Development of a Fourier Domain Low Coherence Interferometry Optical System for

Applications in Early Cancer Detection

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Biomedical Engineering in the Graduate School
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ABSTRACT

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Abstract

Cancer is a disease that affects millions of people each year. While methods for the prevention and treatment of the disease continue to advance, the early detection of precancerous development remains a key factor in reducing mortality and morbidity among patients. The current “gold standard” for cancer detection is the systematic biopsy. While this method has been used for decades, it is not without limitations. Fortunately, optical detection of cancer techniques are particularly well suited to overcome these limitations. This dissertation chronicles the development of one such technique called Fourier domain low coherence interferometry (fLCI).

The presented work first describes a detailed analysis of temporal and spatial coherence. The study shows that temporal coherence information in time frequency distributions contains valuable structural information about experimental samples. Additionally, the study of spatial coherence demonstrates the necessity of spatial resolution in white light interferometry systems. The coherence analysis also leads to the development of a new data processing technique that generates depth resolved spectroscopic information with simultaneously high depth and spectral resolution.

The development of two new fLCI optical systems is also presented. These systems are used to complete a series of controlled experiments validating the theoretical basis and functionality of the fLCI system and processing methods. First, the
imaging capabilities of the fLCI system are validated through scattering standard experiments and animal tissue imaging. Next, the new processing method is validated by a series of absorption phantom experiments. Additionally, the nuclear sizing capabilities of the fLCI technique are validated by a study measuring the nuclear morphology of *in vitro* cell monolayers.

The validation experiments set the stage for two animal studies: an initial, pilot study and a complete animal trial. The results of these animal studies show that fLCI can distinguish between normal and dyplastic epithelial tissue with high sensitivity and specificity. The results of the work presented in this dissertation show that fLCI has great potential to develop into an effective method for early cancer detection.
To my parents, who have taught me more lessons than any
dissertation could ever hold.
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1. Introduction

1.1 Motivation

In December of 2008, the World Health Organization announced that at some point during the next year, cancer was expected to surpass heart disease as the leading killer around the world.\(^1\) The disease, which can develop from a single mutated cell, accounts for nearly a quarter of all deaths in the United States each year\(^2\) and drastically affects the lives of millions of others. Statistics compiled by the National Cancer Institute show an age-adjusted cancer incidence rate in the US of 467 per 100,000 and an age-adjusted death rate of 190 per 100,000.\(^2\) These sobering statistics underscore the gravity of the cancer problem: with an estimated 1500 cancer patients succumbing to the disease each day,\(^2\) it is clear that a solution is needed quickly and a vast amount of time and effort is required to bring that solution about.

With the effects of cancer impacting so many, a substantial amount of research and resources has been focused on developing innovative methods for the prevention, detection, and treatment of the disease. But even as the methods and techniques for treatment continue to evolve and improve, there is one key fact that has remained unchanged: early detection of cancerous development significantly increases a patient’s likelihood of surviving the disease.

Cancers typically develop slowly over time, beginning with just a few abnormal cells that grow and proliferate. The majority of malignancies develop through
precancerous states characterized by varying levels of architectural and cytologic
abnormality. Detecting these structural and chemical changes in tissues at the earliest
possible stages can greatly reduce rates of mortality and morbidity. However, detecting
precancerous development is a great challenge for the available screening techniques.

The current “gold standard” for detecting cancer of the epithelial tissue is the
histopathologic analysis of biopsy samples. Biopsy samples are removed from the
patient before being fixed, stained, and examined by a pathologist for morphological
abnormalities. Although this procedure is the standard practice for cancer diagnosis,
there are several drawbacks to its current form. First, biopsy samples must be examined
and graded by trained pathologists, introducing an element of subjective interpretation.
Studies have shown that the diagnoses of different pathologists often disagree,
especially in the case of low-grade dysplasia. Additionally, there is typically a delay
of days or weeks between the time a biopsy is taken and the time a diagnosis can be
given. A biopsy samples must be fixed, stained, and further prepared before a
pathologist can analyze the sample and render a diagnosis. These procedures usually
take place in a facility separate from the surgical suite, increasing the delay. Finally, the
coverage of a biopsy is very poor. For example, a typical screening protocol for the
esophagus calls for four quadrant biopsies at one centimeter intervals throughout the
length of the esophagus. While this is a large number of biopsies, the procedure covers
only approximately five percent of the organ’s epithelial tissue, leaving the strong possibility of missing a developing neoplasm.\textsuperscript{4}

It is clear that improved screening and diagnostic technologies are needed to overcome these limitations. In recent years, a large amount of research has focused on developing optical, or light-based, detection techniques because such methods hold great promise to overcome the limitations of traditional biopsy listed above. One specific technique, elastic light scattering, is an optical phenomenon that can provide an abundance of information about the structures with which the light interacts. The particular information acquired is dependent upon the type of elastic light scattering measurement collected. The intensity of scattered light can simply be mapped spatially to provide a picture, or it can be measured as a function of wavelength, scattering angle, or polarization to uncover more sophisticated information about a scatterer. Such measurements can reveal an individual scattering particle’s size, shape, or orientation, or the distribution, concentration, or molecular composition of a group of particles.

For decades, elastic light scattering has been utilized in a variety of applications where direct measurement of physical properties is impractical or impossible. Scientific fields ranging from astronomy and meteorology to chemistry and biology have employed these optical measurements in a variety of ways. Astronomy has used light scattering measurements to overcome the vast physical distances that pose a daunting obstacle to other forms of measurement. For example, light scattering has been used to
detect the composition and structure of interplanetary and interstellar dust.\textsuperscript{7,8} The solid grains in interstellar space and the composition of remote environments, such as planetary atmospheres, have also been analyzed using light scattering.\textsuperscript{7} The field of meteorology has also taken advantage of light scattering measurements in order to study a variety of atmospheric and oceanic properties. Light scattering techniques have been applied to measuring air\textsuperscript{9,10} and water\textsuperscript{11} pollution as well as weather and weather systems.\textsuperscript{12,13} Wind sensing has also relied on various light scattering techniques for characterization and velocity measurements.\textsuperscript{14,15} Additionally, elastic light scattering has found great utility in the field of chemistry. Light scattering has been used to study the composition and properties of aerosols\textsuperscript{7} and has also been used for security applications such as explosives detection.\textsuperscript{16}

Most recently, advances in light source and detector technology have allowed elastic light scattering to expand its application into biology and medicine. Using powerful, broadband light sources, elastic scattering spectroscopy (ESS) has been used to investigate the cellular morphology of \textit{in vivo} and \textit{ex vivo} tissue samples. Perelman, \textit{et al.} used elastic scattering spectroscopy to probe cellular morphology by analyzing periodic fine structures in the detected scattering signals.\textsuperscript{17} Later, Backman \textit{et al.} measured the intensity of backscattered light with sensitivity to angle, polarization and wavelength in order to determine the size and distribution of \textit{in vitro} cell nuclei.\textsuperscript{18} This study was significant as it drew attention to nuclear morphology as a strategic target for
subsequent light scattering studies. Not only is the scattering intensity from the nucleus large due to the index of refraction difference between the nuclear membrane and the surrounding cytoplasm, but enlargement of the nuclear diameter is also a key indicator of precancerous growth. Probing the morphology of cell nuclei and other intercellular organelles and clinical applications of ESS were the focus of several subsequent studies.

These advancements have paved the way for a new elastic light scattering technique known as Fourier domain low coherence interferometry (fLCI). fLCI seeks to analyze depth-resolved spectroscopic information in order to recover nuclear morphology from specific subsurface tissue layers. Spectral analysis of these tissue layers aims to detect nuclear diameter enlargement which is characteristic of the earliest stages of precancerous transformation. This biomarker, either alone or in conjunction with other information derived from the light scattering signal, can provide the quantitative information necessary to distinguish between normal and dysplastic epithelial tissue with high sensitivity and specificity.

The fLCI method holds great potential as an optical detection technology that can overcome the key limitations of traditional biopsy. The technique holds promise of a quantitative diagnosis, eliminating the subjectivity of traditional biopsy. fLCI also promises a real time diagnosis, eliminating the delay in diagnosis experienced with current biopsy procedures. And finally, fLCI promises to greatly improve the coverage
of screened tissue, significantly reducing the chances of missing a developing neoplasm. However, for the potential of fLCI to be reached, a great deal of technological advancement must be achieved. The research presented in this dissertation details the theoretical and experimental development of the fLCI technology.

1.2 Project overview

The overall goal of the work described in this dissertation was to develop the fLCI technology from a proof of principle methodology into a technique that can successfully distinguish between normal and dysplastic epithelial tissue with high sensitivity and specificity. In order to achieve this goal, three key milestones were reached.

The first milestone was to establish the ability of fLCI to probe cellular morphology by quantitatively determining nuclear diameter. To achieve this milestone, fLCI validation experiments were extended from polystyrene microsphere phantoms to \textit{in vitro} cell monolayers. The second milestone was to design, build, and verify the functionality of a next-generation fLCI optical system which has the ability to analyze the spectra of subsurface scattering layers in thick experimental samples. In order to reach this milestone, a comprehensive investigation of temporal and spatial coherence was completed. A set of system design criteria and a new data processing method evolved from the study of coherence. The final milestone was to use the next-generation
optical system to complete a validation study in *ex vivo* animal epithelial tissue. The study establishes the promise of fLCI as a method for screening epithelial tissues for cancerous development.

### 1.3 Document organization

The dissertation is organized in the following way. Chapter 2 begins with background information about epithelial cancer. The overall goal of the work is to detect developing carcinomas and therefore a background of the disease is used to frame the steps taken during the development of the technology. A review of the varied optical techniques currently being developed for the detection of carcinomas is presented next. Technical descriptions of each technique are given and *ex vivo* and *in vivo* research highlights are noted. This section is meant to give the reader a frame of reference for where fLCI fits into the technological landscape. Chapter 2 ends with a background description of the fLCI technique. The theoretical basis of the methodology is presented along with results of the first proof of principle experiment.

Chapter 3 delves deeper into the discussion of the theory behind fLCI. The Wigner time-frequency distribution framework for analyzing fLCI and OCT signals is introduced and the formation of several examples of typical optical signals is investigated. Section 3.3 draws connections between time-frequency distributions, the importance of temporal coherence, and improved knowledge of light scattering
structures. The section connects subjects from the vast signal processing literature to the field of coherence imaging and sets the stage for the development of a new processing technique that overcomes long-standing obstacles to the processing of spectroscopic OCT signals.

A new method for processing fLCI and spectroscopic OCT signals known as the Dual Window (DW) processing method is presented in section 3.4. The DW method evolves from the Wigner time-frequency distribution analysis and overcomes the spectral and temporal resolution tradeoff that hampers existing processing methods such as the short-time Fourier transform. Several simulations are presented to demonstrate the resolution improvements and the increase in spectral fidelity of signals processed by the DW method. The temporal coherence effect that can be utilized to gain structural information about experimental samples, introduced in Section 3.3, is also analyzed in the context of the new processing method.

Chapter 3 concludes with an analysis of the role that spatial coherence plays in the formation of white-light interference signals. The differences between low coherence interferometry signal formation in the time-domain and frequency-domain are demonstrated mathematically and the implications for fLCI system design are discussed.

System design and instrumentation is the focus of Chapter 4. The basic first generation common-path system is described first, and its advantages and limitations
are noted. Next, the design and construction of the second-generation imaging fLCI system is described with special attention paid to the design criteria laid out in Section 3.5.

Chapter 5 describes three studies completed to validate theoretical concepts and system capabilities. First, the second-generation imaging fLCI system is validated. Results from a scattering phantom experiment are presented to demonstrate experimentally the theoretical concepts presented in Section 3.5. Additionally, the parallel frequency-domain OCT imaging capabilities of the system are validated with images of a hamster cheek pouch epithelium. Next, the capabilities of the DW processing method are validated experimentally. An absorption phantom is used to show the method’s ability to generate time-frequency distributions with simultaneously high spectral and depth resolution. Finally, the nuclear sizing capability of the fLCI technique is validated with a study determining nuclear diameter for in vitro cell monolayers.

The ex vivo animal tissue studies completed for this work are presented in Chapter 6. First, the pilot animal study which uses the spectral slope metric to distinguish between normal and dysplastic epithelial tissue is presented. This study is followed by the capstone of the dissertation, a complete animal study using nuclear diameter to distinguish between normal and dysplastic ex vivo animal tissue.
Chapter 7 concludes the document with a summary of the presented work.

Potential future directions for research and development of the fLCI technology are also discussed.
2. Background

2.1 Introduction

Fourier domain low coherence interferometry (fLCI) is an optical technology that seeks to distinguish between normal and dysplastic epithelial tissues. In order to more completely understand the objective of the fLCI technique, it is important to have an understanding of the pathology of the disease it seeks to detect. Section 2.2 presents a brief background of epithelial cancer, or carcinoma, and describes the progression through which the disease develops.

A review of optical detection of cancer technologies follows. Several developing techniques are described and pre-clinical and clinical research highlights are noted. Of special interest are two techniques, Light Scattering Spectroscopy and Fourier Domain Optical Coherence Tomography, from which fLCI evolved.

The chapter concludes with the background of the fLCI technique. The theoretical foundations of the technique are described and the original fLCI system is presented. Results of the proof of principle experiment are presented demonstrating the technique’s capabilities in sizing scattering particles from a subsurface layer of an experimental phantom.
2.2 Carcinomas: cancers of the epithelium

Carcinomas are malignant neoplasms that develop in the epithelial tissues of the body. Epithelial tissues are quite varied and perform a number of different functions. These tissues make up many of the body’s surfaces and cavities including the oral mucosa, the lining of the gastrointestinal tract, and ducts within the many glands of the endocrine and exocrine systems among others. Although the different epithelial tissue types share many characteristics, the specific cellular architecture of these body tissues can vary from type to type.

Carcinomas are typically further qualified by the type of epithelial tissue in which they originate. Adenocarcinomas, for example, originate in glandular tissue. Adenocarcinomas are commonly found in the oral mucosa, esophagus, colon, and other organs. Another example, squamous cell carcinoma, is a malignant neoplasm of the squamous epithelium. These carcinomas commonly originate in the oral mucosa, esophagus, and skin. It is not always possible to categorize a carcinoma, however. In such cases, epithelial lesions consisting of undifferentiated cells are simply identified as ‘undifferentiated malignancies.’

With such a vast network of epithelial tissues in the body, it is not surprising that the majority of malignancies originate in the epithelial lining. And quite significantly, most of these carcinomas develop through a pre-malignant state. Incipient epithelial neoplasia tends to follow a sequence of development from hyperplasia to dysplasia to
invasive carcinoma. Although it is convenient to name and discuss discrete stages of development, it is important to remember that, at the morphological level, the progression of a lesion is a continuum.25

Dysplasia is a stage of this continuum characterized by architectural and cytologic abnormalities of varying degrees. Abnormalities of the tissue and cell architecture include exaggerated surface villiform configurations and glandular tissues exhibiting irregular shapes and crowding. Cytologically, cells exhibit several abnormalities including nuclear enlargement, hyperchromasia, decreased mucin production, and nucleoli irregularities.25 Because dysplasia is an important precancerous stage for early disease detection, it is typically further partitioned into low-grade and high-grade dysplasias. Low-grade dysplasia is characterized by several modest aberrations in cellular architecture and function. Cell nuclei enlarge, become hyperchromatic, and focally overlap. However, inflammation is minimal and the capacity for mucin production is maintained. High-grade dysplasia is indicated by major nuclear abnormalities, greatly decreased mucin production, nuclear stratification, and hyperchromatism.25

The overall goal of fLCI is to detect dysplasia at its earliest stages. If fLCI can alert a physician to the presence of a developing carcinoma at its earliest stages, the odds of successful treatment for a patient are substantially improved. In theory, fLCI can target any epithelial tissue that can be reached with an optical instrument. While
long range goals will target the esophagus and colon with fiber-optic probes, the focus of the \textit{ex vivo} tissue study in this dissertation is the oral mucosa as described fully in Chapter 6.

The danger of carcinoma, and of cancer as a whole, is quite clear. The National Cancer Institute (NCI) reports that the lifetime risk of being diagnosed with cancer is now over 40 percent. With the NCI predicting that there will be nearly 1.5 million new cases of cancer in this year alone\textsuperscript{2}, it is clear that current methods of cancer detection must continue to evolve and improve. A great deal of current research seeks to develop optical methods for detecting incipient malignancies in their precancerous states. As stated in the previous chapter, optical techniques are particularly well suited to overcome several of the shortcomings of today’s standard detection methods. Several burgeoning optical detection techniques are examined below.

\textbf{2.3 Optical detection techniques}

\textbf{2.3.1 Fluorescence spectroscopy}

Fluorescence spectroscopy seeks to measure variations in the intensity of emitted fluorescence as a function of wavelength. Fluorescence in epithelial tissue can originate from exogenous stains delivered to the target tissue or from endogenous fluorophores in a process known as autofluorescence. The target tissue is typically illuminated by ultraviolet or short wavelength visible light. In the case of autofluorescence, several
Biomolecules act as fluorophores emitting light of a wavelength longer than that of the illumination light. These biomolecules are found in varying concentrations throughout the tissue layers. The connective tissue of the submucosa typically dominates the fluorescence signal due to the highly fluorescent properties of collagen and elastin.26 However, fluorophore activity can vary depending on the excitation wavelength.

