a topological map of the sample. Figure 6.5(b) illustrates the resulting map for a non-
fluorescent and a fluorescent bead. The sensitivity of the system is 5 nm as determined by the standard deviation of a background region.

Figure 6.5: (a) Unwrapped phase of an interferogram acquired from the center of the fluorescent bead and the line of best fit used to correct for dispersion effects. (b) Topological map of non-fluorescent (clear) and fluorescent (fluoro) beads. Taken from Ref. [105].

Next, the absorption spectra are analyzed by looking into the intensity modulation of the interferometric signals. Note that for these samples, which contain only one strongly reflective surface, the absorption spectra may be computed by demodulating the complex signal rather than processing with the DW method, which is computationally more expensive. This involves taking the absolute value of the Hilbert transformed signal. Figure 6.6 (a) shows two curves corresponding to the negative log of the spectral intensity (equal to $\mu_{tot}(\omega)d$) of the fluorescent and non-fluorescent beads. The ideal extinction coefficient of the fluorescent bead as provided by the manufacturer, is also plotted and shows good agreement with the experimental results, with an extinction maximum located at 540 nm. This procedure is repeated for all points in the sample; then, the normalized spectra are split into red, green, and blue channels using
the CIE color functions to produce the METRiCS OCT image. Figure 6.6(b) shows the results, where the hue and saturation information is superposed on the topological map. Note that the background and clear bead are, as expected, relatively colorless, whereas the fluorescent bead is depicted with a pink hue, consistent with its appearance as seen by the naked eye or under a microscope.

![Figure 6.6: (a) Negative log of the normalized spectrum from the center of each bead. (b) True color representation of the sample superposed on the topological map. Taken from Ref. [105].](image)

Lastly, the dispersive properties of these samples are investigated using NLDS. Here, the residual phase, $\Delta \phi(\omega)$, contains dispersion information from the system, background medium, and sample; thus, the average residual phase of a background region is used as a reference spectrum to eliminate all dispersive contributions originating outside of the samples of interest. The resulting residual phase is used to compute $\Delta n(\omega)$. Figure 6.7(a) shows the dispersion profile for points corresponding to the middle of the clear and fluorescent beads. The ideal dispersion for the fluorescent bead, also shown in Fig. 6.7(a), is calculated using the subtractive KK relation, and
shows good agreement with the measured spectra. Moreover, Fig. 6.7(a) indicates that the amount of dispersion arising from the fluorescent bead is much greater than that of the clear bead, which is expected due to the principles of causality.

![Graph showing refractive index changes](image)

**Figure 6.7:** (c) Changes in the refractive index for a point at the center of each bead. (d) Negative log of the standard deviation of the changes in the real part of the RI superposed with the topological map. Taken from Ref. [105].

The procedure for measuring dispersion using NLDS is repeated for all points on the sample. Figure 6.7(b) shows the results, where the negative log of the standard deviation of Δn is superposed on the topological map. Note that the fluorescent bead is depicted with a dark red hue, corresponding to higher dispersion compared to the background and clear bead, which have lower dispersion and appear in a light yellow hue. These results provide complementary information to the absorption map shown in Fig. 6.6(b).

### 6.4.2 Red blood cells

The second experiment conducted for this proof of principle examines normal, fully oxygenated RBCs. A sample of whole blood was obtained from a healthy donor
and processed as described by Shaked et al. [12], where the silvered cover glasses were treated with 200 µl of 25 µg/ml human fibronectin (BD 356008) for 1 hour at 37°C, then 50 µl of the RBCs suspension was allowed to attach for 30 minutes at room temperature. Attached cells were washed briefly in 2:1 EBM2:HBSS (endothelial basal media 2: Hanks balanced salt solution) before imaging at room temperature and pressure.

The same signal processing method described above was repeated for an isolated RBC. The topological map in Fig. 6.8(a), obtained from the linear phase (SDPM) analysis, illustrates the well-known round biconcave shape of a healthy RBC. Fig. 6.8(b) shows the true color spectroscopic (METRiCS OCT) map, which contains the attenuation-based spectral information. Here, the RBC is depicted to be colorless, which is in fact in good agreement with the appearance of isolated RBCs under inspection with a microscope. These results indicate that the amount of absorption incurred by a single RBC is not sufficient to impart a visibly detectable spectral modulation, thus preventing accurate quantification of important biomarkers, namely Hb concentration and oxygen saturation. These results are consistent with previous findings, where quantification of C_Hb and SO_2 of individual RBCs have shown large variances.[55,58] On the other hand, the results using NLDS, shown in Fig. 6.8(c), clearly demonstrate the higher sensitivity of this method, providing good contrast between the RBC and the background. In fact, based on the sensitivity of the system (~10 mrad), C_Hb as low as ~0.5 g/dL may be
detected, which is two orders of magnitude more sensitive than attenuation-based spectral techniques [93]. Note that normal RBC C_Hb are around 30-36 g/dL.