Autofluorescence is sensitive to biochemical and morphological changes in tissue caused by dysplastic development. Fluorescence spectroscopy systems often deliver excitation light to target tissues through a central fiber surrounded by collection fibers.27 The fluorescence signal is detected by a spectrometer and statistically analyzed by linear discrimination analysis or principle component analysis for significant differences between the fluorescence spectra of normal and abnormal tissue.26

Several research studies have assessed the ability of fluorescence spectroscopy to detect dysplasia in epithelial tissue. Among the first, Panjehpour, et al. used endoscopic laser induced fluorescence (LIF) spectroscopy to differentiate normal and malignant esophageal tissue with 100% sensitivity and 98% specificity.28 The LIF spectroscopy technique was less successful, however, in distinguishing different levels of dysplasia.29 Gillenwater, et al., used fluorescence spectroscopy to differentiate between normal and dysplastic oral mucosa in 23 patients with specificity of 100% and sensitivities ranging from 76.5 to 88.2% depending on the diagnostic algorithm used.30 More recently, Pfefer, et al., used temporally and spectrally resolved fluorescence spectroscopy to detect high-
grade dysplasia in the esophagus. Results of the study showed only moderate levels of sensitivity and specificity.\textsuperscript{31}

2.3.2 Raman spectroscopy

Raman scattering is an inelastic light scattering event in which the energy of the scattered photon is altered by changes in the vibrational and rotational energy of the scatterer’s molecular bonds. The “Raman effect” describes the shift in wavelength that inelastically scattered photons undergo. Because the molecular bonds of biomolecules contain unique vibrational and rotational energies, each molecule has a unique Raman scattering signature. Careful analysis of Raman spectra can reveal the relative composition of specific biomolecules within a tissue. Because dysplastic tissues change biochemically before changing morphologically, Raman spectroscopy holds the potential for detecting precancers at their earliest stages. Raman spectra can reveal the molecularly specific “spectral fingerprint” of the abnormal tissue. However, Raman scattering signals are extremely weak relative to autofluorescence and elastic scattering signals. Only about one in $10^7$ incident photons are inelastically scattered, making the clinical application of Raman spectroscopy challenging.\textsuperscript{26}

Studies assessing the ability of Raman spectroscopy to distinguish normal and abnormal epithelial tissue have been completed both \textit{ex vivo} and \textit{in vivo}. Kendall \textit{et al.} evaluated the use of Raman spectroscopy for the identification of Barrett’s neoplasia in the \textit{ex vivo} esophagus epithelium. Results showed sensitivities ranging from 72% for
fundic-type mucosa to 100% for cardiac-type mucosa and specificities between 90% for adenocarcinoma and 100% for cardiac-type mucosa. Shim et al. reported the first clinical endoscopic Raman spectroscopy trial, though no diagnostically significant results were reported. Raman spectroscopy has also been used to detect oral squamous cell carcinoma. Krishna, et al., reported very significant differences between normal and malignant samples of formalin fixed, ex vivo oral mucosa, though sensitivity and specificity figures were not given.

2.3.3 Light scattering spectroscopy

Light scattering spectroscopy (LSS) is a broadband spectroscopy technique that seeks to determine the density and size distribution of scatterers from elastically scattered light. Because LSS targets elastic scattering, detected photons do not experience a wavelength shift. LSS treats epithelial cell nuclei as Mie scatterers whose scattering cross sections exhibit periodicity with wavelength. The spectral periodicity is proportional to the diameter of the scatterers while the spectral amplitude indicates their density. Nuclear enlargement is one of the first morphological changes that dysplastic cells undergo. LSS targets this biomarker and distinguishes between normal and abnormal epithelial tissue based on measured nuclear diameter distributions.

LSS was first demonstrated by Perelman et al. in both in vitro epithelial cells and ex vivo human esophagus tissue. Both experiments showed clear distinction between the normal and dysplastic nuclear size distributions. Backman et al. used LSS to diagnose
dysplasia and carcinoma in situ in the columnar epithelia of the colon and Barrett’s esophagus, transitional epithelium of the urinary bladder, and stratified squamous epithelium of the oral cavity. The study noted a marked distinction between dysplastic and non-dysplastic epithelium. Wallace et al. used LSS to endoscopically detect dysplasia in 13 patients with Barrett’s esophagus. LSS measurements indicated a dysplastic diagnosis if more than 30% of the scanned cell nuclei exceeded 10 μm in diameter. The study reported 90% sensitivity and specificity, correctly classifying all high-grade dysplasia samples and 87% of low-grade dysplasia samples. A technique combining LSS with fluorescence and reflectance spectroscopy for evaluating patient’s with Barrett’s esophagus was introduced by Georgakoudi et al. The study reported that the combination of techniques improved the sensitivity and specificity for separating high-grade dysplasia from non-high-grade dysplasia and normal tissue.

More recently, Johnson et al. used ESS with a combination of principal component analysis and linear discriminant analysis to determine ex vivo sentinel lymph node status in the breast. The study presented an average sensitivity of 75% and specificity of 89% in test sets that included normal nodes and nodes with partial as well as complete replacement by cancer. ESS was used by Lovat et al. to detect high grade dysplasia and cancer in Barrett’s esophagus. Using leave-one-out and block validation statistical approaches, the study detected high risk sites with 92% sensitivity and 60%
specificity, and differentiated high risk sites from inflammation with a sensitivity and specificity of 79%.

Additionally, Kim et al. have developed depth-resolved low-coherence enhanced backscattering (LEBS) spectroscopy, a modified LSS technique which utilizes self-interference effects in elastically scattered light to detect enhanced scattering intensity in the backward direction.\textsuperscript{39} The technique measures changes in spectral slope to detect preneoplastic transformation in epithelial tissue associated with the field effect.\textsuperscript{40} LEBS was used to accurately risk stratify for colon carcinogenesis in two animal models by measuring microarchitectural changes in colonic epithelium that preceded conventional biomarkers of colon carcinogenesis.\textsuperscript{41} The technique was also used to predict the presence of pancreatic cancer by optically analyzing normal \textit{ex vivo} human periampullary duodenal mucosa.\textsuperscript{42} The study distinguished between control and pancreatic cancer patients with 95% sensitivity and 91% specificity without the need for interrogation of the pancreatic duct. Although LEBS has shown empirical success, the biological phenomena that induce changes in spectral slope measurements are still unknown.

2.3.4 Optical coherence tomography

Optical coherence tomography (OCT) is a biomedical imaging technique based on low coherence interferometry. Light from a low coherence source is split into sample and reference beams which are incident upon an experimental sample and a reference
mirror respectively. The reflected beams are recombined and the interference signal is detected. Initial OCT experiments were completed in the time domain using a photodiode for detection. Because of the low temporal coherence of the light source, interference signals are detected only when the sample and reference path lengths are matched within the coherence length of the source. Depth gating is achieved by scanning the reference mirror such that all depths within the sample are interrogated in sequence. It was soon found that Fourier domain OCT (FDOCT) offered a sensitivity advantage over time domain OCT. In the Fourier domain, an OCT signal is detected as a function of wavelength before being converted to a function of wavenumber. The detected spectrum will contain interference oscillations with frequencies that can each be related to specific depths by a Fourier transform. FDOCT captures information from all depths within a sample without the need for scanning a reference mirror.

With the relatively small penetration depth of only a few millimeters in tissue, the number of imaging applications available for OCT is limited. The modality has found the most success imaging the in vivo human eye. OCT has also shown promise for imaging epithelial tissue of the gastrointestinal tract. Izatt et al. first reported OCT imaging of the gastrointestinal epithelium in the porcine esophagus and human colon with 21 μm depth resolution. Endoscopic OCT imaging of the human gastrointestinal tract was reported by Sivak et al. The novel OCT probe provided a 360 degree radial scan while imaging at 6.7 frames per second with 10 μm depth resolution. Pfau et al.
used a similar system to investigate the diagnosis of dysplasia in the human colon using OCT. The study reported success differentiating adenomas, hyperplastic polyps, and normal tissue, though depth resolution was a limiting factor. Most recently, Isenberg et al. investigated the use of endoscopic OCT (EOCT) for detecting dysplasia in patients with Barrett’s esophagus. The prospective, double blinded study compared the interpretations of endoscopists viewing EOCT images to the histological analysis of pathologists. The study reported 68% sensitivity, 82% specificity, and 78% diagnostic accuracy, while citing increased resolution as a necessity for clinical implementation.

OCT has also been used to image malignancies in the oral mucosa. Clark, et al., compared optical coherence microscopy (OCM) and confocal microscopy images of ex vivo oral neoplasia, reporting an increase in imaging depth with OCM. Tsai, et al., also used OCT to distinguish between normal and dysplastic in vivo oral mucosa by examining the standard deviation of signal profile, the exponential decay constant of the spatial-frequency spectrum, and epithelium thickness.

2.4 Fourier domain low coherence interferometry

Fourier domain low coherence interferometry is an optical technique that combines the sensitivity of LSS with the depth resolution of FDOCT. Like LSS, fLCI seeks to recover structural information about scatterers by examining the wavelength dependence of the intensity of elastically scattered light. However, where LSS compares
light scattering signals to Mie theory to determine the size distribution of scatterers in a probed sample, fLCI determines scatterer sizes by Fourier transforming detected spectra and analyzing the peaks of the resulting correlation plot. Unlike LSS, fLCI seeks to gain depth resolution by employing the coherence gating methods of FDOCT. By exploiting the low temporal coherence length of a broadband light source, fLCI can selectively probe subsurface sample layers for structural information.

Figure 2.1: Schematic of the original fLCI system. L, lenses. BS, beamsplitter. M, reference mirror. S, sample.
The original fLCI system is shown in Figure 2.1. fLCI uses broadband light from a white light source in a Michelson interferometer geometry. Light from the source is coupled into a fiber and recollimated before being incident on a beamsplitter (BS) which splits the light into a reference arm, incident on a mirror, and a sample arm, incident on the experimental sample. Light scattered by the sample is mixed with the reference field at BS and the combined field is focused into a fiber and detected with a spectrometer.

FLCI generates depth resolution by low coherence interferometry in the frequency domain as in FDOCT. The equation for the intensity of the signal detected by the spectrometer is:

\[ I = E^2_S + E^2_R + 2 \cdot \text{Re}[E_S E_R^*] \cos(2 \cdot \Delta z \cdot k + \phi). \]  

(2.1)

It should be noted that the interference information, contained in the third term of Eq. 2.1, is modulated by a cosine which oscillates as a function of \( k \), the wavenumber. As a result, the interference spectrum detected by the fLCI system will feature oscillations with frequencies dependent on \( \Delta z \), or the optical path length difference between the reference and sample arms of the system. The axial spatial cross correlation function between the sample and reference fields can be obtained by Fourier transforming the interference term. The resulting autocorrelation function takes the form of a depth scan with peaks corresponding to the locations of individual reflectors in the experimental sample. FLCI utilizes this method to selectively analyze specific subsurface layers in probed samples.
In order to perform depth resolved spectroscopy, fLCI requires more than simply depth resolution. Data must be processed to simultaneously obtain depth resolution and spectral resolution. In order to generate depth resolved spectroscopic information from data acquired in a single domain, fLCI typically employs a short-time Fourier transform. A Gaussian window is applied to the interference term before Fourier transforming to produce a depth scan centered about a particular center wavenumber. By shifting of the center of the Gaussian window and repeating the process, a data set with both depth and spectral resolution can be generated. It should be noted, however, that with this approach any increase in depth resolution results in the degradation of spectral resolution and vice versa. The windowing procedure and associated resolution trade-off will be analyzed in depth in Chapter 3 of the dissertation.

From the depth resolved spectroscopic information, fLCI seeks to determine scatterer diameter by analyzing wavenumber dependent oscillations at specific depths of interest. More specifically, fLCI seeks to distinguish between normal and dysplastic epithelial tissue by detecting the nuclear enlargement that occurs at the earliest stages of precancerous development. Figure 2.2 (a) shows an illustration representing two nuclei as well as the scattering events that take place at both the front and back surface of each nucleus where an index of refraction change is present. The reflections from the front and back surfaces of the nuclei will interfere with one another, producing constructive or destructive interference, depending on the wavelength of the incident light. If the
Figure 2.2: (a) Cell nuclei with incident and scattered fields indicated. (b) Interference spectra with wavenumber dependent oscillations caused by interference between front and back surface reflections.

The frequency of this oscillation is directly dependent on the diameter of the scatterer with larger particles resulting in a higher frequency of oscillation and smaller particles resulting in a lower frequency of oscillation. FLCI seeks to detect these wavenumber dependent oscillations...
in diagnostically relevant tissue layers in order to measure nuclear enlargement associated with a developing precancer.

Wax et al. first demonstrated the ability of fLCI to obtain depth-resolved spectra for determining scatterer sizes from elastic light scattering.\textsuperscript{52} They reported the ability to isolate the scattering from polystyrene microspheres dried on the back surface of a coverglass. Analysis of the spectral periodicity induced by the NIST certified 1.55 μm diameter microspheres resulted in a measured mean diameter of 1.65 μm ± 0.33 μm. The results of the proof of principle experiment showed the ability of fLCI to size particles with subwavelength accuracy, actually surpassing the imaging resolution of the white light source.

2.5 Summary

Chapter 2 has presented the background information necessary to understand the foundations of the fLCI technique and the technology’s relationship to other optical detection of cancer methods. First, the pathology of carcinoma, the disease targeted by fLCI, was detailed and the precancerous stages through which it develops were discussed.

Additionally, Chapter 2 presented several optical detection of cancer techniques including fluorescence spectroscopy, Raman spectroscopy, light scattering spectroscopy, and optical coherence tomography. The background of each technology was described
and relevant *ex vivo* and *in vivo* tissue studies were noted. The light scattering
spectroscopy and optical coherence tomography techniques were of particular interest
because the fLCI technology utilizes aspects from each of these techniques.

Finally, the theoretical basis of fLCI was detailed and the results of an initial
proof of principle experiment, which measured the diameter of polystyrene
microspheres, were presented. The theoretical ideas discussed in Section 2.4 serve as a
foundation for all subsequent fLCI research. Chapter 3 builds upon these basic
theoretical ideas, detailing a series of extensive studies analyzing the role of temporal
and spatial coherence in depth resolved spectroscopy and white light interferometry.
3. Theory

3.1 Introduction

The development of fLCI technology necessitated an in-depth analysis of the temporal and spatial coherence theory underlying the technique. The analysis developed from the signal processing literature that examines time-frequency distributions (TFDs). These distributions are of particular interest because the depth-resolved spectroscopic data obtained in fLCI is an adapted form of a TFD with depth analogous to time and wavenumber analogous to frequency.

In Sections 3.2 and 3.3, the general relationship between coherence gated signals and bilinear TFDs is examined. Traditional OCT signals in the time domain and frequency domain are considered using the framework of the TFD. Spectroscopic OCT signals are also examined in this framework with particular attention paid to the choice of analysis window and the type of TFD which is generated. The utility of the TFD is shown by processing of numerically simulated data. The Wigner TFD from Cohen’s class of functions\(^{53}\) is used to show that temporal coherence information contained in the Wigner TFD cross-terms can be utilized to gain structural knowledge of samples via SOCT signals.\(^{54}\)

In order to generate depth resolved spectroscopic information from data collected in a single domain, SOCT typically employs a short time Fourier transform (STFT) or a continuous wavelet transform (CWT). However, TFDs generated by the
STFT are limited by the relationship between time and frequency which results in an inherent tradeoff between time (depth) resolution and frequency (wavelength) resolution. In Section 3.4, we present a dual window (DW) method for reconstructing time frequency distributions (TFDs) that applies two orthogonal Gaussian windows that independently determine the spectral and temporal resolution. The effectiveness of the method is demonstrated in simulations of OCT signals that contain fields which vary in time and frequency. The DW method yields TFDs that maintain high spectral and temporal resolution and are free from the artifacts and limitations commonly observed with other processing methods.

Finally, in Section 3.5 the Wigner TFD is used to examine the role of spatial coherence in white light interferometry. The summation of signals in time domain and frequency domain OCT is compared with analysis showing that the two modalities combine signals in a different manner.

3.2 Time Frequency Distributions

The general form of an interferogram can be written as:

$$I_T = |E_R + E_S|^2 = |E_R|^2 + |E_S|^2 + 2 \text{Re}(E_R E_S^* \cos \phi)$$  (3.1)

where $I_T$ is the total detected intensity and $E_R$, $E_S$ are the amplitudes of the reference and sample fields respectively. In general, the sample and reference fields are complex and the relative phase between the two, $\phi$, can be used to isolate the interferometric signal.
Measurements of the total intensity given in Eq. 3.1 may be executed in either the time
or frequency domain. In the time domain, $\phi$ is made to vary linearly in time, allowing
one to create a heterodyne signal which is linear with the sample field. In the frequency
domain, the frequency dependence of $\phi$ is measured and Fourier transformed to yield
the temporal profile of the sample field.

In order to obtain information about the interferogram signal in both the time
and frequency domains simultaneously, the signal must be processed using one of two
main approaches. Linear operations can be applied to the signal to yield a linear TFD or
higher order functions can be calculated such as the bilinear TFDs which comprise the
Cohen class of functions.\textsuperscript{53} In this section we will lay out the basic formalism of each
approach and then examine the relationship between the two.

3.2.1 Linear representation

In order to generate a TFD from data acquired in a single domain, a linear
operation such as the STFT can be applied. Here a window is generated mathematically
with a finite width and center and applied to the acquired signal. In the case of a time
domain signal, $I_T$, a temporal window $W$ of width $T$ and center $t_o$ can be applied,

$$
I_T(t, t_o) = \int I_T(t)W(t, t_o)e^{i\omega t} dt
$$

(3.2)

to yield the spectrum of light associated with the signal at time $t_o$. By executing this
operation at several values of $t_o$ successively, the TFD of the signal is obtained. The one
potential drawback of this approach is that there exists an uncertainty relationship
between the resolution in the frequency domain and that in the time domain such that improved knowledge of the frequency $k$ comes with reduced knowledge of the temporal distribution. Alternately, for data acquired in the frequency domain, a spectral window can be applied to obtain the TFD with a similar tradeoff between time and frequency resolutions.

3.2.2 Bilinear representation

In bilinear signal representations, higher order correlations are analyzed to obtain the joint TFD. As an example, one bilinear representation is the Wigner distribution, which is defined as

$$W(k,t)=\int\langle E(k+q/2)E^*(k-q/2)\rangle e^{-iqt} dq$$  \hspace{1cm} (3.3)

for an electrical field $E$ and where $\langle \cdots \rangle$ denotes a statistical average and $k$ is the wavevector which is related to the frequency as $k = \omega / c$. For a multicomponent signal, such as that found in an interferogram, the Wigner distribution of the total electric field, $E_T = E_R + E_S$, can be written as

$$W_T(k,t) = \int\langle E_T(k+q/2)E_T^*(k-q/2)\rangle e^{-iqt} dq = W_R + W_S + W_{cross}$$  \hspace{1cm} (3.4)

where $W_R$ and $W_S$ denote the individual Wigner distributions for the reference and sample fields respectively and $W_{cross}$ gives the Wigner function for the cross terms.

Although the cross terms are often regarded as undesired artifacts, they contain useful
information about the temporal coherence of the sample field which is useful for coherence gated measurements as we shall explore below.

### 3.2.3 Relationship between linear and bilinear representation

To illustrate the connection between linear and bilinear signal representations, let us consider a frequency domain OCT signal:

\[ I(k) = |E_I|^2 = |E_R|^2 + |E_S|^2 + 2 \text{Re}(E_R E_S \cos(\phi(k))) \]  

(3.5)

Upon elimination of the sample and reference intensities, we are left with the interferometric term:

\[ I_{\text{int}}(k) = E_S^* E_R^* + \text{c.c.} = 2 \text{Re}(E_R E_S \cos(\phi(k))) \]  

(3.6)

As mentioned above, this term is usually processed to give an autocorrelation function:

\[ \Gamma(z) = \int I_{\text{int}}(k) \exp(ikz) dk \]  

(3.7)

which gives the temporal profile of the signal (alternately given here in terms of path length \( z = c \cdot t \), where \( c \) is the speed of light) but with ambiguity about \( t = 0 \) \( (z = 0) \).

Instead of the linear representation of the signal shown above, let’s consider

\[ |\tilde{I}_{\text{int}}(k)|^2, \text{ where } \tilde{\cdot} \text{ is the Fourier transform:} \]

\[ |\tilde{I}_{\text{int}}(k)|^2 = |\Gamma(z)|^2 = \int E_R(k) E_S^*(k) \exp(ikz) dk \int E_R^*(k') E_S(k') \exp(-ik'z) dk' \]  

(3.8)

This can be cast in the form of the bilinear Wigner distribution, through a simple coordinate transform, with:
where the Jacobian of the transform is unity. This substitution yields:

\[
\begin{align*}
\kappa &= \frac{k + k'}{2}, \quad q = k - k', \quad k = \bar{k} + \frac{q}{2}, \quad k' = \bar{k} - \frac{q}{2}
\end{align*}
\]

(3.9)

\[
\int d\kappa dq E_R^\ast(\bar{k} + \frac{q}{2})E_R(\bar{k} - \frac{q}{2})E_S^\ast(\bar{k} + \frac{q}{2})E_S(\bar{k} - \frac{q}{2})\exp(iqz)
\]

(3.10)

Now, according to Eq. 3.3, the Wigner distribution of the sample field is:

\[
W_S(k, z) = \frac{1}{2\pi} \int E_S(\bar{k} + \frac{q}{2})E_S^\ast(\bar{k} - \frac{q}{2})\exp(iqz)dq
\]

(3.11)

which can be inverted to yield the ambiguity function:

\[
2\pi \int W_S(k, z)\exp(-iqz)dz = E_S(\bar{k} + \frac{q}{2})E_S^\ast(\bar{k} - \frac{q}{2})
\]

(3.12)

This form can be inserted into Eq. 3.10 to yield:

\[
|\Gamma(z)|^2 = \int d\kappa dz' W_S(\bar{k}, z')d\kappa dq E_R(\bar{k} + \frac{q}{2})E_R^\ast(\bar{k} - \frac{q}{2})[2\pi \int W_S(\bar{k}, z)\exp(iqz')dz']\exp(iqz)
\]

\[
= 2\pi \int d\kappa dz' W_S(\bar{k}, z')\int dq E_R(\bar{k} + \frac{q}{2})E_R^\ast(\bar{k} - \frac{q}{2})\exp(iq(z + z'))
\]

\[
= (2\pi)^2 \int W_S(\bar{k}, z')W_R^\ast(\bar{k}, z' + z)d\kappa dz' \quad \text{54}
\]

(3.13)

Thus, the bilinear representation of the interferogram signal is given by the overlap of the Wigner Distribution of the sample field with that of reference field.
3.2.4 Bilinear representation of a SOCT signal

The TFD of a SOCT signal can be generated in a similar manner as the bilinear representation given above. Consider a frequency domain SOCT signal which is processed with a window \( W \) to enable joint knowledge of the TFD

\[
\Gamma(z) = \int E_R(k)\varpi(k, k_w)E_S^*(k)\exp(ikz)dk.
\] (3.14)

Here the window can take the form of a Gaussian distribution such as

\[
\varpi(k, k_w) = A \exp\left(-\frac{(k - k_w)^2}{2\Delta k_w^2}\right).
\] (3.15)

For the case that the width of the window is significantly smaller than the bandwidth of the source \( (\Delta k_w << \Delta k) \), we can approximate this signal as

\[
\Gamma(z) \approx \int E_R(k_w)\varpi(k, k_w)E_S^*(k)\exp(ikz)dk.
\] (3.16)

This is a reasonable approximation in most practical cases, as the aim of processing the SOCT signal is to obtain frequency resolution.

The squared magnitude of the signal can again be cast in terms of the Wigner distribution by using a transform similar to that given above in Eq’s 3.8-3.13. The processed signal

\[
S(k_w, z) = |\Gamma(z, k_w)|^2
\]

\[
= |E_R(k_w)|^2 \int \varpi(k, k_w)E_S^*(k)\exp(ikz)dk\int \varpi^*(k', k_w)E_S(k')\exp(-ik'z)dk'
\] (3.17)

upon coordinate transformation becomes
\[ S(k_w, z) = 2\pi |E_R(k_w)|^2 \int W_S(k, z') W_{\tilde{w}}(k - k_w, z + z') d\bar{k} dz' \]  

(3.18)

In Eq. 3.18, the SOCT signal has been processed to yield the temporal (depth) profile of the sample field at the specific frequency (wavelength) given by the center of the window \( k_w \). By systematically varying the center frequency of the window, the TFD of the SOCT signal is generated. The resultant signal is given as the overlap of the Wigner distribution of the sample field with the effective Wigner distribution of the window function.

### 3.3 Temporal coherence and time frequency distributions

The utility of the TFD can be seen by examining a few simple cases which demonstrate its properties. Analysis of the distributions for time and frequency domain OCT signals shows that the knowledge of temporal coherence obtained with a TFD can clearly illustrate the advantage of frequency domain OCT measurements versus data acquired in the time domain. In this section, we analyze the TFDs for OCT signals and relate the results to the signal acquired in each domain. This line of analysis is then extended to examine SOCT signals to demonstrate that knowledge obtained from TFD analysis reveals temporal coherence properties which can improve measurements of the structure of a sample.
3.3.1 TFD of a two-component field

Let us consider a total optical field at frequency $\omega_0$ composed of two components separated by a time delay $T$, as one might find for a Michelson interferometer with the arms possessing a pathlength mismatch, such that $E_T = E_S + E_R$ where each component is a Gaussian pulse of width $a$ and amplitude $E_0$:

$$E_S = E_0 \exp\left(-\frac{t^2}{a^2}\right) \exp(i\omega_0 t) \quad E_R = E_0 \exp\left(-\frac{(t-T)^2}{a^2}\right) \exp(i\omega_0 t) \quad (3.19)$$

The TFD of this total field can be determined using the Wigner distribution,

$$W(t, \omega) = \frac{1}{2\pi} \int E_T^* \left(t - \frac{\tau}{2}\right) E_T \left(t + \frac{\tau}{2}\right) \exp(i\omega\tau) d\tau \quad (3.20)$$

to yield:

$$W(t, \omega) = \frac{E_0^2 a}{\sqrt{2\pi}} \exp\left(-\frac{-2t^2}{a^2} - \frac{a^2(\omega_0 + \omega)^2}{2}\right)$$

$$+ \frac{E_0^2 a}{\sqrt{2\pi}} \exp\left(-\frac{-2(t-T)^2}{a^2} - \frac{a^2(\omega_0 + \omega)^2}{2}\right)$$

$$+ \frac{E_0^2 a}{\sqrt{2\pi}} \cos(T(\omega_0 + \omega))$$

$$\times \exp\left(-\frac{-2(t-T)^2}{a^2} - \frac{a^2(\omega_0 + \omega)^2}{2}\right) \quad (3.21)$$
We can see from Eq. 3.21 that the Wigner distribution develops into the sum of three terms as presented above in Eq. 3.4. The first two terms are Gaussian terms that we will call \( W_R \) and \( W_S \). \( W_R \) is delayed by \( T \) with respect to \( W_S \). The third term is a cross-term \( (W_{\text{CROSS}}) \) which has a Gaussian envelope and a sinusoidal oscillation. Figure 3.1 shows how the cross-term emerges as the time delay \( T \) increases from zero. This term is distinct only when \( T \neq 0 \) and is localized between \( W_R \) and \( W_S \) with respect to the time axis with all three terms centered about the frequency \( \omega_0 \).

### 3.3.2 Time-domain OCT signals

Let us now consider axially scanning the reference mirror of the Michelson interferometer, as in a time-domain OCT system, through all time delays \( \tau \) corresponding to a full depth scan. Figure 3.2 shows the signal for six selected time delays as a distribution in time and frequency, represented by the Wigner distribution.
Figure 3.2: Wigner distributions for time delay $T$ and a center frequency of $\omega_0=12.5$.

The cross term is seen to oscillate with a frequency characteristic of the time delay between pulses. At each time delay the signal is detected using a photodiode, effectively integrating the TFD across frequency. This reduces the distribution to the time marginal defined as the intensity per unit time,

$$\int W(t, \omega) d\omega = |s(t)|^2. \quad (3.22)$$
Figure 3.3 (a) presents the time marginals corresponding to the delays shown in Figure 3.2. As T moves closer to zero, the path length mismatch of the sample and reference arms also gets smaller. The cross-term appears between the two Gaussian peaks. As T crosses the zero point the path length mismatch grows once again.

The time-domain OCT signal is recorded as intensity as a function of the path-length difference. For each path length difference, the signal detected is the time average of the time marginal. In terms of the Wigner distribution, each point of an OCT depth scan is simply the two dimensional integral of the distribution for the corresponding value of T. The plots in Figure 3.3 (a) show the time marginals for six different depths of an OCT scan. Integrating each plot with respect to time will leave us with six discrete points which help make up the interferogram seen in Figure 3.3 (b).

![Figure 3.3](image)

**Figure 3.3:** (a) Time marginals for the Wigner distributions of Figure 3.2. (b) Interferogram from the distributions in Figure 3.2 and (a).
3.3.3 Frequency-domain OCT signals

Figure 3.4 shows another example of a Wigner distribution for an optical signal from the Michelson geometry with mismatched path lengths \( T = 5 \), as well as the corresponding time and frequency marginals. When the distribution is integrated with respect to frequency in order to obtain the time marginal, the sinusoidal cross-term integrates to zero. If we instead integrate the distribution with respect to time, we generate the frequency marginal given as the intensity per unit frequency,

\[
\int W(t, \omega) dt = |S(\omega)|^2.
\]  

(3.23)

Fourier domain OCT systems\(^{44, 45, 55}\) seek to measure the frequency marginal. However, most detection schemes recover the wavelength distribution of the signal where the wavelength is inversely proportional to the frequency. In such arrangements, the signal can be interpolated and rescaled to obtain the frequency distribution.

In its most simple form, Fourier domain OCT uses a Michelson interferometer geometry like that of time domain OCT. However, in Fourier domain OCT the reference mirror is not scanned to selectively probe depths of the sample. Instead, the detected intensity is dispersed with respect to wavelength by using a diffraction grating. The recorded intensity is a frequency spectrum. Figure 3.4 (c) shows the \( |S(\omega)|^2 \) marginal of the Wigner distribution, corresponding to a typical Fourier domain OCT scan. This scan is a frequency spectrum which exhibits an oscillation due to the cross term. Unlike time domain OCT, in Fourier domain OCT it is not necessary to integrate the frequency
marginal, although some averaging occurs due to the resolution limit imposed by the finite pixel size of any practical detector. The frequency dependent oscillation of the spectrum can be Fourier transformed to yield the time delay between the two components, effectively producing a depth scan with a single measurement.

### 3.3.4 TFD for a SOCT signal

In order to analyze the TFD for a SOCT signal, it is more useful to use an example in which the sample field induces a spectral modulation. Let us consider a sample field consisting of two components, \( E_s = E_1 + E_2 \). The corresponding Wigner distribution can be found using Eq. 3.4 to contain three components, one for each of the two field components and a cross term. As shown above, the cross term will produce an oscillation with a frequency characteristic of the delay between the two components.
The sample field given above can be generated by probing a sample containing two reflectors. However, if these two reflectors are spaced too closely, such that the delay between the components is less than the pulse duration \((a)\) in Eq. 3.19, the individual components cannot be resolved using conventional OCT, regardless of frequency or time domain signal acquisition. On the other hand, by reconstructing the TFD of the sample field from the detected signal, knowledge of the induced temporal coherence of the field due to the sample can be obtained. This knowledge can enable one to determine the structure of the sample on finer scales than is possible with the usual resolution associated with the pulse duration.

Figure 3.5 shows the Wigner distribution of the two component sample field given above. To reiterate, with either frequency or time domain OCT, knowledge of the sample field is not obtained directly but rather the detected signal is the convolution of the signal field with a reference field, as shown in Eq. 3.13. In order to determine the delay between the two components of the sample field, we can analyze its TFD to obtain knowledge of the induced temporal coherence of the field due to the sample.

To construct the TFD for this sample field, the signal is processed using a window, as in the STFT. For a Gaussian window, as given in Eq. 3.15, there exists a tradeoff between frequency and time resolution. Figure 3.5 illustrates this tradeoff by showing windows based on two possible frequency widths. For the window with a narrow frequency width (green), high frequency resolution is obtained but at the cost of
poorer time (depth) resolution. On the other hand, a window with a wider frequency width (red) can be used which preserves higher time (depth) resolution but yields poorer frequency resolution.

Processing via the use of the window produces another form of TFD. In the linear representation, the windowed signal gives the spectrogram of the signal. In the bilinear representation, the TFD of the sample field is smoothed by that of the window function, as given by Eq. 3.18. Here, the Choi Williams distribution for the sample field is obtained. Figure 3.6 illustrates the TFD resulting from the use of each of the two
Figure 3.6. Comparison of processing of SOCT signals using (a) broad and (b) narrow spectral windows. Using a broad spectral window maintains high temporal resolution but cannot resolve two closely spaced peaks. (c) In comparison, using a narrow spectral window does not permit the peaks to be resolved but achieves spectral resolution (d) that shows a modulation of the spectral profile (blue/solid) compared with the original spectrum (green/dashed).

Gaussian windows described above. Here we see that upon using a broad spectral window with good temporal resolution (Figure 3.6(a) and (c)) the two peaks are just barely resolved. When using a narrow spectral window, the temporal resolution is
degraded so that the two components of the sample field are not resolved (Figure 3.6(b)). However, the temporal coherence which is induced due to the structure of the sample causes a spectral modulation (Figure 3.6(d)). This modulation can be made more apparent by dividing the processed spectral profile by the source spectrum (Figure 3.7(a)). Fourier transforming this spectral modulation yields a correlation function (Figure 3.7(b)) which shows a sharp peak at the round trip distance between the two sample interfaces. The correlation distance of 2 in Figure 3.7(b) corresponds to two spectral oscillations (as seen in Figure 3.7(a)) over the range of 12 frequency units. Multiplying this ratio by 2π, we recover the approximately 1 spatial unit spacing seen in the original sample field distribution (Figure 3.5).

Figure 3.7 (a) Spectral modulation due to temporal coherence induced by sample. (b) Fourier transforming spectral modulation yields a correlation function with the peak indicating the spacing of the two peaks in the signal field.


3.3.5 Discussion

In the above analysis, the relationship between bilinear TFDs and OCT signals was examined. Conventional OCT measurements reveal one aspect of the TFD of the total field, comprised of the sum of the sample and reference fields. Time domain OCT signals are described by integrating the TFD for the total field over frequency to yield the time marginal. The time marginal is then further integrated over time to yield the interference at a particular delay (depth) of the reference field relative to the sample field, with the complete delay (depth) scan requiring multiple successive measurements. In contrast, Fourier (or frequency) domain OCT signals are obtained by integrating the total field TFD over time. Here no additional integration is needed but instead a Fourier transform is used to yield the delay (depth) scan. The increase in fidelity of Fourier domain OCT\textsuperscript{44, 45, 55} can be seen to originate from this difference in measurement. While these OCT measurements can be viewed as generated from the TFD, it is important to note that the true TFD of the total field may not be a physical quantity, making it inaccessible with a direct measurement such that it must be generated by other means.