Figure 6.8: (a) Topological map of a healthy RBC. (b) True color representation superposed with the topological map. (c) Negative log of the standard deviation of the changes in the real part of the RI superposed with the topological map. (d) Representative spectral profile of the real part of the RI. Taken from Ref. [105].

The full potential of NLDS is realized by analyzing the detailed dispersion spectra, which allows assessment of C_Hb. The procedure for assessing this parameter is the same as that described in Sections 6.3. Figure 6.8(d) shows a typical spectrum along with the ideal fit corresponding to C_Hb = 31.9 g/dL. Note that the measured and ideal spectra are in excellent agreement. The average Hb concentration for the entire cell was C_Hb = 33.4 +/- 7.7 g/dL, in good agreement with expected values of healthy RBCs [93,106].
As a final demonstration, a group of four RBCs are analyzed using the same method described above. Figures 6.9 (a) and (b) illustrate the topological maps using the molecular information from METRiCS OCT and NLDS, respectively. As previously demonstrated, the resulting maps show that the molecular information provided by NLDS achieves higher sensitivity, as observed by the sharp contrast in the topological map. Further, the concentrations computed using NLDS (from left to right) yield 34.6 +/- 9.1 g/dL, 36.0 +/- 11.9 g/dL, 31.6 +/- 13.0 g/dL, and 36.7 +/- 8.2 g/dL, in good agreement with normal values.

![Figure 6.9: Four healthy RBCs. (a) True color representation superposed with the topological map. (b) Negative log of the standard deviation of the changes in the real part of the RI superposed with the topological map. Taken from Ref. [107].](image)

### 6.4.3 Discussion

The results presented in this section demonstrate the ability to detect different dispersion-inducing molecules—specifically a synthetic fluorophore and oxy-Hb—along
with the capability to quantify concentration. Further, NLDS and METRiCS OCT were integrated with SDPM to produce quantitative topological maps with nanometer depth sensitivity that also provide spatially resolved molecular information. It was shown that the absorption information extracted using NLDS is two orders of magnitude more sensitive than attenuation-based analysis, such as METRiCS OCT and SOCT, thereby permitted quantification of Hb concentrations from single RBCs.

The application of NLDS in SDPM provides important advantages over other phase imaging methods that seek to measure the wavelength-dependent real part of the RI. Specifically, other methods require multiple light sources or narrow band-pass filters, consequently limiting the spectral information to sparsely sampled spectral regions [106,108,109]. In contrast, NLDS has access to a wide spectral bandwidth with high resolution. As described in the motivation of this dissertation (Section 1.1.2), the approach may enable new avenues for detecting diseases, for example sickle cell anemia, thalassemia, and malaria, which afflict RBCs.

### 6.5 Summary

NLDS is a novel technique that achieves wide-band, high spectral resolution, dispersion profiles. As described in section 6.2, this information is acquired from the non-linear phase term in LCI signals. Additionally, the same sampled signal contains information regarding the total attenuation coefficient and spatial information that allows three-dimensional imaging. In section 6.3, the causal relationship between the
real and imaginary part of the RI was exploited to separate the absorption and scattering coefficients, which are typically coupled in spectroscopic methods. Lastly, in Section 6.4 NLDS and METRiCS OCT were utilized to provide molecular contrast using SDPM, which achieved ~0.5 µm lateral and 5 nm axial resolution. The methods described in this chapter provide valuable insight into the light scattering and absorptive properties of biologically relevant samples and may enable new directions for diagnosing diseases at the cellular and molecular level.
7. Conclusions and future directions

The work presented in this dissertation describes novel spectroscopic methods that quantitatively assess the spatially varying scattering and absorptive properties of biological samples. These methods are derived from LCI signals, which permit integration of imaging methods, such as OCT and SDPM, with spectroscopic information to achieve functional/molecular imaging in three dimensions.

The basis for a great deal of this work originates from the DW method, due to its ability to provide simultaneously high spectral and spatial resolution from LCI signals. As described in Chapter 3, this method combines two linear time frequency distributions, each with different spatial and spectral properties, to form a new type of bilinear distribution. It was shown mathematically that the DW method probes the Wigner bilinear distribution with two orthogonal, independent windows, thus overcoming the resolution tradeoff associated with linear distributions and avoiding common artifacts found in bilinear distributions. The capabilities of this method were demonstrated through simulations and an experiment that analyzed an absorbing phantom.

Equipped with this tool, scattering signatures in depth resolved spectra were analyzed using fLCI. This method looks at the high frequency oscillations of local spectra, resulting from induced temporal coherence effects, to ascertain the size of dominant scatterers. Analysis of these spectral features in complex, three-dimensional
samples was not possible prior to the DW method due to the fact that high resolution is needed in both the spectral and spatial dimensions. However, with the DW method, the local oscillations can be recovered and quantitative analysis is enabled.