Although the TFD is not directly measured in OCT, the measured OCT signal can be easily related to the individual TFDs of the sample and reference fields. In Fourier domain OCT, the detected signal is given by the overlap of the Wigner distributions for the sample and reference fields. Although not presented here, a similar derivation can be written for time domain OCT signals. In SOCT, the window function
takes the place of the reference field in the overlap integral. In this case, the detected signal takes the form of a different type of TFD. The overlap relations presented here are newly introduced to the field of OCT but have been known in other fields for years. For example, similar relations are used in quantum mechanics and quantum optics.\textsuperscript{57}

In the Cohen class of functions,\textsuperscript{53} an infinite number of distributions can be generated with the appropriate choice of kernel. As an example, the Wigner distribution has a kernel equal to unity. In describing OCT signals, the Wigner function for the sample field is convolved with the Wigner function of the reference field. In the typical case where the reference field is a Gaussian distribution in time and frequency, the detected signal is then given by the Choi Williams distribution,\textsuperscript{53} which can be viewed as a smoothed Wigner distribution. The concept of the TFD was only recently introduced to analysis of OCT signals,\textsuperscript{58} however, this introductory work did not give explicit forms for the TFDs discussed nor did it relate them to detected OCT signals which may explain its limited impact.

The smoothing property of the Choi Williams distribution results in a distribution that is positive definite, an essential feature for representing a detected signal. The Choi Williams distribution is often applied to quell the cross terms that arise in the TFD represented by the Wigner distribution. As such, its use can eliminate the cross terms in the TFD of an OCT signal which can reveal the existence of temporal coherence. For a measured signal given as a Choi Williams distribution, the cross terms
which describe temporal coherence generated due to the structure of a sample are often not readily identified in the detected signal.

Knowledge of this temporal coherence can be used to improve structural information obtained from OCT measurements. As shown above, processing the signal using a window function can generate a TFD for the detected signal. By judicious choice of the window parameters, features in the frequency spectrum can be uncovered which are characteristic of the temporal coherence induced in the field due to structures in the sample. This approach has been applied in fLCI to enable the size of scatterers to be determined with a precision and accuracy that exceeds that possible with conventional OCT images.52,56 More recently, this type of processing has been introduced to OCT imaging to improve knowledge of the sample scattering features.59–61

Although the TFDs presented here are instructive for understanding the role of temporal coherence in analysis of SOCT signals, they have not provided a means to skirt the tradeoff between time and frequency resolutions associated with application of a Gaussian window. However, this analysis has led to the development of a new method for processing SOCT signals with simultaneously high spectral and temporal resolution, described fully in Section 3.4.
3.4 Dual window processing method

As previously mentioned in Section 3.3, TFDs generated by using the STFT to analyze signals from a single domain are severely limited by the tradeoff between time (depth) resolution and frequency (spectral) resolution. Work in the fields of signal processing and quantum physics have paved the way for a new SOCT processing technique that ameliorates the detrimental effects of the time-frequency resolution tradeoff. Thomson, for example, developed a method particularly well suited for stationary Gaussian signals using orthogonal windows as a means for estimating weighted averages for spectral approximations to achieve high-resolution spectral information. Later, Bayram and Baraniuk expanded on Thomson’s method by implementing two Hermite-function-based windows to provide a robust analysis of the time-varying spectrum of non-stationary signals, which are pertinent to fields such as radar, sonar, acoustics, biology, and geophysics. More recently, Lee et al showed that using multiple windows simultaneously can avoid a similar resolution tradeoff in measurement of the position and momentum of a light field.

Here, the dual window (DW) method for processing SOCT signals is introduced. Unlike radar, sonar, etc., the spectral analysis of SOCT is inherently restricted to signals that are quasi-stationary, with a frequency content that does not vary sharply in time as a chirped signal would. Instead, the DW method is applied here to detecting modulation of OCT signals due to scattering or absorption; thus posing a well-conditioned problem.
for the DW method. The technique allows the reconstruction of the Wigner TFD of an
SOCT signal using two orthogonal windows which independently determine spectral
and temporal resolution, avoiding the time-frequency resolution tradeoff that limits
current SOCT signal processing.

3.4.1 Principles of the DW processing method

The DW method is based on calculating two separate STFTs and then combining
the results. The first STFT uses a broad spectral Gaussian window to obtain high
temporal/depth resolution while the second STFT uses a narrow spectral window to
generate high spectroscopic resolution. The two resulting TFDs are then multiplied
together to obtain a single TFD with simultaneously high spectral and temporal
resolutions.

To understand what the DW method is revealing, let us consider the FDOCT
signal

\[ I(k) = I_R(k) + I_S(k) + 2 \text{Re}(E_R^*(k)E_S(k) \cdot \cos(k \cdot d)), \]

where \( I(k) \) is the total detected intensity, \( I_R \) and \( I_S \) are the intensities of the reference and
sample fields, respectively, and \( d \) is a constant optical path difference between the
sample and reference arms. The STFT of the cross correlation term,

\[ 2 \text{Re}(E_R^* \cdot \cos(k \cdot d)), \]

can be expressed as

\[ S(k, z) = \int 2 \text{Re}(E_R^*(\kappa') E_S(\kappa') \cdot \cos(\kappa' \cdot d)) \cdot e^{-\frac{(\kappa - k)^2}{2\sigma^2}} e^{-i\kappa'z} d\kappa'. \]
Note that \( u \), the width or standard deviation of the Gaussian window, must be chosen carefully in order to obtain acceptable spectral or temporal 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 that has good temporal/depth 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 temporal resolution. The DW method, however, can avoid this resolution tradeoff.

Consider the TFDs resulting from two STFTs, \( S_1 \) and \( S_2 \), generated by a narrow spectral window and a wide spectral window, respectively. Assuming that the reference field in Eq. 3.24 is slowly varying over the frequencies of interest, the processed signal is given by

\[
DW(k, z) = S_1(k, z) \cdot S_2^*(k, z)
\]

\[
= \iint 4 \text{Re}(E_s^*(k_1)E_s(k_2) \cdot \cos(k_1 \cdot d) \cdot \cos(k_2 \cdot d)) \cdot \exp\left(-\frac{(k_1 - k)^2}{2a^2}\right) \cdot \exp\left(-\frac{(k_2 - k)^2}{2b^2}\right) \cdot \exp(-i(k_1 - k_2)z) \, dk_1 \, dk_2
\]

(3.26)

where \( a \) and \( b \) are independent parameters that set the widths of the windows, and \( b \gg a \). In order to obtain a more insightful form of the processed signal, consider a coordinate change such that
\[
\Omega = \frac{k_1 + k_2}{2}, \quad q = k_1 - k_2, \quad k_1 = \Omega + \frac{q}{2}, \quad k_2 = \Omega - \frac{q}{2},
\]

(3.27)

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

\[
DW(k, z) = \int \int \Re \left( E_s^* \left( \Omega + \frac{q}{2} \right) E_s \left( \Omega - \frac{q}{2} \right) \cdot \cos \left( \left( \Omega + \frac{q}{2} \right) \cdot d \right) \cdot \cos \left( \left( \Omega - \frac{q}{2} \right) \cdot d \right) \right) \cdot e^{\frac{-(\Omega - \frac{q}{2})^2}{2a^2}} \cdot e^{\frac{-(\Omega + \frac{q}{2})^2}{2b^2}} \cdot e^{-iq \cdot d \Omega \cdot dz}.
\]

(3.28)

The term \( E_s^* \left( \Omega + \frac{q}{2} \right) E_s \left( \Omega - \frac{q}{2} \right) \) from Eq. 3.28 can be expressed in terms of a Wigner TFD by utilizing the ambiguity function defined in Eq. 3.12. After substituting Eq. 3.12 into Eq. 3.28 and simplifying, the processed signal yields

\[
DW(k, z) = \int \int \int \int \Re \left( W_s(\Omega, \zeta) \cdot e^{i\zeta \cdot d \zeta} \cdot \cos(2\Omega \cdot d) \right) \cdot e^{\frac{-(\Omega - \zeta)^2}{2a^2}} \cdot e^{\frac{1}{2a^2}} \cdot e^{\frac{1}{2b^2}} \cdot e^{-i\zeta \cdot d \Omega \cdot dz}.
\]

(3.29)

By integrating Eq. 3.29 with respect to \( q \) and assuming \( a \) is small compared to \( b \), such that \( a^2/b^2 \ll 1 \), the DW signal simplifies to

\[
DW(k, z) = 4b\sqrt{\pi} \int \int \Re \left( W_s(\Omega, \zeta) \cdot e^{\frac{2(\Omega - \zeta)^2}{b^2}} \cdot e^{-2(d + \zeta \cdot z)^2} \cdot \cos(2\Omega \cdot d) \right) \cdot d\Omega d\zeta.
\]

(3.30)

Eq. 3.30 shows that the DW method is equivalent to probing the Wigner TFD of the sample field with two orthogonal Gaussian windows, one with a standard deviation of \( b/2 \) in the spectral dimension and another with a standard deviation of \( 1/(2a) \) in the
spatial/temporal dimension. Furthermore, $a$ and $b$ independently tune the spectral and spatial/temporal resolutions, respectively, thus avoiding the tradeoff that hinders the STFT. Eq. 3.30 also shows that the processed signal is modulated by an oscillation that depends on the constant path difference, $d$, between the sample and reference arms. This phenomenon is also observed in the cross terms of the Wigner TFD, which have been identified to contain valuable information about phase differences and is discussed in Section 3.3.4. We explore the utility of this oscillatory term below in section 3.4.3.

Another interesting result is obtained if $a$ approaches zero and $b$ is taken to be much larger than the bandwidth of the source, $\Delta k$. In these limits, the window with standard deviation $a\to0$ approaches the delta function, while the second window whose standard deviation $b>\Delta k$, becomes a constant across the spectrum. If our signal

$$F(k) = 2 \text{Re}(E_R E_S \cdot \cos(k \cdot d)), \text{ and } f(z) \leftrightarrow F(k) \text{ is a Fourier transform pair, Eq. 3.26 yields}$$

$$DW(k,z)|_{a\to0,b>\Delta k} = S_1(k,z)|_{a\to0} S_2(k,z)|_{b>\Delta k} = \frac{1}{\sqrt{2\pi}} f(z)F(k)e^{-ikz}.$$

Eq. 3.31 is equivalent to the Kirkwood & Rihaczek TFD, and if the real part is taken, it is equal to the Margenau & Hill (MH) TFD. Either of these two distributions can be simply transformed to produce any of the Cohen’s class functions, such as the Wigner TFD.

### 3.4.2 Simulations

To illustrate the power of the DW method, two different simulations are presented. In the first, a signal consisting of two optical fields separated in depth and
center wavenumber is simulated. The total sample field is given by \( E_s = E_1 + E_2, \)
where \( E_1 = E_0 \exp(-z^2)\exp(i \cdot k_1 \cdot z), \) \( E_2 = E_0 \exp(-(z - z_0)^2)\exp(i \cdot k_2 \cdot z), \) and \( k_1 > k_2. \)

The Wigner distribution of the total sample field is given by
\[
W(k,z) = \frac{1}{2\pi} \int E_s^*(z - \zeta)E_s(z + \zeta)e^{\zeta} d\zeta,
\]
and the MH distribution of the total sample field is given by
\[
MH(k,z) = \text{Re} \frac{1}{\sqrt{2\pi}} \overline{E_s(k)} E_s(z) e^{-iz},
\]
where \( \overline{E_s(k)} \leftrightarrow E_s(z) \) is a Fourier transform pair. Figure 3.8 illustrates the resulting TFDs.

The ideal TFD, shown in Figure 3.8 (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 prior knowledge of the individual fields. The ideal TFD in Figure 3.8 (a) contains two pulses with Gaussian shapes in both the temporal and spectral dimensions. The pulses are well separated in each dimension. Figure 3.8 (b)-(d) show different TFDs that can be generated from this single mixed field. The Wigner distribution, shown in Figure 3.8 (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. More often, however, these cross terms are viewed as undesirable artifacts as they yield non-zero values at times/depths and frequencies that
Figure 3.8: (a) Ideal TFD with E₁ centered at $z_0 = 5$ and $k_1 = 13$ and E₂ centered at $z_0 = 0$ and $k_2 = 26$. (b) Wigner TFD. (c) MH TFD. (d) Dual Window method.

do not exist in the field. Moreover, as more components are added to the field, the cross terms may interfere with the local signals. The MH distribution, shown in Figure 3.8 (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.’53 As is the case with the Wigner distribution, these artifacts yield non-zero intensities at times and frequencies that should contain no signal.

The TFD generated using the DW method is presented in Figure 3.8 (d). The TFD is generated by simply computing the product of two STFTs processed with wide
and narrow spectral windows respectively. In Figure 3.8 (d) the cross terms that are present in the Wigner and MH distributions are suppressed as a result of the use of two orthogonal windows.

The second simulation models a SOCT signal from a Michelson interferometer 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. 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 3.9 (a) shows the ideal TFD of the simulated signal while Figure 3.9 (b) and (c) show the TFDs generated by the STFT using narrow and wide spectral windows, respectively. In each case, the effects of the time-frequency resolution tradeoff are obvious. The TFD generated with the wide spectral window suffers from degraded temporal resolution while the TFD generated with the narrow spectral window suffers from degraded spectral resolution. As Xu et al. showed, the STFT window can be optimized for specific applications, but regardless of the window size, a resolution tradeoff must be made.58 Figure 3.9 (d) shows the TFD generated using the DW method which computes the product of the TFDs shown in Figure 3.9 (b) and (c). Figure 3.9 (e)
Figure 3.9: (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 $t = 4.5$ in (a), (c), and (d).

shows the time marginals computed from Figure 3.9 (b)-(d) which demonstrate that the DW method resolves the two sample surfaces with a resolution comparable to that of the ideal case, whereas the narrow spectral window STFT does not. Figure 3.9 (f) shows the spectral profile of the rear surface reflection in Figure 3.9 (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 wavenumbers, while the wide spectral window STFT reveals no absorption information. The DW frequency
profile also reveals the same 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 time and is analyzed further in Section 3.4.3.

### 3.4.3 Local oscillations

It has been shown previously in Section 3.3.5 that temporal coherence information from Wigner TFD cross-terms can be utilized to gain structural knowledge of samples via the SOCT signal. However, these cross terms are typically viewed as undesirable artifacts as they yield non-zero values at times/depths and frequencies that do not actually exist in the field.

Eq. 3.30 shows that signals processed by the DW method are modulated by a cosine term whose frequency depends on the constant path difference, $d$, between the sample and reference arms. This is the same phenomenon that is observed in the cross terms of the Wigner TFD, and these oscillations can be used to gain valuable information about phase differences.

Figure 3.10 (b) shows the frequency profile from the front reflecting surface of the sample in simulation 2 (Figure 3.9). This frequency spectrum is taken from depth 3 (red dashed line) of the TFD shown in Figure 3.10 (a) which was generated by the DW method. The spectral modulation that is present can be further processed to reveal structural information about the simulated experimental sample. Fourier transforming
the spectrum from Figure 3.10 (b) generates the correlation plot shown in Figure 3.10 (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 of the sample.

### 3.5 Spatial coherence in white light interferometry

The utility of the Wigner distribution for analyzing optical signals is not confined to merely investigations of temporal coherence. The Wigner distribution can also be used to analyze the role that spatial coherence plays in the combination of OCT signals. While the vast majority of current OCT systems have employed a spatially coherent light source such as a laser or superluminescent diode (SLD), as an alternative, white light sources offer the advantage of a significantly larger spectral bandwidth centered at
a lower wavelength, enabling superior depth resolution. White light sources can also
provide access to spectral windows that are not readily available using coherent sources.
However, performing OCT or interferometry using a white-light source poses a unique
challenge due to the extremely low spatial coherence of the source light.

OCT with a white light source has been previously demonstrated in both the
time and frequency domains, but to this point OCT images of biological samples
using a white light source have only been demonstrated using time domain (TD) OCT.
The fact that coherence imaging of a biological sample using a white light source in the
frequency domain is a great challenge, while imaging the same sample using a white
light source in the time domain is not is illustrative of the fundamental differences
between TD and FD OCT signals. The Wigner TFDs of these detected signals can be
analyzed to show that multicomponent signals combine differently in FDOCT compared
to TDOCT, leading to specific design criteria for white light FDOCT systems.

Consider the interference signal from a basic Michelson interferometer such that
the total electric field is equal to the sum of the electric field from the sample arm and
the electric field from the reference arm:

\[ E_T = E_S + E_R. \]  (3.34)

As demonstrated above in Section 3.2, the Wigner distribution of the total electric
field can be represented as the sum of the Wigner distributions of the sample field \( E_S \)
and reference field (E_r), along with that of the cross terms representing the interference between them:

\[ W_T = W_R + W_S + W_{\text{cross}}. \] (3.35)

If we consider a sample field of \( n \) components, the Wigner function for the total field will consist of the sum of the Wigner distribution of the reference field, \( n \) sample Wigner distributions (which we combine into \( W_S \)), and the Wigner distributions of the \( n \) cross terms between the reference field and each component of the signal field:

\[ W_T = W_R + W_S + \sum_{n} W_{\text{cross}}^n. \] (3.36)

To examine the differences between FDOCT and TDOCT we need to consider the interference information which is contained in the Wigner distributions of the cross-terms. In the frequency domain, our detected signal is the frequency marginal of the Wigner distribution:

\[
S_{FD}(\omega) = \int dt \sum_{n} \cos(T_n(\omega + \omega_0))
\times \exp\left(\frac{-a^2}{2}(\omega + \omega_0)^2\right) \exp\left(\frac{-2}{a^2}\left(t - \frac{T_n}{2}\right)^2\right)
= a\sqrt{\frac{\pi}{2}} \exp\left(\frac{-a^2}{2}(\omega + \omega_0)^2\right) \sum_{n} \cos(T_n(\omega + \omega_0)).
\] (3.37)

Here, \( a \) is the temporal coherence length of the source, \( \omega_0 \) is the center frequency, and \( T_n \) is the time delay of the \( n^{th} \) component of the signal field relative to the reference
field. The result is a sum of truncated cosine terms. Because these terms add coherently, phase differences between terms will reduce the fringe visibility. In OCT, reduced fringe visibility equates to a degradation of depth scan signal strength.

Alternatively, in the time domain the detected signal is a function of the time delay or path-length difference between the sample and reference fields. Each point of the detected signal is the time-averaged time marginal of the Wigner distribution. Here we have taken the power spectrum of the signal by including the signal from both the in-phase (cos) and out of phase (sin) quadratures, as is commonly done in TDOCT:

\[
S_{tt}(T_n) = \int \int dt d\omega \sum_n \left( \cos(T_n(\omega + \omega_0)) \right)
+ i \sin(T_n(\omega + \omega_0)))\exp\left(-\frac{a^2}{2}(\omega + \omega_0)^2\right)\exp\left(-\frac{2}{a^2}\left(t - \frac{T_n}{2}\right)^2\right)
\]

\[
= \sqrt{\frac{\pi}{2}} \sum_n \exp\left(-\frac{T_n^2}{2a^2}\right). \tag{3.38}
\]

The resulting expression is a sum of Gaussian functions of \(T_n\), the time delay. Each Gaussian pulse represents the power contained in the interference signal due to the \(n\)th field component. Unlike the coherent sum of energy signals in the frequency domain (Eq. 3.37), the power signals in the time domain sum \textit{incoherently} and are less vulnerable to phase differences between field components.

These differences in signal combination between TDOCT and FDOCT lead to the increased challenge of performing OCT with a white light source in the frequency
domain. In FDOCT, the resolution of the detected signal by mode is critical in minimizing the destructive effects that phase differences between field components can have on the coherent sum of energy signals. For many FDOCT systems, this is not a great concern as the spatial coherence length of light from a superluminescent diode, the light source most often used in FDOCT, can be an order of magnitude larger than that from a thermal source. However, for white light OCT the resolution of the detected signal by mode is absolutely necessary.

It is important to note, however, that this effect is the exact opposite of that seen with TDOCT. As seen in Eq. 3.38, signals in the time domain combine incoherently and as a result, interference efficiency is improved as more modes are combined. Therefore, resolution of the detected signal by mode is not necessary.

Figure 3.11 (a) shows a simulation of raw interference spectra collected from 255 adjacent spatial points on a scattering sample. The random phases of adjacent spectra occur as a result of the random location of scatterers within the simulated sample. Figure 3.11 (b) shows two depth scans generated by taking the magnitude of the Fourier transformed spectra, as is typically done in FDOCT. The simulated depth scans illustrate the important difference between coherent and incoherent summation of signals.
Figure 3.11: (a) Simulated spectra from a scattering sample with random phase differences between adjacent spectra. (b) Depth scans generated from raw spectra in part (a). Depth information is degraded by summing spectra before Fourier transforming (solid, red) but preserved by summing channels after Fourier transforming (dashed, blue).

In the case of the solid curve, the simulated spectra from the different spectral channels were summed together before the Fourier transform was performed. The result is a coherent sum of energy signals in which the phase differences between signal components reduce interference efficiency and therefore degrade depth information. Alternatively, the dashed curve in Figure 3.11 (b) shows the simulated depth scan generated by summing the information from different spectral channels after the spectra had been individually Fourier transformed. In this case, the result is an incoherent sum and is therefore less vulnerable to phase differences between individual spectra. The
individual energy signals maintain their interference efficiency such that the signals add incoherently without degrading the depth information.

The analysis above has compared the summation of signals for TDOCT and FDOCT based on the Wigner time-frequency distribution. This analysis shows that the two modalities combine signals in a different manner. The coherent summation of signals in FDOCT can result in decreased interference efficiency when many modes are present. We note that this effect is exactly the opposite of that seen with TDOCT, where the incoherent summation results in improved interference efficiency when many modes are combined.

3.6 Summary

Chapter 3 has presented an extensive analysis of the temporal and spatial coherence theory underlying the fLCI technique. First, the relationship between interferometry signals and bilinear TFDs, such as the Wigner distribution, was examined. The formation of time and frequency domain OCT signals was investigated using the Wigner distribution framework. Additionally, the TFDs generated in spectroscopic OCT and fLCI were analyzed, showing that added structural information about experimental samples could be obtained from the temporal coherence information contained in TFD cross-terms. These theoretical concepts were shown both mathematically and through simulations.
Next, the DW method for processing SOCT and fLCI signals was introduced. The TFDs generated by typical SOCT processing techniques, such as the STFT, suffer from an inherent tradeoff between depth and spectral resolution. Section 3.4 described the theory behind the DW processing method which generates TFDs with simultaneously high depth and spectral resolution. The method was detailed mathematically, and simulations were presented to demonstrate its utility in processing depth resolved spectroscopy signals.

Finally, the role played by spatial coherence in white light interferometry signal combination was considered. Like the previous sections, the Wigner formalism was utilized to illustrate the difference between coherent and incoherent summation of interferometry signals. These theoretical concepts were used to show that white light interferometry systems require the spatial resolution of uncorrelated modes in the optical signal. Simulations were also presented showing that the coherent sum of uncorrelated interference spectra can lead to signal degradation.

In the next chapter, fLCI instrumentation is discussed. First, Section 4.2 details the early generation, common path fLCI system. In Section 4.3, the next generation imaging fLCI system is presented. The imaging fLCI system design utilizes lessons from the spatial coherence study of Section 3.5 to overcome the presented limitations of the common-path system. The imaging fLCI system is capable of measuring depth resolved
spectra from thick experimental samples and of generating ultra-high depth resolution parallel frequency domain optical coherence tomography images.
4. Instrumentation

4.1 Introduction

In this chapter, the instrumentation of two fLCI optical systems is presented. First, the design and development of the common path fLCI system is discussed. This optical system is based on a Michelson interferometer modified for the common path geometry. The system utilizes frequency domain low coherence interferometry to achieve depth resolution, and a common path geometry is employed for its design simplicity and ease of alignment. The absence of a reference arm eliminates the need for complex alignment procedures and path-length matching. While the system is effective as a tool for performing depth resolved spectroscopy, its effectiveness is limited to thin experimental samples.

In Section 4.3, the next generation imaging fLCI system is presented. The design and development of the optical system evolves from the theory discussed in Chapter 3. The system spatially resolves the optical signal from source to detector in order to meet the design criteria identified in Section 3.5, the study of spatial coherence in white light interferometry. Unlike the common path fLCI system, this updated design allows the imaging fLCI system to operate effectively with thick experimental samples up to approximately 1 mm. Additionally, the optical system is capable of parallel FDOCT imaging with ultra-high depth resolution. The imaging capabilities and specifications of this fLCI system are also discussed.
4.2 Common path Fourier domain low coherence interferometry system

The common path Fourier domain low coherence interferometry scheme, shown in Figure 4.1, is based on a modified Michelson interferometer.\textsuperscript{56} White light from a Xe arc lamp source (250W, Newport Oriel, Stratford, CT) is coupled into a multimode fiber (200 \(\mu\)m core diameter). The output of the fiber is collimated by an achromatic lens (L1, \(f_1 = 10 \text{ mm}\)) to produce a pencil beam with a spot size of approximately 5 mm in diameter. The diameter of the spot size can be restricted to adjust the sample illumination if desired. The white light beam is transmitted to the sample by the beamsplitter (BS, 50/50).

Unlike the original fLCI scheme,\textsuperscript{52} where the BS was used to generate separate reference and signal beams, this fLCI system uses a common path configuration where the signal and reference fields travel the same optical path until reaching the sample. The generation of a reference field is achieved by placing the experimental sample on a glass coverslip. As shown in the inset of Figure 4.1, the reflection from the front surface of the coverglass is used as the reference field while the light that is backscattered by the experimental sample comprises the signal field. The two combined fields are directed by the beamsplitter to a second achromatic lens (L2, \(f_2 = 10 \text{ mm}\)) which collects the light and couples to a second multimode fiber (200 \(\mu\)m core diameter). The output of the fiber is coincident with the input slit of a high resolution spectrometer (InSpectrum, Princeton Instruments, Trenton, NJ).
Figure 4.1: Schematic of the common-path fLCI system. Light source – 250 W Xe arc-lamp. L1 and L2 – Lenses. BS – beamsplitter. Inset: Sample arm geometry. Reflection from front surface of coverslip is used as reference field with which light backscattered by the sample can interfere.

The spectrometer utilizes a 1200 lines/mm diffraction grating with a blaze wavelength of 500 nm and a 150 mm light path to produce a spectral resolution of 0.1 nm. The dispersed fields are detected with an integrated, front-illuminated CCD (Hamamatsu, Bridgewater, NJ) which is cooled to -20° C. The system incorporates 16 bit digitization, and data is downloaded in real time to a laptop PC via the USB interface. Spectrometer control and data acquisition is controlled using custom LabVIEW (National Instruments, Austin, TX) software. This system was used to generate the experimental results discussed in Section 5.4.
4.3 Imaging Fourier domain low coherence interferometry system

The second generation imaging fLCI system, shown in Figure 4.2, is based on a modified Michelson interferometer geometry. The system utilizes a 4f interferometer first demonstrated by Wax, et al. White light from a Xenon arc-lamp (150W, Newport Oriel, Stratford, CT) is coupled into a multimode fiber (200 μm core diameter) before being collimated by an achromatic lens ($L_1, f_1 = 10 \text{ mm}$). The resulting pencil beam spans 5 mm in diameter and enters the 4f interferometer formed by lenses $L_2, L_3, L_4, \text{ and } L_5 (f_2, s = 10 \text{ cm})$ along with the beamsplitter, BS. The light is separated by BS into a reference arm, incident upon a reference mirror, and a sample arm, incident on the experimental sample. Light scattered by the sample is recombined with the reference signal by BS and re-imaged onto the detection plane by the 4f imaging system formed by lenses $L_3$ and $L_5$.

The 4f interferometer uses two 4f imaging systems to spatially resolve light from the source to the detector. A schematic of each 4f imaging system is shown in Figure 4.3 (a). For clarity, the beamsplitter has been removed and the light paths have been unfolded. Light scattered by the experimental sample is re-imaged onto the detection plane by the 4f system formed by lenses $L_3$ and $L_5$. Light reflected by the reference mirror is re-imaged onto the detection plane by the 4f system formed by lenses $L_4$ and $L_5$. 

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Figure 4.2. Schematic of the imaging fLCI system. Light source – 250 W Xe arc-lamp. L1 through L5 – lenses. BS – beamsplitter. M – reference mirror. Inset: Incoming light incident on the spectrometer slit. Slit allows only a small slice of incoming light to enter the imaging spectrometer.

The detection plane of the imaging system coincides with the entrance slit of an imaging spectrometer (Shamrock 303i, Andor Technology, South Windsor, CT) which spatially resolves 255 detection channels. The spectrometer utilizes a 1200 lines/mm diffraction grating with a blaze wavelength of 500 nm. Detection is accomplished with a front-illuminated CCD camera (iDus DV420, Andor Technology, South Windsor, CT)
Figure 4.3: (a) Unfolded imaging optics of the 4f imaging interferometer showing 4f systems in sample arm (top) and reference arm (bottom). L3, L4 and L5 – lenses. S – experimental sample. M – reference mirror. D – detection plane. (b) Sample arm operating in scatter-mode in which specular reflection is angled away from collection optics.

with a 1024 by 255 pixel array. The camera uses thermoelectric cooling to operate at -50°C resulting in a negligible dark current. The imaging spectrometer optics, along with the combination of the 1200 lines/mm grating and the 1024 pixel CCD array, limits the detected spectrum to the 500-625 nm range. The high resolution of this grating is necessary to achieve the depth range desired in the coherence images. Other spectral regions can be accessed by tuning the center wavelength of the span or employing a
Figure 4.4: Photograph of the imaging fLCI system. Much of the system is mounted vertically on an optical breadboard to accommodate the experimental sample in a horizontal position. The input of the imaging spectrometer is visible on the far left of the photograph.

A multi-channel spectrometer for simultaneous measurements. Data from the spectrometer is downloaded in real time to a laptop PC via the USB 2.0 interface, and spectrometer control and data acquisition is controlled using custom LabVIEW (National Instruments, Austin, TX) software.
4.3.1 Parallel frequency domain OCT imaging

The imaging fLCI system is also capable of ultra-high depth resolution parallel frequency domain OCT (pfdOCT) imaging. By spatially resolving the optical signal from the source to the detector, the system is capable of generating depth scans from 255 lateral spatial points in the experimental sample. The FDOCT capabilities are referred to as “parallel” because B-mode images are produced in a single acquisition, without the need for scanning the beam spatially or scanning a reference mirror.

As discussed in Section 3.5, in order to obtain OCT images in the frequency domain with a thermal source, it is necessary to limit the number of modes that contribute to each detected signal. Here each mode is considered to span an area given by the square of the transverse coherence length. We limit the contributing modes by spatially resolving the detected signal by coherence area at the detection plane using the 4f imaging system and imaging spectrometer. A similar optical system was used by Grajciar et al.\textsuperscript{66} to execute parallel OCT but used a cylindrical lens between lenses L1 and L2 to focus the light in one dimension. As noted in Section 3.5, this serves to add more coherence areas to the detected signal which lowers the interference efficiency. This may be a reason that satisfactory FDOCT images were not obtained using a thermal light source in that work.

The ultra-broad bandwidth and low center wavelength of the thermal light source yields a theoretical axial resolution of 1.03 μm which is comparable to today’s
best ultra-high resolution TD and FDOCT systems. The experimental depth resolution of the system was determined using a mirror for a sample to be 1.22 μm FWHM. The lateral resolution of the system is 26 μm, equal to the width of the detector pixels. This is poorer than many current TD and FDOCT systems, but it is achieved in a single shot without the need for beam scanning. The SNR of the presented image was determined by a separate experiment to be 89 dB, slightly worse than the SNR of typical coherent-source TD and FDOCT systems.

### 4.3.2 Scatter-mode imaging

Unlike most TD or FDOCT systems, the sample arm of the presented system was designed to operate in “scatter-mode.” Figure 4.3 (b) illustrates the scatter-mode imaging schematic. In this geometry, the light incident on the sample is collimated rather than focused and the sample is tilted such that specularly reflected light is not directed back to the beamsplitter. Instead, only light backscattered by the sample is collected by lens L3 and mixed with the reference field at BS. This adjustment of sample arm geometry allows for the analysis of scattering media by avoiding saturation of the detector pixels by the large surface reflections. These reflections can be orders of magnitude greater than the signal originating from deeper tissue layers.

The imaging fLCI system of Section 4.3 was used to generate the experimental results of Sections 5.2, 5.3, 6.2, and 6.3.
4.4 Summary

The instrumentation of two fLCI systems was presented in Chapter 4. First, Section 4.2 detailed the early generation, common path fLCI system. The system was based on a Michelson interferometer modified for the common path geometry. The simplicity of use and ease of alignment were key benefits of the common path design. However, its usefulness was limited to thin experimental samples as a result of the combination of uncorrelated spatial modes discussed in detail in Section 3.5.

Section 4.3 presented the next generation, imaging fLCI system. The optical system utilized a 4f interferometer and an imaging spectrometer for detection to spatially resolve the optical signal throughout the system. As a result, the system was capable of performing depth resolved spectroscopy on thick experimental samples. Additionally, the spatial resolution of the optical signal allowed the system to generate ultra-high depth resolution pfdOCT images.

Chapter 5 presents a series of controlled studies that experimentally validate the functionality of the presented systems. The imaging capabilities of the imaging fLCI system are validated with scattering standard measurements and pfdOCT images animal tissue. The DW method for processing fLCI signals is also validated by a study using an absorption phantom as an experimental sample. Additionally, the nuclear sizing capabilities of the common path system of Section 4.2 are validated by a study measuring nuclear morphology in in vitro cell monolayers. These experiments both
validate the functionality of the presented fLCI systems as well as set the stage for the animal tissue studies presented in Chapter 6.
5. Validation

5.1 Introduction

In this Chapter the theoretical foundations and experimental capabilities of the fLCI technology described in Chapters 3 and 4 are validated through controlled experiments. First, the imaging capabilities of the fLCI system described in Section 4.3 are discussed. As theorized in Section 3.5, frequency domain white-light interferometry systems such as the fLCI system presented in Section 4.3 require the spatial resolution of uncorrelated modes to prevent the degradation of depth information. Section 5.2 demonstrates this theory empirically using data collected from a scattering standard.

The parallel frequency domain OCT imaging capabilities of the fLCI system are also demonstrated in Section 5.2. A pfdOCT image of a hamster cheek pouch epithelium is presented and several tissue layers and morphological features are identified. This is the first OCT image of a biological sample collected in the frequency domain using a thermal light source.