In Chapter 4, depth resolved scattering by a phantom consisting of agar and polystyrene beads in two distinct layers, which emulates cell nuclei in different layers of tissue, was used to demonstrate fLCI in samples with complex structure. Further, experiments with an ex-vivo rat carcinogenesis model were used to assess fLCI’s ability to detect early signs of cancerous development, where the cell nuclear diameter was used as a surrogate biomarker of disease. It was found that fLCI can distinguish between normal and treated tissues, with results showing high statistical differences between the two groups, in addition to providing evidence of the field effect of carcinogenesis. The advantages of using fLCI compared to other optical methods, as discussed in Chapter 2, include that fact that the information may be gathered from specific regions in tissue rather than incorporating all the information along the trajectories of the photons, and processed data do not need to be interpreted by trained experts nor using computational models. These results show that fLCI can assess tissue health quantitatively, with sensitivity to precancerous states, which holds important implications for screening and diagnosing cancer.

The DW method was also used to gain access to spatially varying spectral modulations due to absorption. Unlike the local oscillations in fLCI, absorption from
chromophores, such as Hb, produce a spectral modulation that is lower in frequency, thereby permitting separation of the two effects. In Section 5.3, the feasibility of detecting Hb absorption in tissues was assessed using absorbing phantoms. Here, the choice of wavelengths, which span the visible spectrum, was an important factor in enabling this method. Since light is subject to high degrees of absorption in this spectral region, the measured spectra are sensitive to low concentrations and small changes of Hb; additionally, the oxy- and deoxy- Hb coefficients are significantly different in the visible spectrum, thus allowing facile assessment of oxygen saturation.

In Section 5.4, a larger emphasis was placed on imaging, where the spectral information was incorporated with tomographic imaging using METRiCS OCT. This method achieves molecular imaging in three dimensions; in addition, the spectral data are processed to yield a true color representation of samples, which provides an intuitive display of the vast spectral information. The detailed spectral profile at each voxel may also be accessed for quantitative analysis to gain insight to parameters of interest, such as Hb oxygen saturation. The method was demonstrated in-vivo using a chick embryo model and a mouse dorsal skinfold window chamber model. Here, quantitative molecular imaging from endogenous and exogenous chromophores was achieved. As the results show, METRiCS OCT is a powerful tool that can enable new directions in early cancer detection, monitoring the delivery of therapeutic agents, and fundamental research, such as improving the understanding of angiogenesis and hypoxia.
The last method described in this dissertation, NLDS, does not employ the DW method, instead spectral information is drawn from the non-linear phase of LCI signals. NLDS probes the real part of the RI rather than the total attenuation coefficient, thus, by using principles of causality, this method leads to an independent measurement of absorption, free from scattering effects. NLDS may also be combined with the DW method to isolate the scattering coefficient of turbid samples. Phantoms that scatter and absorb light were used to demonstrate the method. Further, NLDS was integrated with SDPM and METRiCS OCT to provide molecular imaging based on absorption and dispersion, with sub-micrometer spatial resolution. The results show that NLDS is more sensitive than attenuation based measurements, which permits assessment of Hb concentrations from single RBCs. While the application of this method is limited to sparse samples, NLDS yields molecular contrast in phase microscopy, which can be applied to imaging of functionalized cell receptors or to diagnose diseases that, for example, afflict RBCs.

The tools developed in this dissertation enable a number of different directions, each facing different challenges that will direct future work. In fLCI, an optical fiber based system will have to be developed in order to apply the method \textit{in-vivo}. Automated algorithms that contour the surface of tissues should be implemented in order to facilitate analysis of large data sets corresponding to wide areas of tissue. Future work on METRiCS OCT will consist of establishing other exogenous
chromophores, such as gold nanoparticles, as viable molecular contrast agents with this method. Also, animal models should be explored to determine the utility of METRiCS OCT for understanding tumorigenesis. As with fLCI, an optical fiber based system should be implemented in order to improve upon the pfdOCT system’s SNR, which would also permit endoscopic applications. For both fLCI and METRiCS OCT, faster parallel computing methods and/or use of graphic processing units should be explored to expedite processing with the DW method. In NLDS, disease mechanisms should be studied to determine the diagnostic power of this method. For sickle cell anemia, for example, novel system designs should be considered in order to probe the birefringence properties of these RBCs.
References


61. Wang, P., Bista, R., Khalbuss, W., Qiu, W., Uttam, S., Staton, K., Zhang, L., Brentnall, T., Brand, R. and Liu, Y. Nanoscale nuclear architecture for cancer diagnosis beyond


Biography

Francisco Eduardo Robles was born to Francisco A. Robles and Ariadne Guerrero in Mexico City on July 5, 1985. Twelve years later, he and his family moved to North Carolina, where they lived in Wilmington and Charlotte. In 2003, Francisco went to North Carolina State University, and graduated *summa cum laude* in May, 2007. He received two Bachelor of Science degrees, one in Physics and another in Nuclear Engineering, and a minor in Mathematics. Upon graduating, he entered Duke University seeking a Ph.D. in Medical Physics. Francisco has ten peer reviewed publications, three book chapters, and numerous conference presentations and proceedings. He received his Ph.D. in the fall of 2011.

Peer reviews publications