The Dual Window processing method introduced in Section 3.4 is validated in Section 5.3 with several experiments using an absorption phantom as an experimental sample. The absorption phantom was designed such that the experiments in Section 5.3 parallel the SOCT simulation presented in Section 3.4. The phantom data is processed using both the DW method and the STFT. The spectral and temporal resolution characteristics of the resulting TFDs are compared showing that the DW method avoids
the trade-off between temporal and spectral resolution associated with the STFT.

Additionally, the experiments demonstrate the increase in spectral fidelity achieved in TFDs generated by the DW processing method.

Section 5.3 also presents experimental validation of the local oscillation analysis simulated in Section 3.4. The local oscillations of TFDs generated by the DW method are processed by the Fourier transform to generate a correlation plot which reveals added structural information about the experimental sample.

In the final section, the fLCI system’s ability to accurately size in vitro cell nuclei is validated. An experiment using monolayers of human epithelial cancer cells as an experimental sample is presented. The results of the experiment demonstrate the ability of the fLCI system to accurately and precisely determine the diameter of cell nuclei.

5.2 Imaging system validation

In order to validate the imaging capabilities of the fLCI system, an experiment demonstrating the signal degradation caused by mixing uncorrelated spatial modes was conducted. The experiment uses a scattering standard as an experimental sample and contrasts the depth signals achieved by the coherent and incoherent summation of detected spectra. As described mathematically and simulated in Section 3.5, the experiment shows that because the system uses a spatially incoherent white light source
and performs the coherence imaging in the frequency domain, it is necessary to spatially resolve the optical signal throughout the system.67

Data from a scattering standard sample were acquired using the imaging fLCI system described in Section 4.3. The raw data consisted of spectra from 255 adjacent spatial points in the sample. These interference spectra exhibited random phase changes between the spectra from adjacent spatial points. The randomly changing phase differences between the components of the signal field provide a good basis for the demonstration of coherent and incoherent summation of the signal components.

Figure 5.1 (a) shows the raw interference spectra collected from the scattering sample by the imaging spectrometer. The random phases of the interference fringes occur as a result of the random location of scatterers within the sample. Figure 5.1 (b) shows depth scans generated by taking the magnitude of the Fourier transformed wavenumber spectra, as is typically done in FDOCT. The signals have been summed across the image in different ways. The vast difference in depth information retained by each curve illustrates the necessity of the spatial resolution of modes.

In the case of the solid curve, the raw spectra from the different spatial points were summed together before the Fourier transform was performed. This operation is analogous to a FDOCT detection scheme which sums many modes at the detection plane. As a result of the coherent sum of the energy signals, the phase differences
Figure 5.1: (a) Experimental spectra obtained from a scattering standard sample. (b) Depth scans generated from raw spectra in part (a). Depth information is degraded by summing spectra before Fourier transforming (solid) but preserved by summing channels after Fourier transforming (dashed).

between the signal components reduce interference efficiency and therefore degrade depth information.

Alternatively, the dashed curve in Figure 5.1 (b) shows the depth scan generated by summing the information from different spatial points after the individual wavenumber spectra had been individually Fourier transformed. This is analogous to an incoherent sum where the power from each spatial coherence channel is combined. By utilizing the imaging spectrometer to maintain the spatial resolution of the sample field in the detected signal, the individual energy signals maintain their interference
efficiency. A summed depth scan is obtained by first transforming the cosines so that the signals add incoherently without degrading the depth information.

The analysis above shows that the coherent summation of uncorrelated signals in FDOCT can result in decreased interference efficiency when many modes are present. The system presented in Section 4.3 was designed specifically to spatially resolve the optical signal and avoid this problem. The spatial resolution of the signal not only enables the system to detect scattering from deep layers of thick samples, but it also enables it to perform high resolution parallel FDOCT imaging.

Figure 5.2 shows a scatter-mode image of the hamster cheek pouch epithelium obtained from the presented parallel FDOCT system with a thermal light source. After the animal was sacrificed, the cheek pouch tissue was excised, placed on a coverglass, and moistened with phosphate buffered solution (PBS) before being scanned by the imaging fLCI system. The presented image contains the average of 25 successive scans in order to improve the signal to noise ratio. The image was obtained without the need for scanning a reference mirror or scanning the light beam spatially on the sample.

The pfdOCT image in Figure 5.2 shows several tissue layers indicated by arrows including the epithelium (E), mucosa (M), and submucosa (S). Because the cheek pouch was not stretched during imaging, the tissue also contained folds (F) at points where the sample did not adhere to the coverglass. The included scale bars correspond to 50 μm in the depth (vertical) dimension and 125 μm in the lateral (horizontal) dimension. To our
knowledge, this is the first OCT image of a biological sample collected in the frequency domain using a thermal light source.\textsuperscript{67}

### 5.3 Dual Window processing method validation

To validate the ability of the DW processing method to generate TFDs with simultaneously high spectral and temporal resolution, several experiments were completed using an absorption phantom as an experimental sample.\textsuperscript{69} All data were collected using the imaging fLCI system presented in Section 4.3. The absorption phantom consisted of a glass wedge filled with an absorbing dye as shown in Figure 5.3 (a). Figure 5.3 (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
Figure 5.3: (a) Illustration of absorption phantom. (b) pfdOCT image of absorption phantom. (c) Transmission spectrum of absorbing dye used in absorption phantom.

attenuated at the thicker end of the wedge due to considerable signal absorption from the greater 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 5.3 (c) shows the transmission spectrum of the absorbing dye which shows strong absorption in the high wavenumber range of the detected spectrum. We would expect signals returning from the front surface of the phantom to exhibit a relatively flat spectrum, while signals reflected by the back surface of the phantom would exhibit spectra with significant attenuation of the higher wavenumbers, mirroring the absorption spectrum of the dye through which it passed.
5.3.1 Depth and spectral resolution

The raw data corresponding to the position of the dashed red line in Figure 5.3 (b) was processed with four different methods to yield the four TFDs shown in Figure 5.4. Figure 5.4 (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, showing a spectrum which emulates the source spectrum at the depth corresponding to the front surface of the phantom. The sharp spectral cut-off at high wavenumbers, characteristic of the dye absorption, is evident at deeper depths. However, the narrow spectral window used to generate this TFD yields very poor temporal resolution,

![Figure 5.4: TFD of absorption phantom reconstructed with (a) Narrow spectral window STFT (b) Wide spectral window STFT (c) Moderate spectral window STFT (d) Double window method.](image-url)
resulting in an inability to resolve the two surfaces of the phantom. Figure 5.4 (b) was also processed using the STFT method, but in this case a wide spectral window of 0.665 μm⁻¹ was used. The resulting TFD has excellent temporal 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 for the rear surface spectrum. Figure 5.4 (c) shows the TFD generated using the STFT method with a window of moderate spectral width, 0.048 μm⁻¹. As expected, the spectral and temporal resolutions of the resulting TFD fall between those of Figure 5.4 (a) and (b), illustrating the temporal-spectral resolution tradeoff 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 Figure 5.4 (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 spectral and temporal resolution. The front phantom surface exhibits a spectrum which emulates the source spectrum 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.

The utility of the DW processing method is further demonstrated by examining spectral cross-sections and time marginals of the generated TFDs. Figure 5.5 (a) displays
Figure 5.5: (a) Spectral cross-section at depth 245 μm in Figure 5.4 (c) and (d), along with dye transmission spectrum. (b) Spectral cross-section at depth 220 μm in Figure 5.4 (c) and (d), along with source spectrum. (c) Time marginals from Figure 5.4 (c) and (d), along with corresponding A-scan.

Spectral profiles from depths corresponding to the absorption phantom’s rear surface in the TFDs of Figure 5.5 (c) and (d). For reference, the absorbing dye transmission spectrum is displayed as well. Figure 5.5 (b) shows spectral cross-sections from depths corresponding to the phantom’s front surface, along with the phantom’s reflectance spectrum for reference. The time marginals of each TFD are displayed in Figure 5.5 (c) along with the corresponding A-scan from Figure 5.3 (b). 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. Because
the DW method produces a bilinear TFD, the noise floor of the resulting time marginal is
lower than that of the time marginal produced by the STFT.

5.3.2 Spectral fidelity

In addition to limiting the resolution tradeoff associated with the STFT, the DW
method also achieves an increase in the spectral fidelity of generated TFDs. The
normalized spectra from Figure 5.5 (a) and (b) are plotted in Figure 5.6 with the high
frequency modulation removed by a low-pass filter. By separating the low frequency
content from the high frequency local oscillations, we can assess the fidelity with which
each processing method recreates the ideal spectrum. Chi-squared values for each
processing method were calculated to assess goodness-of-fit.

Table 1 summarizes the chi-squared values. For both the rear surface spectra in
Figure 5.6 (a) and the front surface spectra in Figure 5.7 (b), the chi-squared values
associated with DW method are lower than those of the STFT indicating that the DW

<table>
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<th>STFT</th>
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Figure 5.6: (a) Spectral profiles of Figure 5.5 (a) with high frequency modulations removed. (b) Spectral profiles of Figure 5.5 (b) with high frequency modulations removed.

The processing method recreates the ideal signal with greater spectral fidelity. In addition, we calculated the goodness-of-fit for the square of the STFT to account for the fact that the DW method produces a bi-linear distribution. The DW method is also seen to produce superior spectral fidelity than the STFT squared.
5.3.3 Local oscillations

As with the simulated SOCT signals, the local oscillations seen in the TFD obtained from probing the absorption phantom (Figure 5.4) can also be analyzed to gain structural information about the experimental sample. Figure 5.7 (b) shows the spectral profile from the front surface of the absorption phantom indicated by the dashed red line in Figure 5.7 (a). Fourier transforming this spectrum produces a correlation plot as shown in Figure 5.7 (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. Here the measurement uncertainty is larger than the 1.22 μm depth resolution due to the fact that the glass surface was slightly abraded to increase the signal, producing a broader range of path lengths.

By comparing the performance of the DW and STFT processing methods in analyzing SOCT signals from an absorption phantom, we have shown that the DW method recovers TFDs with superior fidelity while simultaneously maintaining high spectral and temporal resolution. In addition, we have shown that local oscillations contained in the TFDs generated by the DW method contain valuable information about the structure of experimental samples. Unfortunately, processing methods such as the STFT and CWT are limited by an inherent tradeoff between spectroscopic and depth resolution. This time-frequency tradeoff greatly reduces the utility of the analysis by
Figure 5.7: (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.

degradating either the depth or spectral resolution to the point that important features cannot be accurately reconstructed. We expect that by avoiding this tradeoff, the DW processing method will enable new directions in SOCT and depth resolved spectroscopy.

5.4 Nuclear sizing validation

The strength of fLCl as a depth-resolved spectroscopic analysis method lies in its ability to quantitatively determine the mean diameter of scatterers in a specific subsurface layer of an experimental sample. In order to distinguish between normal and abnormal epithelial tissue, fLCI capitalizes on this capability by detecting enlargement
of epithelial cell nuclear diameter, a key biomarker for developing neoplasms. In order to validate the technology’s ability to accurately measure nuclear size, a series of experiments which probed the nuclear morphology of in vitro cell monolayers was conducted.56

5.4.1 Experimental sample

To demonstrate the applicability of the fLCI technique to biological structures, experiments were executed using in vitro samples of T84 epithelial cell monolayers, a cell line derived from a human colonic adenocarcinoma. The T84 cells (from Duke Cell Culture Facility) were grown in LabTek chambered coverglasses (Nalge Nunc International, Naperville, IL) using a growth medium consisting of a 1:1 mixture of high-glucose Dulbecco’s modified Eagle medium and Ham’s F12 medium containing 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES and 0.5 mM sodium pyruvate supplemented with 5% fetal calf serum, 95 units/ml penicillin and 95 μg/ml streptomycin (all Gibco BRL products, Life Technologies, Grand Island, NY). Cells were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. For the experiments, cell monolayers were transferred to Hank’s Balanced Salt Solution (HBSS, Sigma, St. Louis, MO).

T84 cells were prepared for microscopy imaging with two different nuclear stains. To accomplish the nuclear tagging, the cells were fixed in 3.7% formaldehyde in Tris Buffered Saline (TBS) for 20 minutes at 37°C, rinsed with PBS and incubated with a
solution of 8 μg/mL Hoechst 33342 stain (Molecular Probes, Carlsbad, CA) for 20 minutes. T84 samples were similarly prepared for confocal Z-stack imaging, but were incubated with a solution of 1.67 μg/mL LDS-751 (Molecular Probes, Carlsbad, CA) nuclear stain in ethanol.

5.4.2 Data Processing

A typical spectrum of light backscattered by the sample is shown in Figure 5.8 (a). The data are presented as a function of wavenumber, over the range of 10 μm⁻¹ to 12 μm⁻¹, corresponding to a wavelength range of 525-625 nm. The data show a prominent oscillation which arises from the interference between the reflection from front of the coverglass (reference field) and that from the rear surface (signal field). Because this study was completed before the development of the DW processing method, the STFT was used to obtain depth resolved spectroscopic information. Figure 5.8 (b) shows a contour plot for the processed data resulting from this transform. The processed data are given as a function of depth on the horizontal axis and as a function of wavenumber on the vertical axis. The lighter areas correspond to higher field strength. The signal localized at the depth of 450 μm, corresponding to the roundtrip optical pathlength through the coverglass, gives the interference between the front and back surface reflections.

In order to exclusively examine the contribution from the T84 cells, the depth resolved spectrum of the field scattered by the cells is compared to that obtained from a
blank coverslip. By taking the ratio of the two, we are able to examine the scattering efficiency of the cells (Figure 5.9 (a)) separately from the source spectrum and the transmission through the coverglass. The ratio shows a periodic oscillation in the scattering efficiency of the cells. This oscillation can be understood by considering that the field scattered by the front of the nuclei will interfere either constructively or destructively with that from the rear of the nuclei depending on the number of wavelengths which will fit inside of the nucleus diameter.

**Figure 5.8** (a) Typical spectrum of light scattered by the *in vitro* cell sample. (b) Contour plot showing the depth resolved spectral data for the T84 cell sample.
Figure 5.9 (a) Spectrum showing the scattering efficiency of the T84 cell nuclei. (b) Correlation function obtained by Fourier transforming ratioed data shown in (a).

The size of the nucleus can be determined by Fourier transforming this ratio and analyzing the correlation information which is obtained (Figure 5.9 (b)). The correlation function shows a sharp peak at a round trip path of 19.15 μm which is equal to $2 \cdot n \cdot d$ where $n$ is the index of refraction of the cell nuclei (1.395) and $d$ is the nuclear diameter. This analysis yields a nuclear diameter of 6.86 +/- 1.37 μm, with the uncertainty given by the pixel size of this correlation function. In the experiments, the spectra from 11 different cell samples are analyzed, with the results given below in Section 5.4.3.

In order to gauge the accuracy of the fLCI measurements, the nuclear diameters of T84 cells were also measured using fluorescence and confocal microscopy. Cells were fixed and treated with nuclear dyes as described in Section 5.4.1. Standard fluorescence microscopy was used to generate X-Y images which were analyzed using ImageJ software to manually measure the nuclear diameters. Results from 50 randomly selected
cells were averaged to determine the mean transverse diameter of the cell nuclei. A typical fluorescence microscopy image of T84 nuclei can be seen in Figure 5.10 (a).

Confocal Z-stacks were also acquired to assess the nuclear dimensions in the X-Z plane. Z-stacks were assembled into 3D volumes from which X-Z slices were selected and measured manually. The nuclear diameter in the Z direction was measured using confocal microscopy for comparison to the information obtained with the fLCI system. Longitudinal diameter measurements were obtained from 25 randomly selected T84 cells and averaged to determine the mean longitudinal diameter of the nuclei. A typical Z-stack image is shown in Figure 5.10 (b).

Figure 5.10 (a) Fluorescence microscopy image of T84 cell nuclei. Nuclei were stained with Hoechst 33342 nuclear stain. (b) Confocal Z-stack image of T84 cell nuclei with all three axes of resolution visible. Nuclei were stained with LDS-751 nuclear stain.
5.4.3 Results

The nuclear size determined using fLCI yielded consistent results across measurements of all 11 samples. The fLCI measurements generated a mean longitudinal diameter of 6.87 +/- 0.81 μm. Image analysis of confocal microscopy Z-stack images yielded a longitudinal diameter of 6.8 +/- 1.1 μm and a transverse diameter of 10.5 +/- 1.9 μm. Fluorescence microscopy was also used to investigate the transverse profile of the cell nuclei, yielding a mean transverse diameter of 10.4 +/- 1.4 μm. The standard deviation of the fLCI measurements is lower than that of the microscopy measurements due to the high number of cells probed with each fLCI measurement compared to that examined using image analysis. The distinction between the longitudinal and transverse diameters of the cell nuclei is discussed in depth in Section 5.4.4.

<table>
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5.4.4 Discussion

Experimental results show that fLCI is an excellent technique for accurately and precisely probing the nuclear morphology of epithelial cells. Although previous light scattering experiments with T84 cells have yielded larger nuclear sizes than those measured here using fLCI, we believe that this discrepancy is caused by differing measurement geometries and analysis methods. We confirm this assertion through comparison to confocal X-Z images.

The discrepancy between the fLCI measurements and those of standard fluorescence microscopy image analysis can be understood by considering that the light microscopy results are measuring the transverse profile of the cell nuclei while fLCI results probe the longitudinal profile of the cells. The difference between the two profiles can be explained by realizing that epithelial cells, such as the T84 cells, tend to flatten (Figure 5.11 (a)) upon contact with a substrate. In addition, examination of confocal microscopy images of cell nuclei in the X-Z plane (Figure 5.11 (b)) reveals that epithelial cells also tend to have different horizontal and vertical dimensions. This parameter is often quantized as ‘nuclear roundness’ in image analysis of histology slides.

Because the fLCI system is designed to only detect light directly backscattered within a narrow angular range, fLCI necessarily probes only the longitudinal dimension
of the cells. Therefore, it is most appropriate to compare the fLCI measurements with the longitudinal confocal microscopy measurements. This comparison reveals that fLCI is capable of measuring the longitudinal diameter of epithelial cell nuclei with high precision and accuracy. Although the fLCI technique is sensitive to reflections from cellular structures other than the nucleus, we are confident that our analysis method specifically obtains the features of the nucleus, rather than those of the entire cell. This specificity arises from the greater refractive index variation between the nucleus and the cytoplasm (3-5%) compared to that between the cytoplasm and the culture medium (<1%) which
would give the overall size of the cell body contained by the cell membrane. Further, epithelial cells do not contain any structures besides the nucleus that are large enough to produce a peak in the correlation distance graph corresponding to a size of 6-7 μm. In contrast, other sub-cellular organelles, such as mitochondria and lysosomes have a diameter of 0.2-2 μm.

The validation experiments presented in Section 5.4 demonstrate fLCI’s capability to accurately and precisely determine the longitudinal diameter of in vitro cell nuclei. The fLCI method’s ability to make quantitative nuclear morphology measurements demonstrates the potential feasibility of the technique for clinical diagnostic applications such as detecting early cancer development.

5.5 Summary

In Chapter 5, experiments validating the theoretical foundations and experimental capabilities of fLCI, described in Chapters 3 and 4, were presented. First, the imaging capabilities of the next generation, imaging fLCI system were validated. Using a scattering standard as an experimental sample, the results of the simulations in Section 3.5 are experimentally confirmed. The study showed the deleterious effects that the coherent summation of uncorrelated spatial modes can have on depth information. Additionally, the pfdOCT imaging capabilities of the imaging fLCI system were
demonstrated with ultra-high depth resolution images of a hamster cheek pouch epithelium.

In Section 5.3, the DW processing method was validated with experiments using an absorption phantom as an experimental sample. The experiments demonstrated the DW method’s ability to generate TFDs with simultaneously high depth and spectral resolution. In addition, the results showed an increase in spectra fidelity in TFDs generated by the DW method compared to those generated by the STFT. Finally, the local oscillations contained in generated TFDs, first explored in Section 3.3, were also measured. The measurements revealed increased knowledge of the experimental sample structure.

Section 5.4 experimentally validated the nuclear sizing capabilities of the fLCI technique. The study used the common path fLCI system to measure nuclear morphology in *in vitro* cell monolayers. The measurements were compared to fluorescence and confocal microscopy images, showing that fLCI is capable of determining nuclear diameter with high precision and accuracy.

The experiments presented in Chapter 5 set the stage for the culmination of the dissertation: the measurement of animal tissue health. Chapter 6 presents a pilot animal study and a complete animal trial which seek to distinguish between normal and dysplastic epithelial tissue in an animal model of carcinogenesis. The knowledge gained
from the previously presented theoretical and experimental studies is drawn upon to guide the completion of each animal study.
6. Animal studies

6.1 Introduction

Chapter 5 detailed a series of experiments validating the functionality and theoretical foundations of the fLCI technique in a variety of controlled experiments. While these experiments were essential to the development of the fLCI technology, the overarching goal of the technique is the measurement of tissue health. Chapter 6 follows from previous work, detailing animal tissue studies that were conducted to demonstrate the ability of the fLCI system to distinguish between normal and dysplastic epithelial tissue.

Section 6.2 details the animal model used in both studies, the hamster cheek pouch carcinogenesis model. The background of the model and its relationship to carcinoma of the human oral mucosa is discussed. Additionally, the progression of carcinogenesis in the animal model is described. The treatment plan and timeline used to prepare the animals for the study are described and the procedures used to excise and prepare the tissue samples for measurement are also detailed.

Section 6.3 discusses an initial, pilot animal study. The materials and methods used to complete the experiments are described and the data processing algorithm is discussed in depth. The pilot animal study utilizes the spectral slope metric discussed previously in Section 2.3.3 to distinguish between treated and untreated animal tissue samples. The basis of spectral slope is described and explained, and the algorithm for
extracting the measurement is presented. The results of the pilot study, which show statistically significant differences between the spectral slopes of normal and dysplastic epithelial tissue samples, are also presented and discussed.

A more complete animal study is detailed in Section 6.4. Like the pilot study, the materials and methods and data processing algorithms are explained. In the complete animal study, the DW processing method (discussed previously in Section 3.5) is utilized to generate depth resolved spectroscopic plots with simultaneously high depth and spectral resolution. Wavelength dependent oscillations in the spectra detected from specific subsurface tissue layers are detected and analyzed. These measurements are used to obtain nuclear diameter measurements from the basal tissue layer. Results are presented showing fLCI’s ability to distinguish between normal and dysplastic epithelial tissue with high sensitivity and specificity. The impact of the study’s results is also discussed.

6.2 Animal model

The animal studies were completed using the hamster cheek pouch carcinogenesis model. Past studies have linked the etiology of squamous cell carcinoma to environmental factors such as chemical carcinogens.71 As a result, carcinogen induced animal models are excellent systems for studies of squamous cell carcinoma because chemical agents are the dominant etiologic factor in each. The Syrian golden hamster
cheek pouch carcinogenesis model is closely correlated to the development of cancers of
the human oral mucosa.\textsuperscript{71} It has been shown that the squamous cell carcinomas
produced by application of carcinogens to the cheek pouch of Syrian golden hamsters
are histologically similar to the premalignant and malignant human oral squamous cell
carcinoma.\textsuperscript{71} Additionally, this particular animal system is one of the best known and
best characterized models of squamous cell carcinoma.

Oral cancers typically originate in a process of ‘field cancerization’ in which the
epithelial tissue is preconditioned by a carcinogenic chemical agent.\textsuperscript{72} The development
of oral cancer is ideal for investigating multi-step carcinogenesis because initiation,
promotion, and progression are all essential steps in the advancement of the disease.\textsuperscript{73}
As a result, the Syrian golden hamster cheek pouch carcinogenesis model is an excellent
model for investigations of such multi-step carcinogenesis systems. In this model,
malignant tumors are preceded by a sequential progression of hyperplasia to dysplasia
to carcinoma.\textsuperscript{73} The premalignant lesions are similar to human leukoplakia and
carcinoma \textit{in situ}. The sequence of progression in the hamster cheek pouch
carcinogenesis model is listed in Table 6.1.\textsuperscript{71}

There are several key advantages to using the hamster buccal pouch as a model
system for studies of squamous cell carcinoma. First, the cheek pouch is a convenient
tissue due to its easy access for delivery of carcinogenic chemical agents. Another
advantage is the histological similarity between the hamster cheek pouch mucosa and
Table 6.1: Pathology progression in the hamster cheek pouch carcinogenesis model

<table>
<thead>
<tr>
<th>Number of weeks</th>
<th>Pathology</th>
</tr>
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<tbody>
<tr>
<td>4 to 6</td>
<td>hyperkeratosis, chronic inflammation</td>
</tr>
<tr>
<td>6 to 8</td>
<td>hyperkeratosis, dysplastic hyperplasia</td>
</tr>
<tr>
<td>8 to 10</td>
<td>carcinoma in situ</td>
</tr>
<tr>
<td>10 to 16</td>
<td>invasive squamous cell carcinoma</td>
</tr>
</tbody>
</table>

the keratinizing human oral mucosa. And finally, both the absence of spontaneous tumors and the susceptibility to hormones, micronutrients, and other systemic influences are advantages of the model.71

For the animal studies, all experimental protocols were approved by the Institutional Animal Care and Use Committees of Duke University and North Carolina Central University and in accordance with the National Institutes of Health (NIH). Male Syrian golden hamsters, six weeks of age, were obtained from Harlan Laboratories (Indianapolis, IN) and housed at North Carolina Central University. The animals were housed four per cage in a room with controlled temperature and humidity and in a twelve hour light/dark cycle. Regular cage changes ensured maintenance of hygienic conditions. All animals were given the AIN-93M diet (Research Diets, New Brunswick,
The diet consisted of 14% casein, 0.18% l-cystine, 49.5% corn starch, 12.5% maltodextrin 10, 10% sucrose, 5% cellulose, 4% soybean oil, 0.0008% t-Butylhydroquinone, 3.5% mineral mix, 1% vitamin mix, and 0.25% choline bitartrate. Tap water was available *ad libitum*. After an acclimatization period of one week, the left cheek pouch of each animal was topically treated with 100 μl of 0.5% 7,12-dimethylbenz[a]anthracene (DMBA) (Sigma Chemical Company, St. Louis, MO) in mineral oil with a paintbrush three times per week for six weeks. The right cheek pouch was left untreated and served as the control group.

At 24 weeks after the initial treatment of DMBA, the hamsters were shipped to Duke University for optical spectroscopic analysis. The hamsters were euthanized by CO₂ asphyxiation before being subjected to gross necropsy. The entire left and right cheek pouches were excised and cut into two pieces. The samples were laid flat between two coverglasses, moistened with PBS, and immediately scanned by the fLCI system. Following the optical scanning, scanned areas were marked with India ink and the tissue samples were fixed in 10% PBS buffered formalin. The fixed samples were later embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for histopathological analysis.
6.3 Pilot animal study

The objective of the pilot animal study was to assess the ability of the fLCI technique to distinguish between normal and dysplastic epithelial tissue in a small number of animals. Successful results in the pilot study would demonstrate the potential of the fLCI technique and pave the way to undertaking a full scale animal study.

6.3.1 Materials and methods

The pilot study was conducted using the hamster cheek pouch carcinogenesis model described in Section 6.2. All tissue preparation procedures described in Section 6.2 were followed. Tissue samples from seven hamsters were analyzed in the pilot animal study. Tissues were scanned using the imaging fLCI system detailed in Section 4.3.

6.3.2 Data processing

The processing algorithm for the pilot animal study included several steps as illustrated in the flow chart of Figure 6.1. The raw data acquired by the imaging fLCI system consisted of 120 spectra, each detected by a spatially resolved detection channel. The detected signals originated from adjacent spatial points on the experimental sample. As mentioned in Chapter 4, although the imaging spectrometer used for detection was capable of detecting 255 spatially resolved channels, the diameter of the signal beam was only large enough to illuminate 120 channels.
Figure 6.1: Flow chart of the data processing algorithm used for the pilot animal study. The included steps transform raw data into depth resolved spectroscopic plots and measure spectral slopes of specific tissue layers.

Because the pilot animal study was completed before the development of the DW processing method, the STFT was utilized to generate the TFDs used to analyze the spectral characteristics of the experimental sample at depths of interest. Processing was accomplished by custom Matlab (The MathWorks, Natick, MA) software. The spectrum from each channel of the imaging spectrometer was processed by the STFT individually to generate 120 depth resolved spectroscopic plots. The STFT utilized a Gaussian window with a FWHM of 0.112 μm⁻¹ and a spacing of 0.0534 μm⁻¹ between
successive applications of the window. The STFT degrades spectral resolution by a factor of 41 and depth resolution by a factor of 25. The plots generated from each channel were then summed in order to maximize the spectral signal-to-noise ratio. The resulting depth resolved spectroscopic plot for a typical sample from the pilot animal study is shown in Figure 6.2 (c).

Figure 6.2 (a) PfdOCT image of the hamster cheek pouch epithelium with E, epithelium L, lamina propria. (b) Depth scan generated by summing transverse axis of image in part (a). (c) Depth resolved spectroscopic data generated by the STFT.

In order to determine the depths of interest for further spectroscopic analysis, the raw experimental data was converted to a pfdOCT image by a line-by-line Fourier transform. Figure 6.2 (a) shows a typical pfdOCT image from the pilot animal study with two tissue layers, the epithelium (E) and the lamina propria (L), labeled. Due to the relatively low signal-to-noise ratio of the B-mode image in Figure 6.2 (a), it is useful to generate a single depth scan, as shown in Figure 6.2 (b), by summing the image across
the transverse axis. For each depth plot, the keratinized tissue layer was identified as the first, narrow scattering peak. The transition from this peak to the next, broader scattering peak was identified as the epithelial surface. The pilot animal study spectroscopically analyzed two depth segments within each tissue sample. First, the ‘surface layer’ consisted of the first 50 μm of depth beneath the epithelial surface. This tissue segment contained the epithelial and basal tissue layers. The ‘sub-surface layer’ consisted of the tissue 51-100 μm beneath the surface. This segment primarily contained the lamina propria and submucosa tissue.

For each depth segment of interest, the average spectrum was computed and normalized by the spectrum from a known scattering standard in order to isolate the scattering effects of the experimental sample. To generate the normalization spectra, fLCI data was collected from a scattering standard and processed using the STFT. The average spectrum for each 50 μm depth segment was computed and used to normalize experimental spectra from the corresponding depth. For the pilot animal study, spectral oscillations such as those analyzed in Section 5.4 could not be measured. This can be attributed to resolution tradeoff associated with the STFT. In order to generate sufficiently high spectral resolution, depth resolution is degraded to the point that the spectral oscillations from specific tissue layers are washed out.

Instead, a metric known as spectral slope was analyzed to distinguish between normal and dysplastic epithelial tissue. Previous studies have indicated that
precancerous development in tissue alters spectral slope and that the metric can be used to distinguish between normal and diseased tissue. In order to measure spectral slope, a line was fit to each normalized experimental spectrum. The spectral slope for each sample was simply the slope of the linear fit.

**6.3.3 Results**

Typical experimental data collected during the pilot animal study is shown in Figure 6.3 along with the corresponding histology images. Results from untreated (normal) tissue are shown in part (a) and from treated (dysplastic) tissue in part (b). Note the darkening and thickening of the basal layer in part (b) indicating the presence of dysplasia. For each tissue sample, the average spectrum from each of the surface and sub-surface layers is shown along with the corresponding linear fit and associated slope and $r^2$ values.

The results of the pilot animal study are summarized in Figure 6.4. Part (a) shows the average spectral slope values measured from the surface layer of all scanned normal and dysplastic samples, respectively. For the surface layer, the normal tissue samples had an average spectral slope of 0.20 with a standard deviation of 0.010, and the dysplastic samples had an average spectral slope of 0.177 with a standard deviation of 0.018. This resulted in a statistically significant p-value of 0.011. Figure 6.4 (b) shows the corresponding data for the sub-surface tissue segment. In this case, the normal
Figure 6.3: (a) Typical experimental data from untreated hamster cheek pouch epithelium showing spectra from surface and sub-surface tissue layers along with linear fit and spectral slopes. Corresponding histology image is also presented. (b) Typical experimental data from treated tissue. Corresponding histology image is also presented.

Tissue samples had an average spectral slope of 0.178 with a standard deviation of 0.022, and the dysplastic samples from the sub-surface layer had an average spectral slope of 0.157 with a standard deviation of 0.021. The resulting p-value was 0.135 which is not statistically significant.
Figure 6.4: Summary of results from the pilot animal study. (a) Mean surface layer spectral slope values for normal and dysplastic tissue. (b) Mean sub-surface spectral slope values for normal and dysplastic tissue.

6.3.4 Discussion

The results of the pilot animal study show that fLCI has the potential to be an effective technique for distinguishing between normal and dysplastic epithelial tissue. Based on the experimental results, spectral slope can be an effective biomarker for detecting neoplastic transformation in buccal pouch tissue. It should be noted, however, that based on the results of the pilot study, depth resolution is a key aspect to effectively using spectral slope as a metric for distinguishing between normal and dysplastic animal tissues. The analysis of the surface tissue layers shows a statistically significant p-value of 0.011 between normal and dysplastic tissue samples whereas the analysis of
the sub-surface tissue layers shows a not statistically significant p-value of 0.135 between the tissue types. The difference in results from the surface layer and the sub-surface layer is important, but not unexpected.

Neoplasia typically develops in the basal tissue layer before expanding toward the tissue surface into the epithelium. Therefore, a greater difference between the spectral slopes of normal and dysplastic tissue is expected when analyzing the surface layer which interrogates the basal layer and epithelium. The dysplastic transformation would alter the cellular architecture of the sub-surface tissue layer less drastically, and therefore smaller differences between the spectral slopes of dysplastic and normal tissue would be expected for this layer.

6.4 Complete animal trial

Although the pilot animal study was successful in showing statistically significant differences between normal and dysplastic epithelial tissue samples, it did not achieve the goal of quantitatively assessing nuclear diameter. Section 6.4 details a larger scale animal study that utilizes a new data processing algorithm to extract nuclear morphology measurements from experimental samples. The complete animal trial seeks to distinguish between treated and untreated epithelial tissue with high sensitivity and specificity by measuring differences in the nuclear diameter of cells in the basal tissue layer.
6.4.1 Materials and methods

Like the pilot animal study, the complete animal trial was conducted using the hamster cheek pouch carcinogenesis model described in Section 6.2. All tissue preparation procedures described in Section 6.2 were followed. The complete animal trial analyzed tissue samples from 21 hamsters. All samples were scanned using the imaging fLCI system described in Section 4.3. Although a treated and an untreated sample from each animal was scanned by the fLCI system, only 16 of 21 untreated samples were used in the study. The signal-to-noise ratio of the scans from the remaining five untreated samples was too low to provide useful data. Therefore, these scans were discarded before any spectroscopic analysis took place.

6.4.2 Data processing

The data processing algorithm for the complete animal trial is summarized by the flow chart shown in Figure 6.5. The raw data acquired by the imaging fLCI system consisted of 120 spectra, each of which originated from an adjacent spatial point on the experimental sample. The raw data along with the plots of three such spectra are shown in Figure 6.6 (a). The diameter of the signal beam was small enough to illuminate only 120 of the 255 spectral channels of the imaging spectrometer.
Figure 6.5: Flow chart of the data processing algorithm used for the complete animal trial. The included steps transform raw data into depth resolved spectroscopic plots and determine the scatterer size from specific tissue layers by analyzing spectral oscillations in the detected signal.

In order to spectrally analyze specific tissue layers, the spectrum detected by each channel of the imaging spectrometer was processed using the DW processing method described in Section 3.4. A custom Matlab program was used to process the data with both a narrow spectral window of 0.0405 μm⁻¹ FWHM and a wide spectral window of 0.665 μm⁻¹ FWHM. The TFDs generated by each window were multiplied to produce a TFD with simultaneously high spectral and depth resolution. The resulting 120 depth resolved spectroscopic plots were summed together to maximize the signal-
Figure 6.6: (a) Raw data from the complete animal trial with spectra from three spectrometer channels shown. (b) Three depth resolved spectroscopic plots produced by DW processing the spectra in part (a). Summing the plots from all channels produces the final TFD as shown.
to-noise ratio, producing a total depth resolved spectroscopic plot for each tissue sample as shown in Figure 6.6 (b).

As described in Chapter 2, carcinomas first develop in the basal layer of the epithelial tissue. In hamster buccal pouch tissue, the basal layer tends to be approximately 30 to 50 μm beneath the surface of normal tissue, and approximately 50 to 150 μm beneath the surface of dysplastic tissue. Because the basal layer offers the earliest opportunity for detecting developing dysplasia, it is the target tissue layer for the fLCI technique and for this study.

In order to target the basal layer of the epithelium, first the raw data was processed into a pfdOCT image by a line-by-line Fourier transform. The B-mode images were summed across the transverse axis to generate depth plots like those presented in Figure 6.7. Several important histological features can be identified in the depth scans and co-registered with the corresponding histopathology images. Figure 6.7 indicates the location of the keratinized layer (green arrow), the basal layer (red arrow), and the underlying lamina propria (blue arrow) in the histopathology images of an untreated and treated tissue sample. Scattering peaks corresponding to the same tissue layers were identified in each depth scan. To correlate the distances in the histology images with distances in the depth scans, the index of refraction of the tissue had to be taken
Figure 6.7: Histopathology image and corresponding depth plot for (a) untreated and (b) treated epithelium. Arrows indicate keratinized layer (green), basal layer (red), and lamina propria (blue).

into account. An average refractive index for the tissue of $n = 1.38$ was used to convert depth scan distances to optical path lengths.\textsuperscript{75,76} Variation of the index within the tissue is a potential limitation of the current method and is discussed further in Section 6.4.4.
Figure 6.8: (a) Depth resolved spectroscopic plot with basal layer indicated by dashed box. (b) Spectrum from basal tissue layer along with power law fit. (c) Residual spectrum from basal tissue layer. (d) Correlation plot generated by Fourier transforming spectrum in (c). Peak correlation distance can be related directly to scatterer size.

A 15 μm depth segment corresponding to the location of the basal layer was selected and the corresponding depths were identified in the depth resolved spectroscopic plot as shown in Figure 6.8 (a). The selected spectra were averaged to generate a single spectrum for the basal layer. As shown in Figure 6.8 (b), a power law
curve of the form \( y = b \cdot x^\alpha \) was fit to each spectrum using Matlab’s Curve Fitting Toolbox. The \( \alpha \) term of each power law curve was recorded to analyze fractal dimension information. This is discussed further in Section 6.4.4. The residual of each spectrum was calculated by subtracting the power law curve from the experimental spectrum to produce a normalized spectrum as shown in Figure 6.8 (c).

The normalized spectra showed clear oscillations resulting from interference produced by scattering from the front and back surfaces of basal cell nuclei. The normalized spectrum was Fourier transformed to generate a correlation plot like that shown in Figure 6.8 (d) which showed a clear peak corresponding to the dominant frequency in the normalized spectrum. Peak detection was carried out by an automated, custom Matlab program. The script first high-pass filtered the spectrum with a cutoff of 4 cycles in order to remove any low frequency content corresponding to extremely small scattering particles. The location of the correlation plot peak was then automatically detected by the Matlab script and related to scatterer diameter with the simple equation
\[
d = \frac{\text{correlation distance}}{(2 \cdot n)}
\]
where \( n \) is the refractive index and \( d \) is the diameter of the cell nuclei. An nuclear index of refraction of \( n = 1.395 \) was used.\textsuperscript{56}

**6.4.3 Results**

The results of the complete animal trial are summarized in Table 6.2, Table 6.3, and Figure 6.9. The 16 untreated tissue samples had a mean basal layer nuclear diameter of 4.28 \( \mu \text{m} \) with a standard deviation of 0.69 \( \mu \text{m} \). The 21 scanned treated tissue
samples had a mean basal layer nuclear diameter of 9.50 μm with a standard deviation of 2.08 μm. A statistical t-test revealed a p-value of less than 0.0001, indicating an extremely statistically significant difference between the basal layer nuclear diameters of the two populations.

Assessing fractal dimension, the 16 untreated samples had a mean $\alpha$ value of 0.33 with a standard deviation of 0.19. The mean $\alpha$ value of the 21 treated samples was 0.44 with a standard deviation of 0.22. A statistical t-test resulted in a not statistically significant p-value of 0.15. These results indicate that, in its current form, fLCI is incapable of distinguishing between normal and diseased epithelial tissue using fractal dimension information from the $\alpha$ term. This is discussed further in Section 6.4.4.

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>16</td>
<td>21</td>
</tr>
<tr>
<td>Mean (μm)</td>
<td>4.28</td>
<td>9.50</td>
</tr>
<tr>
<td>Std. Dev</td>
<td>0.69</td>
<td>2.08</td>
</tr>
</tbody>
</table>

p-value < 0.0001**

Table 6.2: Summary of nuclear diameter measurements from the complete animal trial.
Table 6.3: Summary of alpha term measurements from the complete animal trial

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>16</td>
<td>21</td>
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<tr>
<td>Mean</td>
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<td>0.44</td>
</tr>
<tr>
<td>Std. Dev</td>
<td>0.19</td>
<td>0.22</td>
</tr>
</tbody>
</table>

p-value = 0.15

Figure 6.9 plots each treated (blue square) and untreated (red x) tissue sample as a function of its measured basal layer nuclear diameter. The presented decision line results in excellent separation between the normal and diseased samples. Using the indicated decision line, the study results in correctly categorizing 21 of 21 treated samples for a 100% sensitivity and correctly categorizing 16 of 16 untreated samples for a 100% specificity.
6.4.4 Discussion

The experimental results of the complete animal trial show that fLCI has great potential as a technique for distinguishing between normal and dysplastic epithelial tissue. Experimental measurements showed an excellent ability to precisely and accurately distinguish between treated and untreated in situ animal tissue using nuclear diameter as a biomarker. The measured diameters correspond nicely with the nuclear diameter expected for the basal tissue layer of hamster cheek pouch epithelium when
measurements are adjusted to account for fLCI’s measurement of the minor axis of cell nuclei. It should be noted that the development of dysplasia *in situ* results in thickening in the basal tissue layer and a breakdown of cellular organization. As a result, fLCI may measure the major axis of some nuclei in diseased tissue, further contributing to the detected nuclear enlargement when compared with normal tissue.

The use of the DW processing method is an important difference between the pilot animal study and the complete animal trial. The DW processing method permitted the measurement of spectral oscillations induced by nuclear scattering that could not be detected in the pilot animal study. As discussed in Chapter 3, the STFT used to generate TFDs in the pilot animal study suffered from an inherent tradeoff between spectral resolution and depth resolution. As a result of this tradeoff, achieving an acceptable spectral resolution required the degradation of depth resolution to the point that the spectral oscillations induced by nuclear scattering were washed out. This washout was likely due to phase and frequency differences in the spectra from different tissue layers which were combined as a result of the poor depth resolution.

The use of the DW method for processing the experimental data of the complete animal trial produced depth resolved spectroscopic plots with simultaneously high depth and spectral resolutions. The DW method generated satisfactory spectral resolution while maintaining high depth resolution, therefore permitting the spectral analysis of thin tissue segments. By avoiding the combination of signals from many
tissue layers, the oscillatory components of spectra originating from the basal tissue layer were preserved. Therefore, the key issue that plagued the pilot animal study was avoided.

Although the pilot animal study showed that the measurement of spectral slope was an effective way to distinguish between normal and dysplastic epithelial tissue, there are multiple advantages to using nuclear diameter as the distinguishing metric. First, nuclear enlargement is a known biological phenomenon used by pathologists as a biomarker when diagnosing dysplasia, whereas the biological foundation of spectral slope variations is still unclear. Additionally, measuring nuclear diameter offers a quantitative diagnosis which can potentially track the development of carcinoma from hyperplasia to low grade dysplasia to high grade dysplasia. As of now, it is uncertain how these varying states of disease affect spectral slope.

The analysis of power law $\alpha$ terms was expected to yield fractal dimension information that would help to distinguish between normal and dysplastic epithelial tissue. The not statistically significant results of this portion of the study indicate that a more complex model of scatterers within the tissue must be developed for future studies. The results of the pilot study as well as other light scattering research$^{41, 42, 74}$ indicate that, in addition to spectral modulations, spectral shape yields insight into tissue microarchitecture and health. Developing a scatterer model that can capture this information should be a priority as the fLCI technology is further developed.
Though the results of the complete animal study are extremely promising, the current methods are not without limitation. The dependence on refractive index in selecting tissue layers is a challenge that must be studied in the future. The current fLCI data processing algorithm does not account for potential variations of refractive index within a tissue. The current method also does not adjust for potential index changes induced by the onset of dysplasia. In order to accurately measure optical path lengths within a tissue sample, a dynamic model of refractive index must be developed.

Similarly, a robust method to account for the varying thickness and location of the basal layer in developing dysplasia should be implemented.

Although the detection of peaks in the correlation plots for this study was automated to eliminate bias, subsequent analysis of the correlation data revealed that some plots contained multiple prominent peaks. Understanding how correlations between neighboring cellular structures and correlations between tissue layers contribute to generated correlation plots will facilitate the development of an advanced scattering model. As the fLCI technique progresses, an analysis assessing the effectiveness of using Mie theory for fLCI scatterer size determination should also be undertaken.

The results of the complete animal trial presented in Section 6.4 demonstrate fLCI’s capability to distinguish between normal and dysplastic epithelial tissue with high sensitivity and high specificity. The fLCI method’s ability to make quantitative
nuclear morphology measurements demonstrates its potential as an effective technology for optically detecting dysplasia. The results of these experiments lay the groundwork for further development of fLCI into a technique for clinical diagnostic applications such as the detection of early cancer development.

6.5 Summary

Chapter 6 presented the results from two animal studies. First, the animal model used in both studies, the hamster cheek pouch carcinogenesis model, was detailed. Background of the animal model was given, the animal treatment plan and timeline were explained, and all experimental sample preparation procedures were described.

Next, details of the pilot animal study were presented. The materials and methods used and the data processing procedures followed were described in depth. The pilot study utilized the spectral slope metric to distinguishing between normal and dysplastic epithelial tissue in 7 animals. The study analyzed data from two depth segments within each cheek pouch. Experimental results showed statistically significant differences between spectral slope measurements from normal and dysplastic tissue in the surface layer, but not in the sub-surface layer. The results of the pilot study indicated that measuring spectral slope with fLCI has the potential to be an effective biomarker for distinguishing between normal and diseased epithelial tissue, but that depth resolution was of key importance.
Section 4.4 presented the results of the complete animal trial. The study spectroscopically analyzed 21 dysplastic samples and 16 normal samples from the hamster cheek pouch. The materials and methods used and the data processing algorithm followed were described. The complete animal study utilized the DW processing method, introduced in Section 3.4, to generate depth resolved spectroscopic plots from each measured sample. The DW method provided the simultaneously high depth and spectral resolution necessary to analyze spectral oscillations and measure nuclear diameter in the manner utilized in the validation experiments of Section 5.4. The results of the complete animal study showed that fLCI distinguished between normal and dysplastic epithelial tissue with 100% sensitivity and 100% specificity. Future directions and current limitations of the fLCI technique were also discussed.
7. Conclusions and future directions

The work presented in this dissertation has chronicled the development of the fLCl technology from the first proof of principle experiment to the completion of a successful animal study. A number of technical and theoretical hurdles were overcome along the way.

The common path fLCl system described in Section 4.2 was designed for its simplicity and ease of alignment. The system was used for the nuclear sizing validation experiments detailed in Section 5.4. That study showed that fLCl was capable of measuring nuclear diameter in \textit{in vitro} cell monolayers with high precision and accuracy, but the common path system was limited to measurements of thin experimental samples.

The study of spatial coherence in white light interferometry presented in Section 3.5 explained the theoretical foundation of the common path system’s limitations. As the study showed both mathematically and with simulations, the coherent sum of uncorrelated spatial modes can degrade depth information, thus limiting measurements to thin experimental samples. This knowledge was utilized to design and assemble the next generation, imaging fLCl system detailed in Section 4.3. The system used a 4f interferometer and an imaging spectrometer for detection to spatially resolve the optical signal throughout the system. This advancement permitted the measurement of thick experimental samples.
The imaging capabilities of the new fLCI system were validated in Section 5.2 through scattering standard experiments and the pfdOCT imaging of hamster cheek pouch tissue. The scattering standard experiments confirmed the results of the simulations described in Section 3.5. The ultra-high depth resolution pfdOCT imaging capabilities of the imaging fLCI system were established by the presented image of the hamster cheek pouch epithelium, the first OCT image of a biological sample acquired in the frequency domain using a white light source.

Using the imaging fLCI system, an initial, pilot animal study (Section 6.2) was undertaken. The study looked to distinguish between normal and dysplastic epithelial tissue in the cheek pouch tissue from seven hamsters. In processing the data from the study, the tradeoff between depth and spectral resolution in the depth resolved spectroscopic information generated by the STFT prohibited the measurement of nuclear diameter. Instead, the spectral slope metric was measured, showing statistically significant differences between normal and diseased tissue in one of the two targeted depth segments. The results of this study indicated that fLCI is capable of distinguishing between normal and dysplastic tissue using spectral slope, but that depth resolution was essential. Although the results of the pilot study were promising, they did not achieve the goal of measuring nuclear diameter in \textit{ex vivo} animal tissue. In order to accomplish this, it was necessary to develop a means to circumvent the tradeoff between depth and spectral resolution inherent to the STFT.
An analysis of temporal coherence and time frequency distributions, discussed in Section 3.3, was undertaken to provide a more complete theoretical understanding of OCT and fLCI signals. The study showed that temporal coherence information contained in TFD cross terms could provide valuable structural information about experimental samples. The insight gleaned from this study led to the development of the DW processing method. Section 3.4 mathematically described the method and used simulations to show that it was capable of generating TFDs with simultaneously high depth and spectral resolution. These results were validated in Section 5.3 through a series of experiments using an absorption phantom as an experimental sample. The development of the DW method provided a means for overcoming the tradeoff between depth and spectral resolution that has long hindered traditional methods for processing SOCT and fLCI signals, such as the STFT. The development also set the stage for the completion of a complete animal study measuring nuclear diameter using fLCI for the first time in animal tissue.

The complete animal study is presented in Section 6.3. The study measured 21 diseased and 16 normal hamster cheek pouch samples and processed the data using the DW method. The resulting depth resolved spectroscopic plots had the high depth and spectral resolution necessary to measure spectral oscillations induced by nuclear scattering. The resulting nuclear diameter measurements distinguished between normal and dysplastic epithelial tissue with 100% sensitivity and 100% specificity.
Although the results of the complete animal study were extremely promising, current fLCI methods have several limitations which will guide the future development of the technology. First, a study measuring scatterer diameter in controlled thick phantoms is necessary to further validate the results of the complete animal study. Additionally, a more complete scattering model for animal tissue should be developed. Because optical path length measurements are dependent on refractive index, the fLCI technique must account for this in determining the location of specific tissue layers. A deeper understanding of the dynamics of refractive index in dysplastic animal tissue must be developed.

The fLCI method’s nuclear sizing algorithm, while effective in the complete animal study, is likely overly simplistic and should be further developed. Understanding how correlations between neighboring cellular structures and correlations between tissue layers contribute to generated correlation plots will facilitate the development of an advanced scattering model. Additionally, an analysis assessing the effectiveness of using Mie theory for fLCI scatterer size determination should also be undertaken. The measurement of fractal dimension and spectral slope as metrics for distinguishing between normal and diseased tissue should also be explored further. The completion of these projects as well as the conversion to a fiber optic based system will allow the fLCI technology to characterize in vivo animal tissue and move closer towards the goal of becoming a clinically viable early cancer detection technique.
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Biography

Robert Nicholas Graf, or “Nick” as he is known to his friends, was born and raised in Pekin, IL, a town of 30,000 in the heartland of Illinois. He broadened his horizons in the fall of 2000, leaving central Illinois to attend Duke University in Durham, NC. Nick graduated from Duke, magna cum laude in the spring of 2004, receiving a bachelor’s degree in Biomedical Engineering. He immediately entered Duke’s Graduate School seeking a Ph.D. from the same department. Nick has six peer reviewed publications to go along with numerous conference presentations and proceedings publications. He received his Ph.D. in the spring of 2009. Nick is and will forever be passionate about his family, friends, music, and Dear Old Duke.

Peer Reviewed Publications


